universität wien

MASTERARBEIT

Titel der Masterarbeit

„Allelopathic activity of stoneworts“

verfasst von

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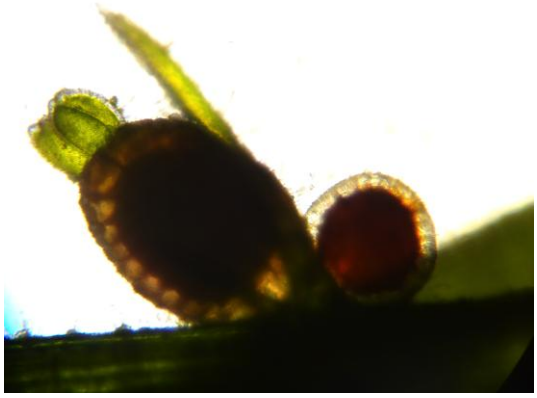
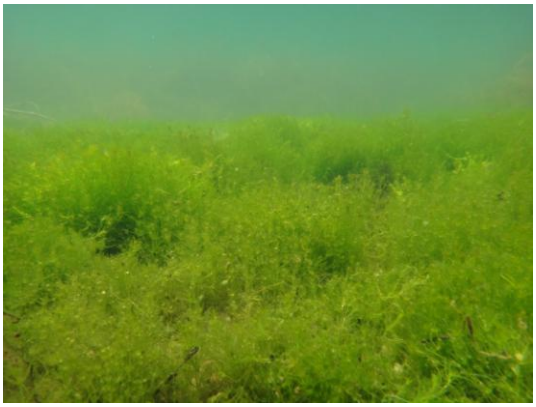
angestrebter akademischer Grad

Master of Science (MSc)

Wien, August 2015

Studienkennzahl lt. Studienblatt:	A 066 833
Studienrichtung lt. Studienblatt:	Masterstudium Ökologie
Betreut von:	Ao. Univ. Prof. Mag. Dr. Michael Schagerl



Ein Einblick in die Welt der Armleuchteralgen.

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1.) Allgemeine Einleitung

Algen sind großteils photosynthetisch aktive, sauerstoffproduzierende Organismen ohne Gefäßbündel und ohne für höhere Pflanzen typische Gliederung in Blatt, Stamm und Wurzel. Algen besiedeln Habitate im Süßwasser, Salzwasser und sogar an Land. Weiters werden sie in mikroskopische und makroskopische Algen (Seetang, Blasentang, Armleuchteralgen etc.) unterteilt, wobei letztere eine gewisse Ähnlichkeit mit den höheren Pflanzen aufweisen (Graham et al. 2009). Makroalgen sind bereits mit freiem Auge erkennbar und werden teilweise mehrere Meter hoch. Sie bilden keine eigentlichen Blätter aus, daher wird der Pflanzenkörper in seiner Gesamtheit als "Thallus" bezeichnet. Algen gelten als Vorgänger der Landpflanzen, wobei noch darüber diskutiert wird, von welcher Gruppe diese genau abstammen (Civáň et al. 2014). Der Begriff der Algen umfasst Gruppen mit echtem Zellkern (Eukaryoten) und solche ohne Zellkern (Prokaryoten), welche auch Blaualgen oder Cyanobakterien genannt werden (Graham et al. 2009).

Eine stetige Konkurrenz um Licht und Nährstoffe im Gewässer führt zur Entwicklung vieler Strategien. Ein Beispiel hierfür ist die Produktion chemischer Abwehrstoffe (Allelopathie), die potenzielle Konkurrenten unterdrücken. Definiert wurde dieser Begriff vom bekannten österreichischen Botaniker Hans Molisch im Jahre 1937 und beschreibt die Wirkung einer Pflanze auf eine andere, wobei diese im Sinne von Molisch hemmend, aber auch fördernd sein kann (Molisch 1937). Allelopathie kommt bei Landpflanzen vor, wie etwa dem Walnussbaum oder Reis. Aber auch Wasserpflanzen, wie das Hornkraut oder Armleuchteralgen (Characeen), weisen allelopathische Wirkungen auf, um ihre Thalli vor aufwachsenden Mikroalgen zu schützen. Auch im Freiwasser lebende Mikroalgen, so genanntes Phytoplankton, können dadurch unterdrückt werden (Gross et al. 2007).

Die Namensgebung „Armleuchteralgen" bezieht sich auf die Anordnung der Seitenäste mit begrenztem Wachstum, welche stockwerksartig übereinander wachsen und an Armleuchter erinnern. Characeen kommen weltweit mit 450 Arten (Europa: etwa 45 Arten) in stehenden Süßgewässern oder Quellbächen vor, wobei einige bis ins Brackwasser vordringen (Krause 1997).

Die Erforschung der Allelopathie gestaltet sich oft schwierig, da zunächst kontrollierte Laborexperimente durchgeführt werden müssen (Gross et al. 2007). Um die Wirkung dieser Stoffe im System selbst zu beweisen, müsste man weitere Versuche im Freiland durchführen.

Aufgrund ihres Vorkommens in artreinen Beständen und der sattgrünen Farbe standen Armleuchteralgen schon früh unter Verdacht, chemische Abwehrstoffe zu bilden (Berger &

Schagerl 2003). Entnimmt man Pflanzen aus dem Wasser, fällt bei vielen Arten ein intensiver, an Knoblauch erinnernder, schwefeliger Geruch auf, welcher charakteristisch für diese Algengruppe ist. Dieser Geruch war ebenfalls ein Grund zur weiteren Untersuchung der allelopathischen Wirkung (Anthoni et al. 1986). Bisher wurden bereits einige chemische Verbindungen isoliert und identifiziert. Dennoch sind noch viele Mechanismen dieses komplexen „chemischen Kampfes" zwischen Armleuchteralgen und Mikroalgen unbekannt.

Ziel dieser Studie war es, vier verschiedene bekanntermaßen allelopathisch aktive Arten von Armleuchteralgen auf ihre hemmende Wirkung gegen Cyanobakterien, Grünalgen und einer heterotrophen Bakterienart (Leuchtbakterien) zu testen. Weiters wurden zwei Trocknungsmethoden angewendet, um etwaige Einflüsse der Probenbehandlung zu prüfen. In der Studie wurden auch flüchtige, wirksame Substanzen berücksichtigt. Durch die Anwendung verschiedener organischer Lösungsmittel zur Extraktion der wirksamen Stoffe konnten Rückschlüsse auf die Polarität der Stoffe gezogen werden.

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2.) Abstract

Allelopathy is a widespread phenomenon among terrestrial and aquatic plants. We focused on allelopathic effects of stoneworts (Characeae), which so far did not receive much attention. For this purpose, we studied four different *Chara* species (*C*. *aspera* Willdenow, *C*. *globularis* Thuillier, *C*. *rudis* (Braun) Leonhardi and *C*. *tomentosa* Linnaeus); each of the species was collected from two sampling locations to consider site differences. We used both live algae shoots and extracts of dry material for bioassays. Two species of green algae (*Chlorella vulgaris* Beijerinck, *Scenedesmus acuminatus* (Lagerheim) Chodat), two strains of Cyanobacteria (*Synechococcus elongatus* Nägeli, *Synechococcus leopoliensis* (Raciborski) Komárek) and one heterotrophic marine, bioluminescent Proteobaterium (*Vibrio fischeri* (Beijerinck) Urbanczyk) were chosen as target organisms. Dichloromethane (DCM), N-butanol and methanol (MeOH) were used for the allelochemical extractions. Two different drying methods, air drying at 30 °C and lyophilisation at -100 °C, were applied for detecting possible differences of treatment. Furthermore the condensed ice of the lyophilisation was collected in order to consider volatile substances with potential allelopathic effects. Inhibitions of different target organism by *Chara* extracts were comparable. Also the two applied drying methods showed only small differences. Strong inhibitions of *S*. *elongatus*, *S*. *leopoliensis* and *V*. *fischeri* by live stoneworts and extracts indicated an antibiotic effect. Green algae were never inhibited by live *Chara* nor extracts, except *S*. *acuminatus*, which showed a strong inhibition by the polar N-butanol ice extracts. Also both Cyanobacteria species were strongly affected by the polar ice extracts revealing presence of volatile allelopathically active substances. Pulse amplitude modulated fluorescence measurements of photosystem II indicated negative effects on the performance of the target algae's photosynthesis. In comparison to the photoautotrophic target organisms, *V*. *fischeri* showed a controversial pattern on plant extracts: *V*. *fischeri* was also affected by extracts, which did not show any inhibition of photoautotrophic target organisms. Our study reveals the high antibiotic potential of allelopathic substances of four stonewort species, which could be further applied in several fields such as lake restoration and pharmacological approaches.

Key words: Chara, allelopathy, stoneworts, methanol, N-butanol, dichloromethane, bioassay, HPLC, extract

3.) Introduction

Characeae, also called stoneworts, are submerged photoautotrophic multicellular macroalgae with an appearance rather similar to higher plants (McCourt et al. 2004). Besides the Zygnematales and the Coleochaetales they have been discussed as ancestors of land plants (McCourt et al. 2004, Wodniok et al. 2011, Civáň et al. 2014). Representatives of stoneworts are able to colonize different habitats, such as spring-fed brooks, lakes (also deeper layers), ponds and fluvial systems; they are found in freshwater to brackish waters. Characeae usually form monospecific submerged meadows (Berger & Schagerl 2004): they act as primary colonisers in disturbed and temporary habitats due to oospores, which have a long lasting viability (Wade 1990). *Chara*-dominated lakes are generally clear with low phytoplankton productivity and great Secchi-depths (Crawford 1977). Furthermore Characeae are often dominant in clear-water states of shallow water bodies (Scheffer 1998). Many of them have a characteristic pungent smell due to sulphuric compounds (Anthoni et al. 1980), which are thought to be allelopathically active. Today it is commonly accepted, that some representatives of the Characeae are allelopathically active (Anthoni et al. 1980, Hilt & Gross 2008).

According to Molisch (1937), allelopathy is defined as an either harmful or beneficial biochemical effect between two plants**.** It is a widely distributed phenomenon among plants and can be found between terrestrial, e.g. walnut trees (Ercisli et al. 2005), and also in aquatic plants, e.g., *Myriophyllum*, *Elodea*, *Ceratophyllum* and *Stratiotes* (Kurashov et al. 2014). Moreover, allelopathy between planktonic taxa occurs (Schagerl et al. 2002, Gross 2003a, Mulderij et al. 2007, Barreiro & Vasconcelos 2014, Mandal et al. 2014). Producing alleochemicals is an advantageous strategy for being highly competitive in terrestrial and aquatic habitats. This might be the reason for the great success of several invasive macrophytes, such as *Ludwigia*, *Impatiens glandulifera* Royle, *Chrysanthemoides monilifera* (Linnaeus) Norlindh, *Brachiaria brizantha* Hochst. ex A.Rich. and *Eichhornia crassipes* (Martius) Solms (Dandelot et al. 2008, Gruntman et al. 2014, Harun et al. 2014, Kato-Noguchi et al. 2014a, 2014b).

Some previous investigations have been undertaken assessing the allelopathic activity of several macrophytes and stoneworts with respect to their potential application for restoring eutrophic water bodies (Berg et al. 1999, Pakdel et al. 2013, Rojo et al. 2013). Growth inhibition of phytoplankton induced by allelopathically active submerged macrophytes (including the Characeae) can occur if the species occupy a certain volume of the lake with a

density of > 100 g m^{-2} dry mass and certain sensitive microalgae species such as diatoms or Cyanobacteria dominate the planktonic community (Hilt & Gross 2008). A loss of submerged vascular macrophyte vegetation followed by a switch to the turbid state due to eutrophication has been documented for temperate shallow lakes (Körner & Nicklisch 2002). Replanted dominating macrophyte meadows (including stoneworts) could be useful in wetland restoration and in achieving stabilized clear-water states by reducing the growth of phytoplankton (Jeppesen et al. 1997, Hilt & Gross 2008, Rojo et al. 2013). Allelopathy of submerged vascular macrophytes is strong enough to inhibit and control the growth of phytoplankton on larger scales up to mesocosm level. Therefore this activity is regarded to be a more reliable management strategy than nutrient competition due to its independence of changing the ecosystem's nutrient inputs (Vanderstukken et al. 2014). Another application of Characeae as ecological engineers could be growth control of harmful Cyanobacteria such as *Microcystis*, which produces cyanotoxins (Chen et al. 2012).

Evidence for allelopathic activity of *Chara* extracts was shown for some species in previous studies (Berger & Schagerl 2003, 2004, Hilt & Gross 2008), but may also occur in other species. In many cases Cyanobacteria and diatoms, but only sporadically Chlorophytes were affected by *Chara* (Berger & Schagerl 2003, 2004, Rojo et al. 2013) and by some vascular aquatic macrophytes (Mulderij et al. 2005, Hilt 2006).

Numerous laboratory experiments have been carried out, but *in situ* allelopathy of submerged macrophytes and stoneworts is difficult to prove. Only few field-studies dealing with allelopathic interactions in aquatic systems exist so far (Forsberg et al. 1990, Gross et al. 2007, Hilt et al. 2012).

According to Gross et al. (2007), a single method is insufficient for proving allelopathic activity of submerged macrophytes; a combination of both laboratory and field experiments is definitely needed and provides much better information. If laboratory and field experiments are compared, differences in strengths of allelopathic effects might be caused by bacterial degradation of labile compounds during the exudation period, fast dilution of allelopathic substances into the surrounding water and the unknown concentration of these substances in the ambient water under *in situ* conditions (Gross et al. 2007, He et al. 2008). Moreover, some studies suggested that epiphytic algae are less sensitive to allelopathic substances of aquatic vascular macrophytes than planktonic algae (Mulderij et al. 2005, Hilt 2006). It is assumed that epiphytic algae developed strategies to become resistant against allelopathic substances of vascular macrophytes due to co-evolution within the same habitat (Reigosa et al. 1999).

Another point of discussion is a species specificity of allelopathic interactions between submerged macrophytes or charophytes and target organisms (Van Donk & Van de Bund 2002, Hilt & Gross 2008, Pakdel et al. 2013). Mixed cultures containing several *Chara* species and *Myriophyllum spicatum* Linnaeus inhibited phytoplankton growth rate much more than monocultures did (Rojo et al. 2013).

Additionally, abiotic factors influence allelopathic interactions. Nutrient limitation might serve as one example, as it increases the sensitivity of phytoplankton to allelopathic substances released by submerged macrophytes (Reigosa et al. 1999). Light availability is another factor which might influence the degree of phytoplankton sensitivity (Gross 2003b). Mulderij et al. (2005) revealed, that exudates of the vascular emerged macrophyte *Stratiotes aloides* Linnaeus had a stronger effect on the growth of green algae at low irradiance compared to algae at high irradiance.

Also the stonewort's age could have a significant effect on the allelopathic intensity. Young stoneworts enhanced the duration of the lag-phase of planktonic green algae cultures, older specimens lowered the growth rate during the exponential phase (Mulderij et al. 2003). The authors further suggested that selective inhibition of some phytoplankton species might be present.

Chemical compounds of different *Chara* species showed inhibitions of the phytoplankton photosystem II activity in several laboratory experiments (Berger & Schagerl 2004, Hilt 2006). For vascular freshwater macrophytes (Körner & Nicklisch 2002, Jiang et al. 2014) and marine macroalgae (Gao et al. 2014, Ye et al. 2014), several investigations on the inhibition of photosystem II of target algae (macro- and microalgae) have been performed by PAM-measurements (pulse amplitude modulated fluorescence).

According to Gross (2003a), allelochemicals released by submerged aquatic macrophytes into the ambient water must possess a hydrophilic character and have to be exuded at certain concentrations for reaching and effectively influencing their target organisms despite dilution in aquatic systems. Exactly these characteristics were found by Bankova et al. (2001), who focused on *Chara globularis* Thuillier and found antibiotic activity of mostly aqueous (polar) extracts. They mentioned that also volatile substances could exhibit allelopathic activity. Substances from a hydromethanol extract of *Chara aspera* Willdenow had strong effects on the growth of Cyanobacteria (Berger & Schagerl 2003). This effect was attributed to low molecular weight substances which might easily pass cyanobacterial cell membranes (Gross 1999).

Some substances of stoneworts were analysed so far, partly probably allelopathically active (Table 1). 5-methylthio-1,2,3-trithiane and 4-methylthio-1,2-dithiolane have been isolated from *Chara globularis* and are assumed to be responsible for photosynthesis and growth inhibition (Anthoni et al. 1980). Both substances are thermolabile and volatile. The same substances were isolated from freshwater and brackish *Chara* in a laboratory experiment (Wium-Andersen et al. 1982). No traces of flavonoids (secondary plant metabolites with defence potential) could be detected so far (Bankova et al. 2001).

Furthermore charamin, a quaternary ammonium salt, was isolated from *Chara globularis*, which showed antibiotic activities (Anthoni et al. 1986). Ortner (2012) and Doblander (2013) found a huge amount of L-tryptophan in different stonewort species which is also proven to be allelopathically active. They however did not test the identified substances. Kurashov et al. (2014) summarised several allelopathically active compounds of submerged aquatic macrophytes belonging to aldehydes and ketones, ethers, terpenoids, phytoecdysteroids, fatty acids and other organic acids, aromatic hydrocarbons and phenol derivatives. Presumably more than one allelochemical of *Chara aspera* is responsible for growth inhibition of other algae (Berger & Schagerl 2003).

Table 1: Volatile (a) and polar substances (b) of Characeae. [1] Bankova et al. (2001), [2] Doblander (2013), [3] Ortner (2012), [4] Wium-Andersen et al. (1982), [5] Anthoni et al. (1986).

(a) Known volatile substances of Characeae		
Class	**Compound**	**References**
Acids	pyruvic acid	[1]
	nonanoic acid, 9-oxo	[1]
	hexanedioic acid	[1]
Chlorinated compounds	chloroacetic acid	[1], [2]
	methane, oxybis [chloro-]	[1]
	chloropyruvic acid	[1], [2]
	dichloropyruvic acid	[1], [2]
	2,3-dichlorobutyric acid	[1]
Terpenoids	dihydroactinidiolide	[1]
	hexahydrofarnesylacetone	[1], [2], [3]
	farnesylaceton	[2]
	geranylaceton	[2]
	phytol	[2]
Ketones	2-pentanone-4-hydroxy-4-methyl	[1]
	2,5-hexanedione	[1]
	2-pentadecanone, 6,10,14-trimethyl	[1]
	2-butanone	[1]
Esters	isopropyl myristate	[1]
Ethers	diphenyl ether	[1]
Hydrocarbons	heptadecane	[1]
	octadecane	[1]
	pentadecane, 2,6,10,14 tetramethyl-	[1]
	cyclohexane, undecyl-	[1]
	nonadecene	[1]
Ionons	β-ionon	[2], [3]
Phenols	2,2'methylenebis (4-methyl-6-tert-butylphenol)	[2]
Ammonium-Ions	charamine	[5]

(b) Known polar substances of Characeae		
Sulphuric compounds	4-methylthio-1,2-dithiolane (Charatoxin)	[2], [4]
	5-methylthio-1,2,3-trithiane	[2], [4]
Nucelosids	uridin	[2]
	adenosin	[2]
	thymidin	[2]
	guanosin	[2]
Amino acids	L-tryptophan	[2], [3]

Gross et al. (2007) compared several approaches to detect the allelopathic effects of submerged aquatic macrophytes (including Characeae) on phytoplankton and discussed pros and contras of these methods. Anthoni et al. (1986) and Ortner (2012) used HPLC (high performance liquid chromatography) and NMR (nuclear magnetic resonance) spectroscopy to identify the antibiotic substance charamin. Today HPLC and GC-MS (gas chromatography and mass spectrometry) are commonly used for identifying allelochemicals (Bankova et al. 2001, Berger & Schagerl 2003, Gao et al. 2011, Doblander 2013, Jiang et al. 2014, Kurashov et al. 2014) and are often combined with bioassays. Water or methanol (MeOH) is often used for the extraction of allelochemicals (Berger & Schagerl 2003, Berger & Schagerl 2004, Gross et al. 2007, Gao et al. 2011, Ye et al. 2014).

We aimed to identify intra- and interspecific allelopathic activities in four Characean species. To consider site-specific differences, each species was sampled from two water bodies. We searched for intergeneric differences of target organisms, which were photoautotrophic eukaryotes and prokaryotes and heterotrophic prokaryotes (possible antibiotic effects). We considered both polar and non-polar *Chara* substances and tested two drying methods (air drying at 30 °C and lyophilisation at -100 °C). To our knowledge, this is the first study, which also included potential effects of volatile substances: we collected ice which condensed during the lyophilisation process and tested also ice extracts for allelopathic activity.

4.) Material and methods

4.1. Collection, drying and treatment of live Chara

Four *Chara* species, each from two sampling sites, were chosen to check for their allelopathic activity (Table 2). According to screening tests of Berger & Schagerl (2004), *C. tomentosa* Linnaeus and *C. rudis* (Braun) Leonhardi showed lower allelopathic activities than *C. globularis* Thuillier and *C. aspera* Willdenow.

Table 2: Sampling sites, geographic coordinates and date of collection of the different *Chara* species.

Species	Date of collection	Geographic coordinates
Chara globularis		
I. Obere Drau	29.06.2014	46°49'20.63"N; 13°23'39.34"E
II. Botanischer Garten	05.06.2014	48°11'38.56"N; 16°23'00.53"E
Chara tomentosa		
I. Lake Attersee	22.05.+ 27.07.2014	47°48'13.88"N; 13°29'22.45"E
II. Lake Neusiedlersee	02.07.2014	47°45'45.43' N; 16°45'12.56"E
Chara aspera		
I. Lake Millstättersee	28.06.2014	46°46'24.16"N; 13°38'32.06"E
II. Lake Attersee	27.07.2014	47°52'56.59"N; 13°31'54.00"E
Chara rudis		
I. Lake Lunzer Untersee	19.06.+ 30.07.2014	47°51'08.75"N; 15°03'30.10"E
II. Lake Erlaufsee	22.06.+ 30.07.2014	47°47'22.31"N; 15°16'34.26"E

Fig. 1: Sampling site of *Chara tomentosa* in the Lake Attersee, Unterach, Upper Austria. Photo provided by Steger J.

Fig. 2: Sampling site of *Chara aspera* in the Lake Attersee, Nußdorf, Upper Austria. Photo provided by Mähnert B.

From each sampling site (examples: Fig. 1 and Fig. 2), several kg of fresh algae were collected either by hand (wading) or snorkelling (1-4 m water depth). The collected stoneworts were identified under a Motic SMZ-168 binocular microscope according to the identification key of Krause (1997). Some *Chara* shoots were treated with citric acid or vinegar for removing calcareous layers on the cortex.

Two different drying methods were applied to compare the stability of the allelochemicals (Fig. 3). The fresh material was divided into two parts, carefully rinsed with tap water and slightly "centrifuged" with a salad spinner to get rid of attached water. One part was prepared for the freeze-dryer (Zirbus technology VaCo 2-E; -100 °C), the other part was dried in a drying cabinet at 30 °C (Binder, Art.No.: 9120-0073; 50 % ventilator-speed). Before starting lyophilisation, the material was placed in a beaker glass, weighed on a A&D electronic balance FY-2000 for fresh mass and frozen for several h in a -80 °C freezer. Before and between lyophilisation, the freeze-dryer was washed with several litres of Milli-Q-water to avoid contamination.

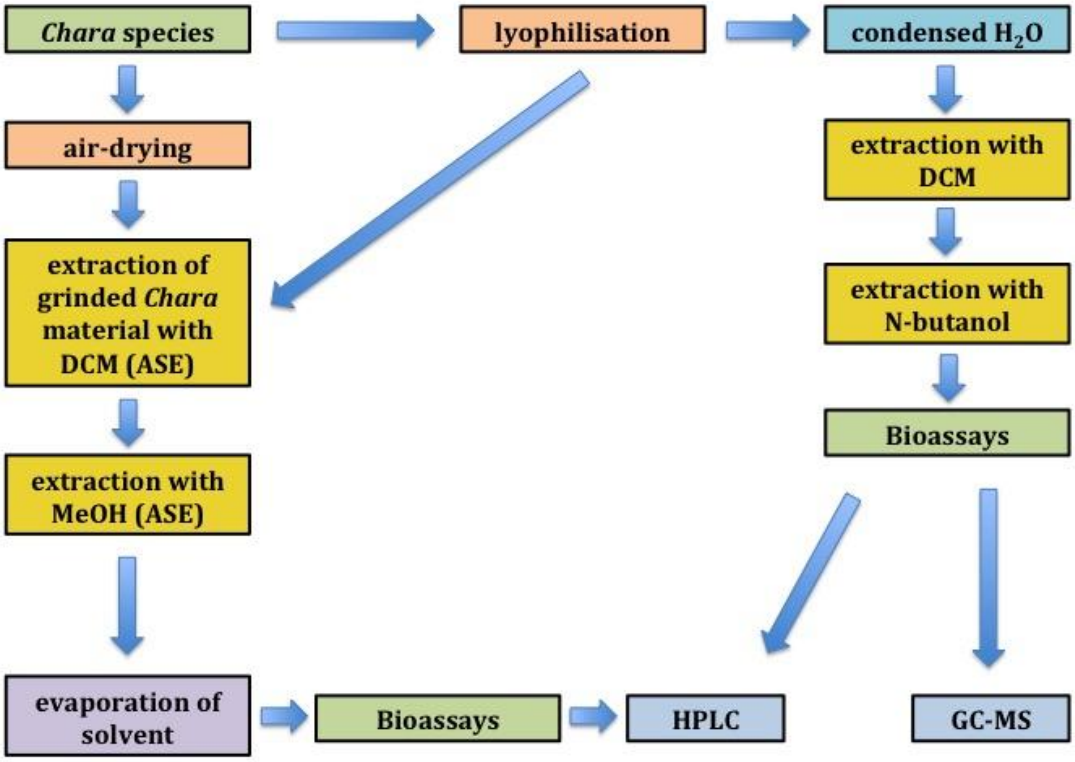

Fig. 3: Workflow for extractions. ASE = Accelerated solvent extraction machine, DCM = dichloromethane, GC-MS = gas chromatography combined with mass spectrometry, HPLC = high performance liquid chromatography, MeOH = methanol.

After drying, tightly attached epizoa (e.g. *Dreissena* or *Bithynia*) were removed. The dry material was then homogenised with a mortar, filled into paper bags, weighed (Sartorius LC 4801 P balance) for dry mass and stored in the -80 °C freezer with a desiccant in plastic boxes. Ice from the freeze drier was molten, collected and stored in 50 ml Greiner tubes in the -80 °C freezer (Dometic Medical Systems UF 755 G) until use for further experiments.

4.2. Analysis of ash mass

For determining the ash mass (inorganic fraction), 1 to 2 g of dried material were combusted in a muffle furnace (450 °C for 6 h; Kittec XR Lehrer Thermocomputer TC 507).

4.3. Extraction with methanol (MeOH), *dichloromethane* (DCM) *and N-butanol*

DCM (VWR, Prolabo, GPR Rectapur, No.: 25631.362) was used for extracting non-polar substances, MeOH (p.a. quality) was taken for extracting polar compounds by means of an accelerated solvent extractor (Dionex ASE 200). 5-13 g of the respective species were filled into cartridges and extracted twice with 30 ml of DCM followed by twice extractions with 30 ml of MeOH of the same sample at room temperature. The two extracts with the same solvent were combined and evaporated in an evaporator (Heidolph VV2011 with water bath WB2001; MeOH: 40 °C, 180-200 mbar; DCM: 40 °C, 600-650 mbar).

Ice from the freeze-drier was thawed and extracted in a first step with DCM for non-polar substances (ratio 1:1) followed by a second step with N-butanol (p.a. quality) for polar substances. DCM was then removed in a Heidolph evaporator, N-butanol in a GeneVac EZ-2-Series Personal evaporator (program: Medium BP, 40 °C). The extract crops were weighed with an analytical balance (Sartorius BP210D) and stored in an exsiccator with desiccant material. The extract concentrations were referred to the organic mass of *Chara* material. For further experiments and analyses, N-butanol ice extracts were re-dissolved in MeOH (p.a. quality). In total 48 different extracts were available for further experiments (Table 3).

Table 3: On all four *Chara* species, two sampling sites each, the following six extraction types were applied. Altogether 48 extracts were available for further tests. MeOH = methanol (polar extracts), DCM = dichloromethane (non-polar extracts).

***Chara* species**	**Obtained extracts**
Chara aspera (Lakes Attersee & Millstättersee) *Chara globularis* (Botanischer Garten & Obere Drau) *Chara rudis* (Lakes Erlaufsee & Lunzer Untersee) *Chara tomentosa* (Lakes Attersee & Neusiedlersee)	Lyophilised dry material extracted with DCM
	Lyophilised dry material extracted with MeOH
	Ice from lyophilisation extracted with DCM
	Ice from lyophilisation extracted with N-butanol
	Air dried material extracted with DCM
	Air dried material extracted with MeOH

4.4. Bioassays with living Chara shoots and extracts

Four different microalgae strains were used as target organisms (Table 4). The strains provided homogenous growth on both solidified incubated agar plates and in liquid media. Cultures were grown in 3NBBM and BG11 media in a light-dark cycle of 16 to 8 h (15 µmol photons m^{-2} s^{-1} PAR) in a climate chamber at 18 °C (http://www.uni-goettingen.de/en/list-of-media-and-recipes/186449.html). All bioassays described in 4.4. were performed in petri dishes on solidified agar.

Table 4: Target organisms for testing the allelopathic activity of living stonewort shoots.

Target species	Group	Culture media (autoclaved at 120 °C)	Origin of culture
Chlorella vulgaris BEIJERINCK	Chlorophyta	3NBBM	SAG 211-11b, Algensammlung Universität Göttingen
Scenedesmus acuminatus (LAGERHEIM) CHODAT	Chlorophyta	3NBBM	ASW 05027, Algenkultursammlung Phycology Vienna (Kusel-Fetzmann & Schagerl 1992)
Synechococcus elongatus NÄGELI	Cyanobacteria	BG11	SAG 89.79, Algensammlung Universität Göttingen
Synechococcus leopoliensis (RACIBORSKI) KOMÁREK	Cyanobacteria	BG11	SAG 1402-1, Algensammlung Universität Göttingen

Healthy *Chara* shoots were cut off, carefully rinsed with distilled water and checked for epizoa under the microscope (Motic SMZ-168 binocular). For each *Chara* sampling site and target species, triplicates plus one control (inoculated only with the target algae) were prepared in three steps by using plastic petri dishes according to Berger & Schagerl (2004): first, a layer with 1 % agar (Fluka Analytical, BioChemika 05039-500G; Lot# 0001438982; Milli-Q water) was prepared for each petri dish. Then, an agar medium with 0.2 % agar (3NBBM for *Scenedesmus acuminatus* and *Chlorella vulgaris*, BG11 for *Synechococcus elongatus* and *Synechococcus leopoliensis*) was prepared, cooled down to 30°C and inoculated with a few ml of the target algae stock. This medium was immediately filled onto the basic agar layer. Finally, one *Chara* shoot per petri dish was placed onto the inoculated second agar layer and pressed down carefully. The petri dishes were tightened with parafilm to avoid drying, placed in the culture room and kept at 18 °C, 16 hours light, 8 hours dark at 30 µmol photons m^{-2} s^{-1} PAR. Photos of petri dishes were taken with a Sony Cybershot DSC-TX20 after 14 and 21 d of growth time, respectively.

The intensity of inhibition (live material and extracts) of the bioassays was defined by the size of the surrounding clearance zone and is listed in Table 5. The effects were categorised

according to the strength of inhibition –, +, ++ and +++ (no inhibition, weakest to strongest). Due to the different experimental design of the *Vibrio fischeri* assays, inhibition was defined as a > 20 % decrement in bioluminescence after addition of *Chara* extracts. For images of inhibition categories, which are shown in Table 5, see Figures 12-18 in 5.1.

No assays with live material and *Vibrio fischeri* were conducted due to the bioluminescence method, where only clear solutions can be measured.

Tab. 5: Definition of the inhibition strength of the bioassays.

Inhibition	**Bioassays with living *Chara* shoots**	**Bioassays with *Chara* extracts**
–	No inhibition of microalgae growth	No inhibition of microalgae growth
+	Small inhibition zone around shoot (ca. 1/3 of petri dish area)	Inhibition zone < 1.5 mm
++	About 2/3 of the petri dish area showed no microalgal growth around the shoot	Inhibition zone 1.5 ≤ 5.0 mm
+++	No microalgal growth observable	Inhibition zone > 5.0 mm

For testing the allelopathic activity of the *Chara* extracts, sterile glass fibre filter discs were used (6 mm in diameter, Munktell MG-C). Target cultures were prepared as described above. Polar extracts, including the N-butanol ice extracts, were re-dissolved in MeOH (p.a.) and non-polar extracts in DCM (p.a.) to a concentration of 5 mg extract ml^{-1} solvent by sonication for 2 min.

For each of the 48 *Chara* extracts, triplicates were prepared by applying 10 µl of the re-dissolved extract on the filters with a 500 µl Hamilton syringe. The filters were dried overnight in an exsiccator with desiccant material for complete removal of the solvents. Additionally, one filter without any extract or solvent and one treated with DCM or MeOH only (10 µl of each) were used as negative controls. Filters were placed with tweezers onto the second agar layer and each petri dish was closed with parafilm. Photos were taken as described above; inhibition zones were measured by using the software ImageJ64 (Rasband 1997-2014).

4.5. Extract tests on heterotrophic, marine, bioluminescent bacterium (Vibrio fischeri)

A complete LumiStox test-kit (LCK 480; Dr. Bruno Lange GmbH & Co KG, Düsseldorf, Germany) was applied as a standardized testing procedure in the laboratories of the Institute for Environmental Biotechnology (BOKU – IFA Tulln). The whole experimental setting

followed the instructions of the manufacturer. All 48 extracts were re-dissolved in MeOH and tested at a concentration of 5 mg extract ml^{-1} solvent. Before the assays took place, all extracts were centrifuged for 5 min with 1340 rpm by using an Eppendorf miniSpin for avoiding disturbances of this optical measurement by floating particles. 20 µl of each solution and a control of 20 µl pure MeOH were transferred into the glass cuvettes of the LumiStox-instrument and the solvents evaporated at room temperature. Positive and negative controls were included for each testing set. An incubation time of 30 min was chosen after adding *Vibrio fischeri* to the extracts.

4.6. PAM-measurements (pulse amplitude modulated fluorescence)

For testing potential effects on photosystem II (PS II) of target organisms, a Waltz measuring device was used (PAM-2500/US, ED-101US/MD, PAM-2500/US/D, PAM-2500/US/ER; software: PAMWin, Walz – Mess- und Regeltechnik, Effeltrich, Germany). The maximum quantum dark yield decreases, if an extract inhibits the target organism by affecting the PS II complex. The calculation for the maximum quantum dark yield of PS II followed the formula $F_v/F_m = (F_m - F_0)/F_m$ after Maxwell & Johnson (2000). F_m is the maximum dark fluorescence, F_0 the minimum and F_v the variable fluorescence.

60 µl of MeOH extracts (concentration 5 mg extract ml^{-1} solvent) which showed inhibitory effects in the bioassays, was evaporated in glass tubes in an exsiccator. Target algae were dark-adapted for at least 15 min. Then 3 ml of culture solution were added to the dried extracts. For better dilution of the extracts the glass tubes were carefully shaken. Triplicates were prepared for each target organism. PAM-measurements were taken at 5, 15, 30, 60, 90 and 120 min incubation. As a reference, each target culture was tested without any extract (100 % PS II activity).

4.7. Chromatographic analyses

Thin-layer chromatography (TLC) was performed for a first overview of the intra- and interspecific similarity of the *Chara* extracts. Silica plates (Merck Analytical chromatography; TLC Silica gel 60 F_{254}; aluminium sheets 20 x 20 cm; No.: 1.05554.0001) were used as stationary phase. All 48 extracts were re-dissolved in MeOH (polar extracts) or DCM (non-polar extracts) to a concentration of 5 mg extract ml^{-1} solvent. 4 µl of each re-dissolved extract were spotted on the silica plate. Two eluent systems and two spraying reagents were used (Table 6). Additionally, two flavonoid markers, rutin for system A and

quercetin for system B, were used for comparison. Photos of the TLC plates were taken with a Sony α NEX-3 under UV_{366nm} and visible light.

Table 6: Eluents and spraying reagents in TLC. System A for polar and system B for non-polar extracts after Wagner & Bladt (2001). Spraying reagents were prepared after Csontala (2004); MeOH = methanol.

	System A (polar)	**System B (non-polar)**
Eluents	Ethyl acetate-methanol-water (75:11:10)	Toluene-ethyl acetate (93:7)
Flavonoid marker	Rutin (0,5 mg ml^{-1} MeOH)	Quercetin (0,5 mg ml^{-1} MeOH)
Spraying reagents	1.) Anisaldehyde-sulphuric acid: heating at 100 °C for 5-10 min - Detection: daylight 2.) Natural product reagent/Polyethyleneglycol - Detection: UV_{366nm}; for phenolic substances	

All extracts were analysed by means of high performance liquid chromatography (HPLC) with specific gradients for polar and non-polar extracts (Table 7) on a Shimadzu HPLC (Autosampler Sil-20AC HT, Parallel Double Micro Plunger Type LC-20AD, Prominence degasser DGU-$20A_5$, HPLC System Controller CBM-20A, HPLC Photo Diode Array Detector SPD-M20-A, Column oven CTO-20AC). All extracts were re-dissolved in MeOH by sonication and analysed at a concentration of 5 mg extract ml^{-1} solvent. Before injection, the extracts were centrifuged for 5 min with 1340 rpm by using an Eppendorf miniSpin in order to avoid clogging of the HPLC instrument caused by remaining particles. The results were analysed with the software HPLCsolution (Shimadzu, Kyoto, Japan).

Table 7: HPLC conditions for the analysis of polar and non-polar extracts.

	Polar extracts Modified after Doblander (2013)	**Non-polar extracts**
Column	Phenomenex Synergi Hydro RP, particle size: 4 µm, 150 x 3 mm, pore size: 80Å	Dionex Acclaim 120, C18, particle size: 3 µm, 150 x 2,1 mm, pore size: 120Å
Solvent A	Water	Water (acidified to pH = 3 with formic acid)
Solvent B	Acetonitrile	Acetonitrile

Gradient	0-5 min: 5 % solvent B 5-10 min: 5 to 25 % solvent B 10-11 min: 25-95 % solvent B 11-15 min: 95 % solvent B 15-16 min: 95-5 % solvent B 16-20 min: 5 % solvent B	0-60 min: 0 to 100 % solvent B 60-70 min: 100 % solvent B 70-71 min: 100-0 % solvent B 71-80 min: 0 % solvent B
Oven temperature	30 °C	25 °C (room temperature)
Flow rate	0.4 ml min^{-1}	0.5 ml min^{-1}
Concentration of extracts	5 mg ml^{-1}	5 mg ml^{-1}
Injection volume	5 µl	20 µl
Detector	UV detector, D2 lamp (Shimadzu SPD-M20)	UV detector, D2 lamp (Shimadzu SPD-M20)

For detecting volatile substances, allelopathically active N-butanol ice extracts and DCM ice extracts of all four *Chara* species were analysed by means of gas chromatography and mass spectrometry (GC-MS) on a Shimadzu GC-2010 gas chromatograph-Shimadzu GCMS-QP2010 (Table 8). The extracts were re-dissolved in DCM, centrifuged at 1340 rpm (Eppendorf miniSpin) and analysed at a concentration of 5 mg extract ml^{-1} solvent. Results were analysed with the software GCMSsolution (Shimadzu, Kyoto, Japan). Two libraries (WILEY229.LIB, NIST147.LIB) and the Kovats retention index were used for characterisation.

Table 8: Conditions for the GC-MS-analyses.

Column	Phenomenex, Zebron ZB-5, GC Capillary Column, 60 m x 0.25 mm x 0.25 µm
Carrier gas	Helium
Total flow of carrier gas	20.2 ml min^{-1}
Injection volume	1 µl
Pressure (GC)	187.7 kPa

Gradient (GC)	0-90 min: 60-330 °C 90-100 min: 330 °C
Solvent cut time (GC)	6 min
Temperature increase per min (GC)	3 °C
Ion source temperature (MS)	250 °C
Interface temperature	270 °C

4.8. Data analysis

Data were analysed using the software package Sigma Plot 11.0 and 12.0 (Systat Software, San Jose, CA, USA), ImageJ 1.46r (Rasband 1997-2014), Microsoft Excel 2011 and PowerPoint 2011, Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA) and GNU Image Manipulation Program 2.8.14 – GIMP, Kimball et al. (1995-2014).

5.) Results

5.1. Bioassays and Vibrio fischeri tests

The polar ice extracts showed the highest potential for inhibitions followed by shoots (Table 9). Polar MeOH and N-butanol extracts resulted in stronger allelopathic activity than the non-polar DCM extracts. As only one DCM extract showed inhibition (Table 9), further experiments focused on MeOH extracts of dry material (Fig. 5). For results of HPLC and GC-MS-analyses see 5.4. and 5.5.

Vibrio fischeri was the most sensitive target organism showing an inhibition in more than 50 % of the total experiments followed by Cyanobacteria; eukaryotic algae showed lowest allopathic effects (Table 9; Fig. 4 and 5). The bioassays indicated only little interspecific differences of *Chara*. When comparing the two sampling sites, *Chara globularis* and *Chara rudis* showed similar allelopathic activities; *Chara aspera* and *Chara tomentosa* resulted in greater differences (Table 9).

The seven most active extracts tested at 20 µl for bioluminescence inhibition were additionally run at a volume of 100 µl to collect information about dose-dependence. Despite adding the fivefold amount of extracts, bioluminescence inhibition was only twice as high as in the previous experiments with 20 µl (data not shown).

Tab 9.: Summary of all tests (bioassays with living material, extracts and *Vibrio fischeri*; mean of the replicates). Chl. vulg. = *Chlorella vulgaris*, Scen. acum. = *Scenedesmus acuminatus*, Syn. elo. = *Synechococcus elongatus*, Syn. leop. = *Synechococcus leopoliensis*, DCM = dichloromethane, MeOH = methanol, ND = not determined, – = No inhibition of microalgae growth (homogenous growth), + = Inhibition zone < 1.5 mm, ++ = Inhibition zone 1.5 ≤ 5.0 mm, +++ = Inhibition zone of > 5.0 mm. Inhibition in *Vibrio fischeri* assays was defined as a > 20 % decrement in bioluminescence.

***Chara* species**	**Extract**	***Chl. vulg.***	***Scen. acum.***	***Syn. elo.***	***Syn. leop.***	***Vibrio fischeri* [inhibition in %]**
Chara aspera	DCM lyophilised	–	–	–	–	28
Lake	MeOH lyophilised	–	–	+	–	33
Millstättersee	DCM air dried	–	–	–	–	21
	MeOH air dried	–	–	–	–	27
	DCM ice	–	–	–	–	5
	N-butanol ice	–	+++	+	++	26
	Living *Chara* shoots	–	–	++	+++	ND
Chara aspera	DCM lyophilised	–	–	–	–	23
Lake Attersee	MeOH lyophilised	–	–	+	–	19
	DCM air dried	–	–	–	–	21
	MeOH air dried	–	–	–	–	30
	DCM ice	–	–	–	–	4
	N-butanol ice	–	–	+	–	3
	Living *Chara* shoots	–	–	+	+++	ND
Chara	DCM lyophilised	–	–	–	–	22
globularis	MeOH lyophilised	–	–	–	–	8
Botanischer	DCM air dried	–	–	–	–	21
Garten	MeOH air dried	–	–	–	–	14
	DCM ice	–	–	–	–	11
	N-butanol ice	–	+++	++	+++	27
	Living *Chara* shoots	–	–	++	+++	ND
Chara	DCM lyophilised	–	–	–	–	30
globuaris	MeOH lyophilised	–	–	–	–	7
Obere Drau	DCM air dried	–	–	–	–	26
	MeOH air dried	–	–	–	–	33
	DCM ice	–	–	–	–	9
	N-butanol ice	–	+++	+	+	30

	Living *Chara* shoots	–	–	++	+++	ND
Chara tomentosa **Lake Attersee**	DCM lyophilised	–	–	–	–	21
	MeOH lyophilised	–	–	+	+++	19
	DCM air dried	–	–	–	–	17
	MeOH air dried	–	–	–	–	25
	DCM ice	–	–	–	–	7
	N-butanol ice	–	+++	+++	++	19
	Living *Chara* shoots	–	–	+	+++	ND
Chara tomentosa **Lake Neusiedlersee**	DCM lyophilised	–	–	–	–	16
	MeOH lyophilised	–	–	–	–	2
	DCM air dried	–	–	–	–	18
	MeOH air dried	–	–	+	–	4
	DCM ice	–	–	–	–	2
	N-butanol ice	–	+++	+	+	27
	Living *Chara* shoots	–	–	+	++	ND
Chara rudis **Lake Lunzer Untersee**	DCM lyophilised	–	–	–	–	28
	MeOH lyophilised	–	–	–	–	27
	DCM air dried	–	–	–	–	23
	MeOH air dried	–	–	–	–	23
	DCM ice	–	–	–	–	11
	N-butanol ice	–	+++	+	++	19
	Living *Chara* shoots	–	–	++	+++	ND
Chara rudis **Lake Erlaufsee**	DCM lyophilised	–	–	–	–	30
	MeOH lyophilised	–	–	–	–	29
	DCM air dried	–	–	–	–	24
	MeOH air dried	–	–	–	–	13
	DCM ice	–	–	–	++	24
	N-butanol ice	–	+++	+	+	28
	Living *Chara* shoots	–	–	++	+++	ND

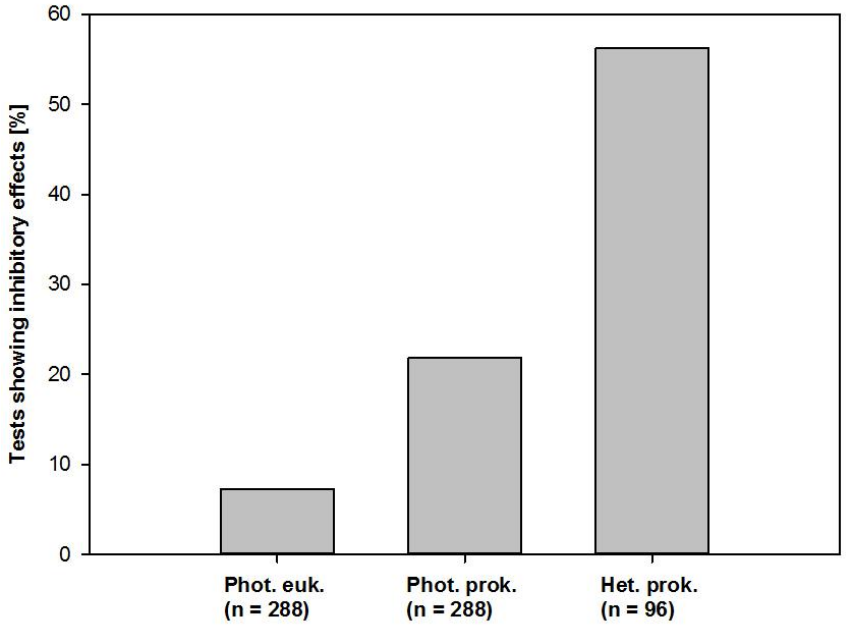

Fig. 4: Proportion of *Chara* extract bioassay tests (in % of total experiments) causing inhibition of target groups (presence/absence of inhibition). n = number of tests including replicates, Phot. euk. = photoautotrophic eukaryotes (*Chlorella vulgaris* and *Scenedesmus acuminatus*), Phot. prok. = photoautotrophic prokaryotes (*Synechococcus elongatus* and *Synechococcus leopoliensis*), Het. prok. = heterotrophic prokaryotes (*Vibrio fischeri*).

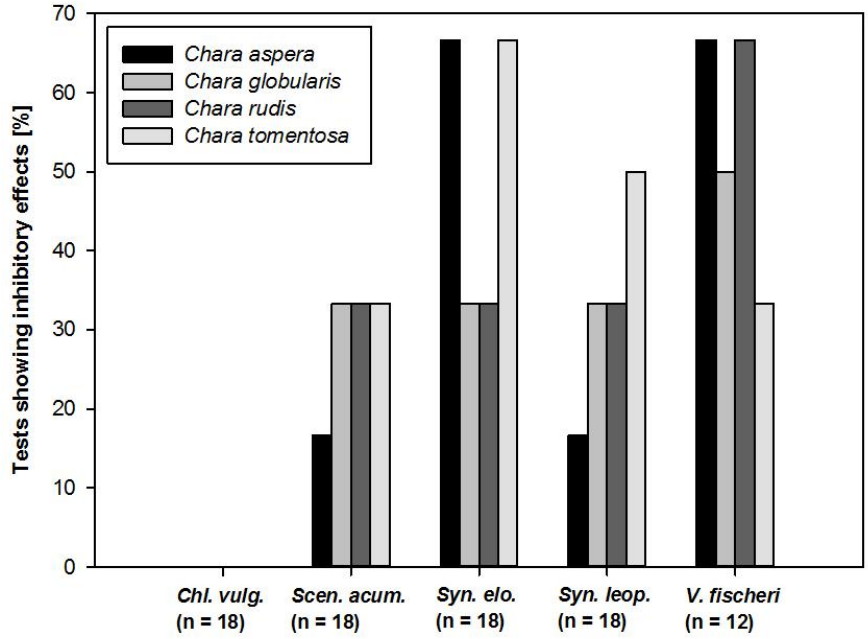

Fig. 5: Inhibitory effects of polar extracts (in % of total experiments) of *Chara* species on the different target organisms (presence/absence of inhibition). Both sampling sites summarised. n = number of tests including replicates, *Chl. vulg.* = *Chlorella vulgaris*, *Scen. acum.* = *Scenedesmus acuminatus*, *Syn. elo.* = *Synechococcus elongatus*, *Syn. leop.* = *Synechococcus leopoliensis*, *V. fischeri* = *Vibrio fischeri*.

The growth of *Chlorella vulgaris* was inhibited neither by living *Chara* shoots nor by their extracts. *Scenedesmus acuminatus* showed a similar sensitivity to all four *Chara* species. *Chara aspera* had the strongest effect on *Synechococcus elongatus* and *Vibrio fischeri*, but less on *Scendesmus acuminatus* and *Synechococcus leopoliensis*. *Chara tomentosa* strongly inhibited *Synechococcus elongatus* and *Synechococcus leopoliensis*. *Chara rudis* also caused strong inhibition of *Vibrio fischeri*.

Vibrio fischeri showed the highest sensitivity to the DCM extracts of dried *Chara* material followed by the polar N-butanol extracts of the lyophilisation ice (less than 20 %; Fig. 6).

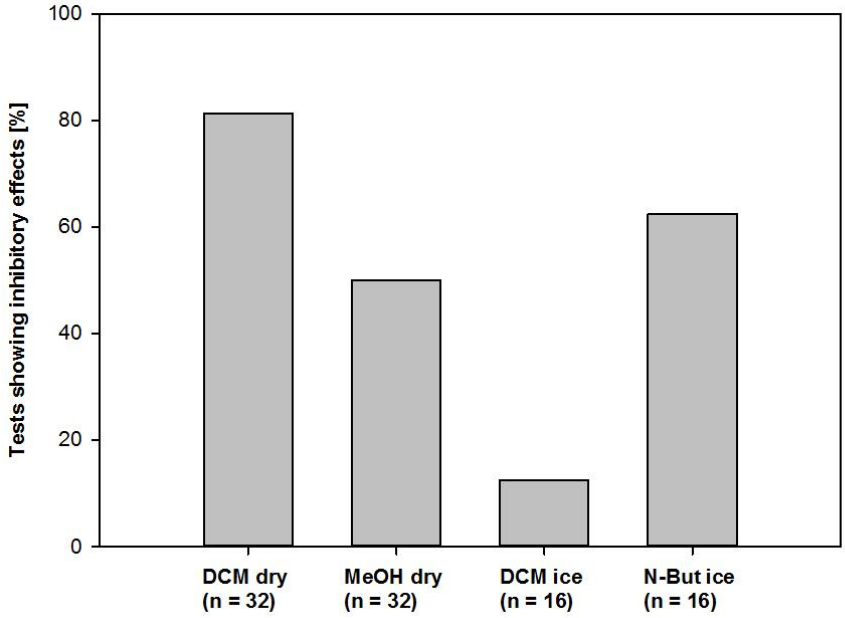

Fig. 6: Inhibition of *Vibrio fischeri* by polar and non-polar *Chara* extracts (in % of total experiments; presence/absence of inhibition). n = number of tests including duplicates, DCM dry = dichloromethane extracts of dried material (lyophilisation and air drying summarised), MeOH dry = methanol extracts of dried material (lyophilisation and air drying summarised), DCM ice = dichloromethane ice extracts, N-But ice = N-butanol ice extracts.

DCM extracts of dried material did not show any inhibition of photoautotrophs (Fig. 7). The N-butanol ice extracts resulted in the strongest inhibition. MeOH extracts of dried material and DCM ice extracts caused inhibitions in less than 10 % of tests (Fig. 7).

Both DCM and MeOH extracts of air dried material showed higher bioluminescence inhibitions of *Vibrio fischeri* than extracts of lyophilised material (Fig. 8).

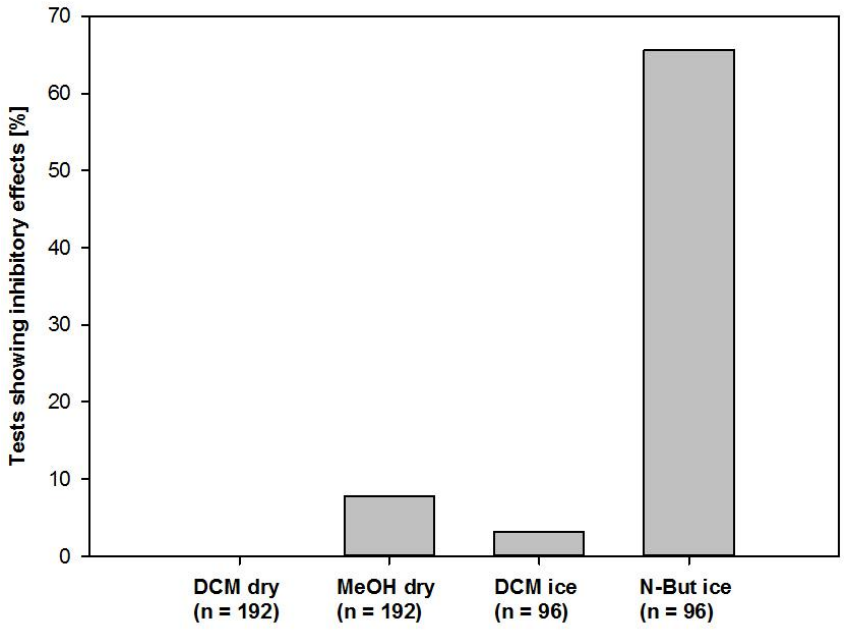

Fig. 7: Inhibition of photoautotrophs (all taxa included) by polar and non-polar *Chara* extracts (in % of total experiments; presence/absence of inhibition). n = number of tests including triplicates, DCM dry = dichloromethane extracts of dried material (lyophilisation and air drying summarised), MeOH dry = methanol extracts of dried material (lyophilisation and air drying summarised), DCM ice = dichloromethane ice extracts, N-But ice = N-butanol ice extracts.

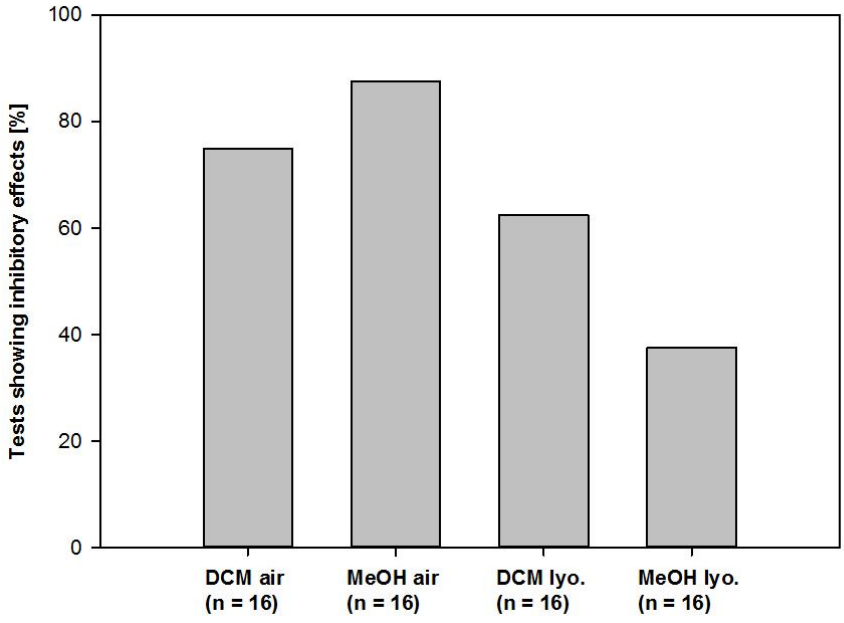

Fig. 8: Comparison of the two drying methods with respect to the inhibitory effects (in % of total experiments; presence/absence of inhibition) on *Vibrio fischeri*. All extract tests, *Chara* species and sampling sites summarised. n = number of tests including duplicates, DCM air = dichloromethane extracts of air dried algae, MeOH air = methanol extracts of air dried algae, DCM lyo. = dichloromethane extracts of lyophilised algae, MeOH lyo. = methanol extracts of lyophilised algae.

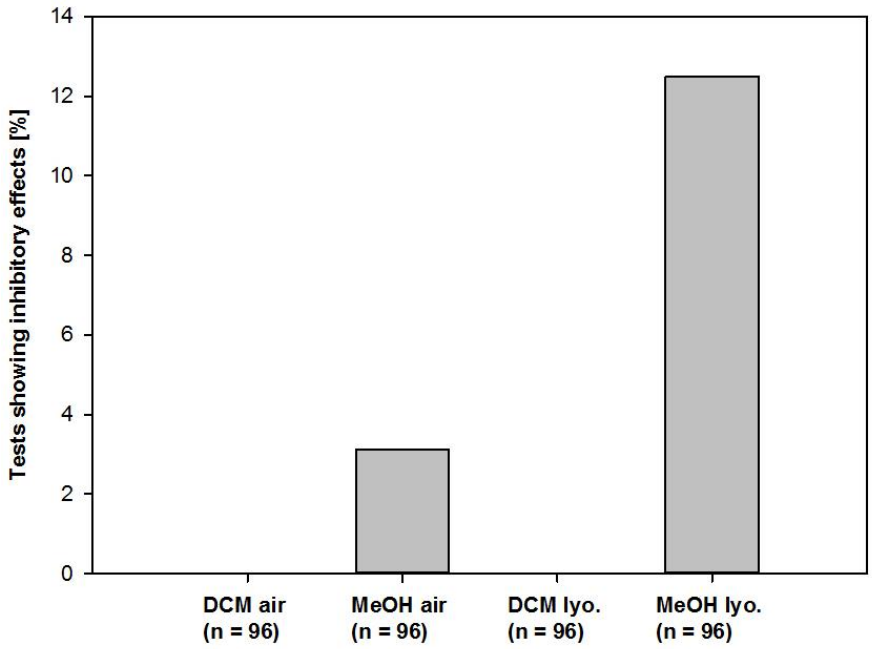

Fig. 9: Comparison of the two drying methods of *Chara* material with respect to the inhibitory effects (in % of total experiments; presence/absence of inhibition) on *Chlorella vulgaris*, *Scenedesmus acuminatus*, *Synechococcus elongatus* and *Synechococcus leopoliensis*. All extract tests, *Chara* species and sampling sites summarised. n = number of tests including triplicates, DCM air = dichloromethane extracts of air dried algae, MeOH air = methanol extracts of air dried algae, DCM lyo. = dichloromethane extracts of lyophilised algae, MeOH lyo. = methanol extracts of lyophilised algae.

Non-polar DCM extracts did not inhibit any of the four photoautotrophic target organisms in the bioassays (Fig. 9). In contrast to the MeOH extracts of air dried *Chara* material the MeOH extracts of lyophilised material showed more than twofold inhibitory activity in the bioassays.

Chara rudis extracts resulted in the highest amount of tests with inhibitory effects (80 %) on the Proteobacterium *Vibrio fischeri*, followed by *Chara aspera* and *Chara globularis* (Fig. 10); extracts of *Chara tomentosa* were least active.

Chara tomentosa caused the highest amount of all inhibitions (almost 20 %) summarised from all tests on photoautotrophic target organisms followed by *Chara rudis* (Fig. 11). *Chara aspera* and *Chara globularis* showed similar amounts of inhibitory effects in the extract bioassays.

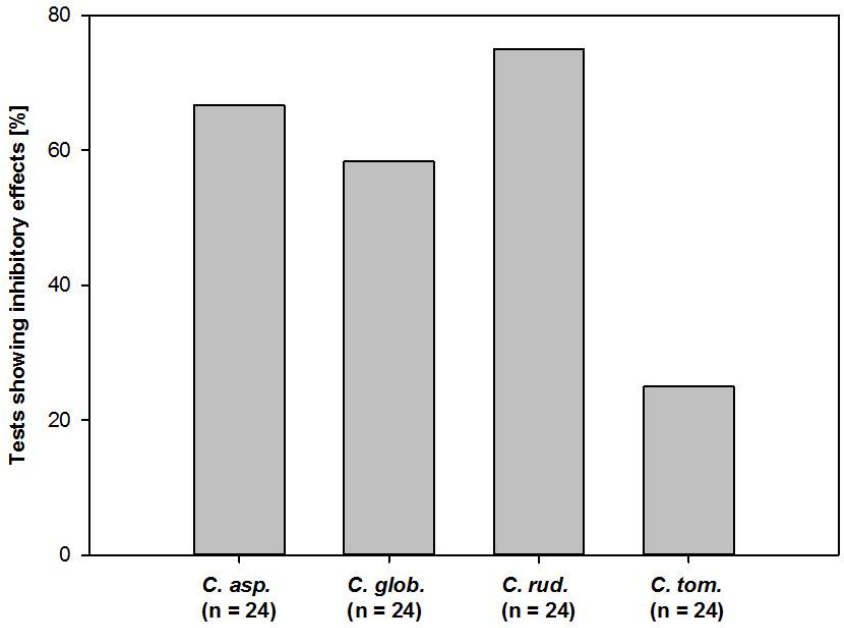

Fig. 10: Inhibitiory effects on *Vibrio fischeri* (in % of total experiments; presence/absence of inhibition). All extracts of all *Chara* species are included. n = number of tests including duplicates, *C. asp.* = *Chara aspera*, *C. glob.* = *Chara globularis*, *C. rud.* = *Chara rudis*, *C. tom.* = *Chara tomentosa*.

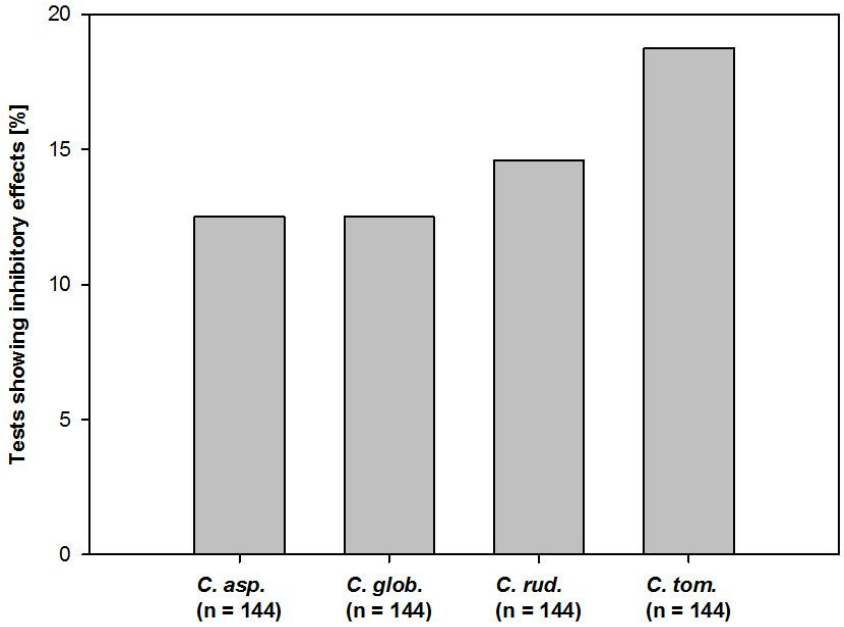

Fig. 11: Inhibitory effects on *Chlorella vulgaris*, *Scenedesmus acuminatus*, *Synechococcus elongatus* and *Synechococcus leopoliensis* (in % of total experiments; presence/absence of inhibition). All extracts of all *Chara* species are included, target organisms are summarised. n = number of tests including triplicates, *C. asp.* = *Chara aspera*, *C. glob.* = *Chara globularis*, *C. rud.* = *Chara rudis*, *C. tom.* = *Chara tomentosa*.

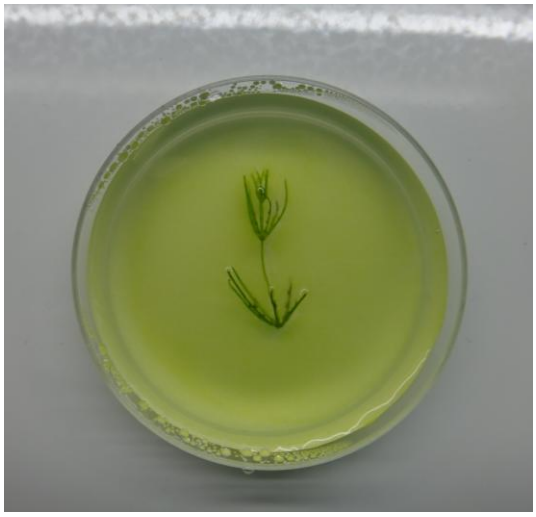

Fig. 12: Example of bioassay and inhibition category –. Living shoot of *Chara globularis* (Botanischer Garten) tested on *Chlorella vulgaris* showing no inhibition zone.

Fig. 13: Example of bioassay and inhibition category +. Living shoot of *Chara globularis* (Obere Drau) tested on *Synechococcus elongatus*.

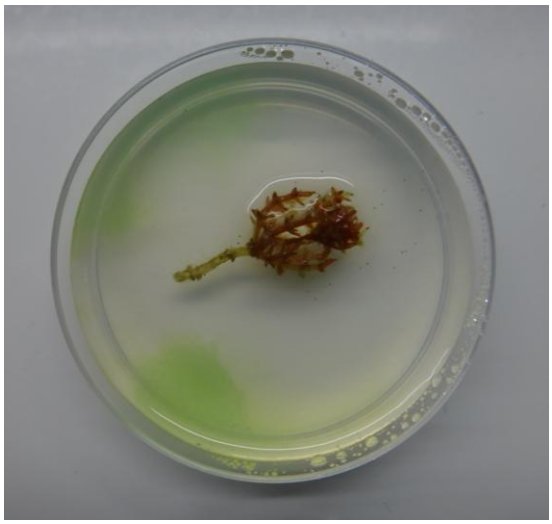

Fig. 14: Example of bioassay and inhibition category ++. Living shoot of *Chara tomentosa* (Lake Neusiedlersee) tested on *Synechococcus leopoliensis*.

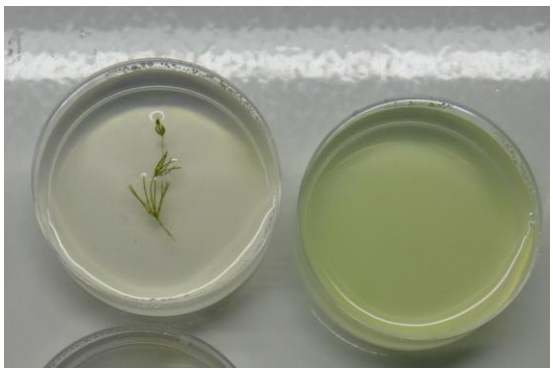

Fig. 15: Example of bioassay and inhibition category +++. Living shoot of *Chara aspera* (Lake Millstättersee) tested on *Synechococcus leopoliensis* on the left side and the negative control on the right side.

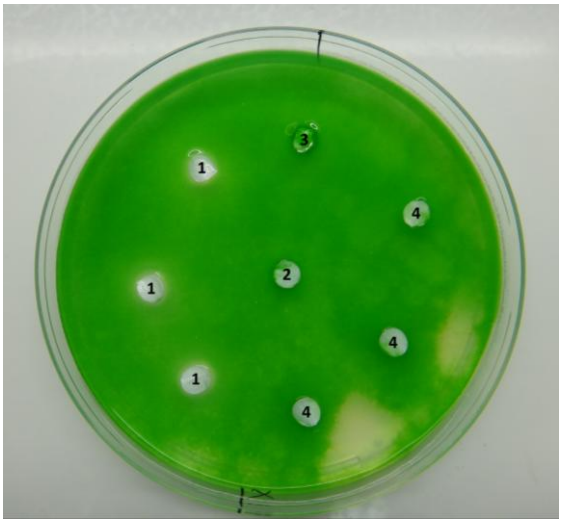

Fig. 16: Polar extracts of lyophilised *Chara globularis* (Obere Drau) tested on *Synechococcus leopoliensis*. 1 = N-butanol ice extract of lyophilisation (inhibition intensity = +), 2 = filter without any extract or solvent (MeOH), 3 = filter with evaporated MeOH, 4 = MeOH extract of lyophilised algae (inhibition intensity = –).

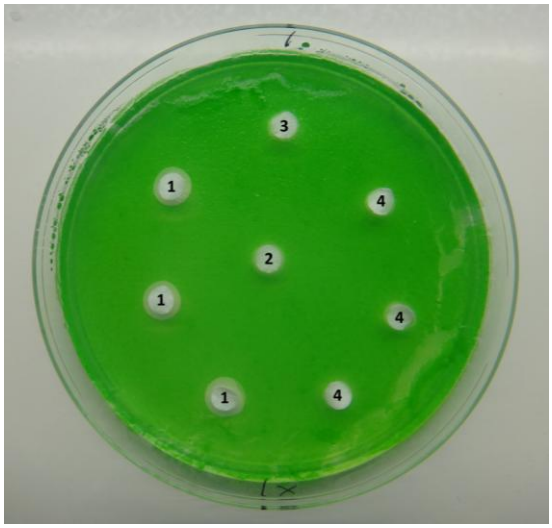

Fig. 17: Polar extracts of lyophilised *Chara globularis* (Botanischer Garten) tested on *Synechococcus elongatus*. 1 = N-butanol ice extract of lyophilisation (inhibition intensity = ++), 2 = filter without any extract or solvent (MeOH), 3 = filter with evaporated MeOH, 4 = MeOH extract of lyophilised algae (inhibition intensity = –).

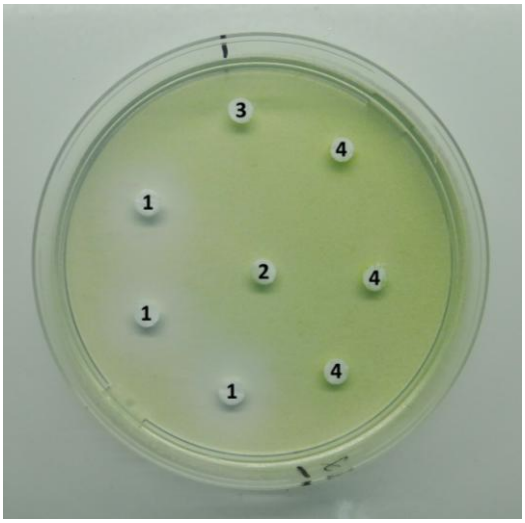

Fig. 18: Polar extracts of lyophilised *Chara rudis* (Lake Erlaufsee) tested on *Scenedesmus acuminatus*. 1 = N-butanol ice extract of lyophilisation (inhibition intensity = +++), 2 = filter without any extract or solvent (MeOH), 3 = filter with evaporated MeOH, 4 = MeOH extract of lyophilised algae (inhibition intensity = –).

Figures 12-15 represent examples of each inhibition category -, +, ++ and +++ in bioassays with living *Chara* shoots. Figures 16-18 show bioassays with *Chara* extracts and the detected inhibition categories -, +, ++ and +++.

5.2. PAM-measurements

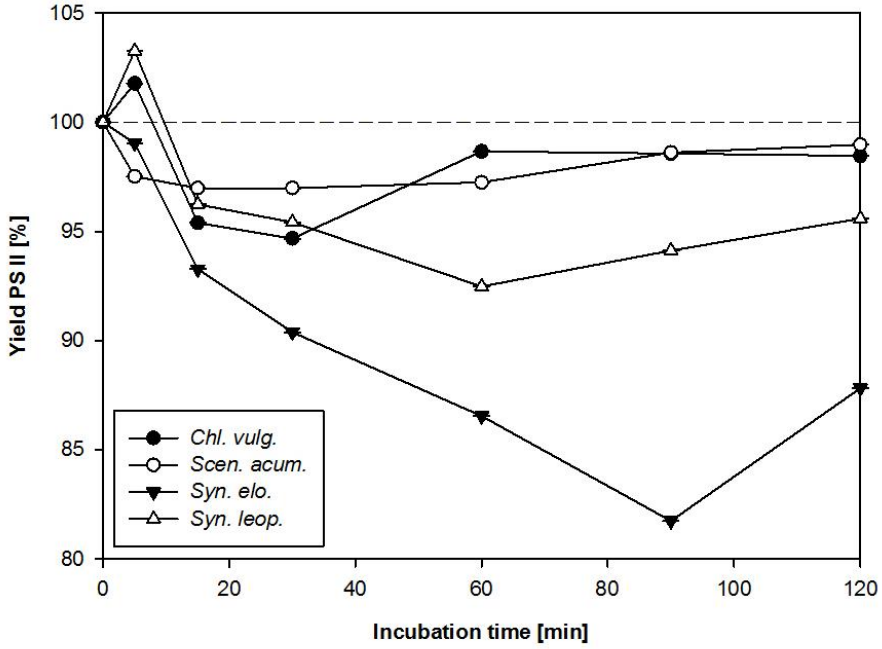

Fig. 19: PAM (Pulse amplitude modulated fluorescence) measurements of MeOH extracts of lyophilised *Chara tomentosa* (Lake Attersee). This extract was chosen due to inhibitory effects in bioassay experiments. Mean maximum quantum dark yield of PS II (triplicates) is given as percentage of the initial quantum yield (dashed line = 100 %) before incubating the target organisms with the *Chara* extract. *Chl. vulg.* = *Chlorella vulgaris*, *Scen. acum.* = *Scenedesmus acuminatus*, *Syn. elo.* = *Synechococcus elongatus*, *Syn. leop.* = *Synechococcus leopoliensis*, Yield PS II = maximum quantum dark yield of photosystem II.

Figure 19 shows a steep decline of the maximum quantum dark yield (afterwards referred to as "yield") of all four target organisms within the first 20 min after incubation with the *Chara* extract (concentration of 5 mg ml^{-1}). After 15 to 30 min incubation, the yield of *Chlorella vulgaris*, *Scenedesmus acuminatus* and *Synechococcus leopoliensis* remained quite constant. *C. vulgaris* regenerated again after 30 min and *S. leopoliensis* after 60 min, respectively. The yield of *Synechococcus elongatus* decreased within the first 90 min followed by subsequent, but incomplete recovery.

5.3. TLC experiments

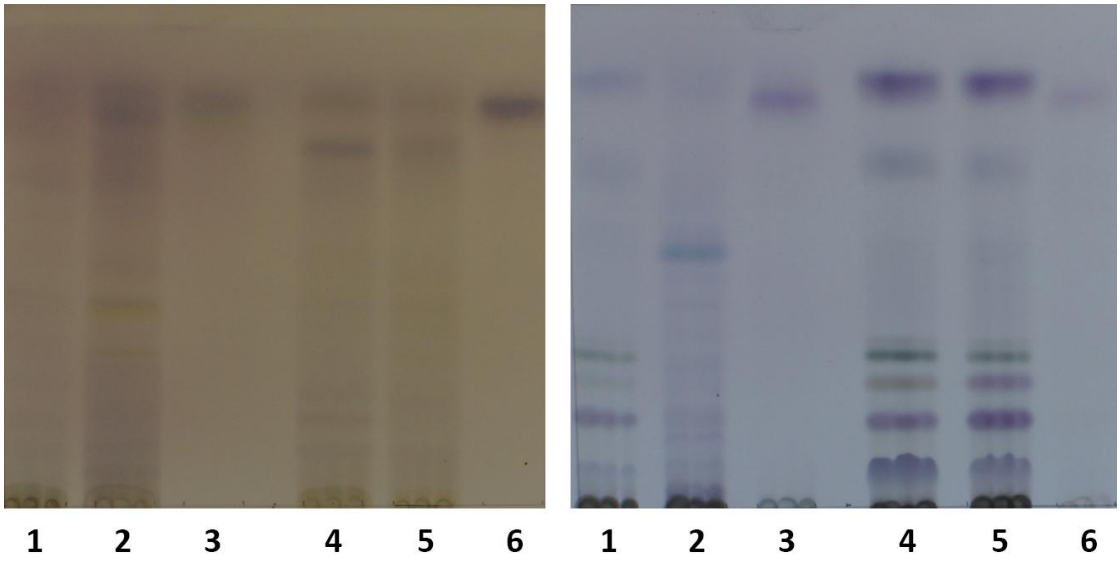

Fig. 20: Example of thin-layer chromatograms of *Chara aspera* extracts under visible light. Detection: anisaldehyde-sulphuric acid reagent and heating at 100 °C for 5-10 min. Left: DCM extracts in system B (Table 6); right: MeOH and N-butanol extracts in system A (Table 6). 1 = MeOH extracts of lyophilised algae from Lake Millstättersee, 2 = MeOH extract of air dried algae from Lake Millstättersee, 3 = N-butanol ice extract from Lake Millstättersee, 4 = MeOH extracts of lyophilised algae from Lake Attersee, 5 = MeOH extract of air dried algae from Lake Attersee, 6 = N-butanol ice extract from Lake Attersee.

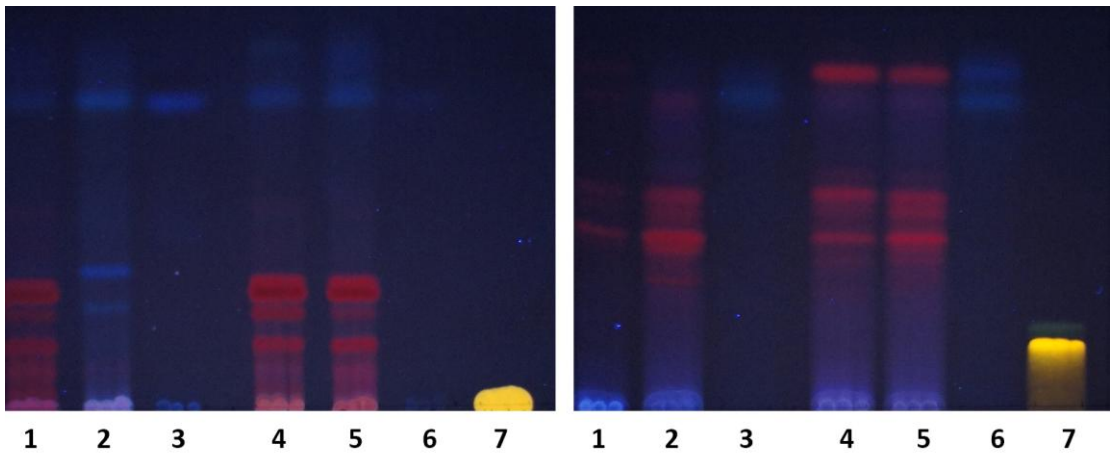

Fig. 21: Example of thin-layer chromatograms of *Chara aspera* extracts under UV_{366nm}. Detection: natural product reagent and polyethyleneglycol. Left: DCM extracts in system B (Table 6); right: MeOH and N-butanol extracts in system A (Table 6). 1 = MeOH extracts of lyophilised algae from Lake Millstättersee, 2 = MeOH extract of air dried algae from Lake Millstättersee, 3 = N-butanol ice extract of lyophilised algae from Lake Millstättersee, 4 = MeOH extracts of lyophilised algae from Lake Attersee, 5 = MeOH extract of air dried algae from Lake Attersee, 6 = N-butanol ice extract of lyophilised algae from Lake Attersee, 7 = flavonoid markers (rutin in system A and quercetin in system B).

The chromatographic fingerprint in the thin-layer chromatograms (examples of *Chara aspera;* Figure 20 and 21) shows that the composition of the extracts from two sampling sites is quite similar. No evidence for flavonoids could be observed (Fig. 21). The red bands in Figure 21 pointed to chlorophylls. The three other stonewort species *Chara globularis*, *Chara rudis* and *Chara tomentosa* showed rather identical band patterns (Figs. 28-32 in 12. Appendix).

5.4. HPLC-analyses

The polar N-butanol ice extracts could not be separated by means of HPLC. Due to their high polarity, they resulted in only one peak at the solvent front (chromatograms not shown). None of the DCM ice extracts showed any peak under UV-detection. Thus, only non-polar and polar extracts of the dried material are plotted in the Figures 22, 23, 24 and 25.

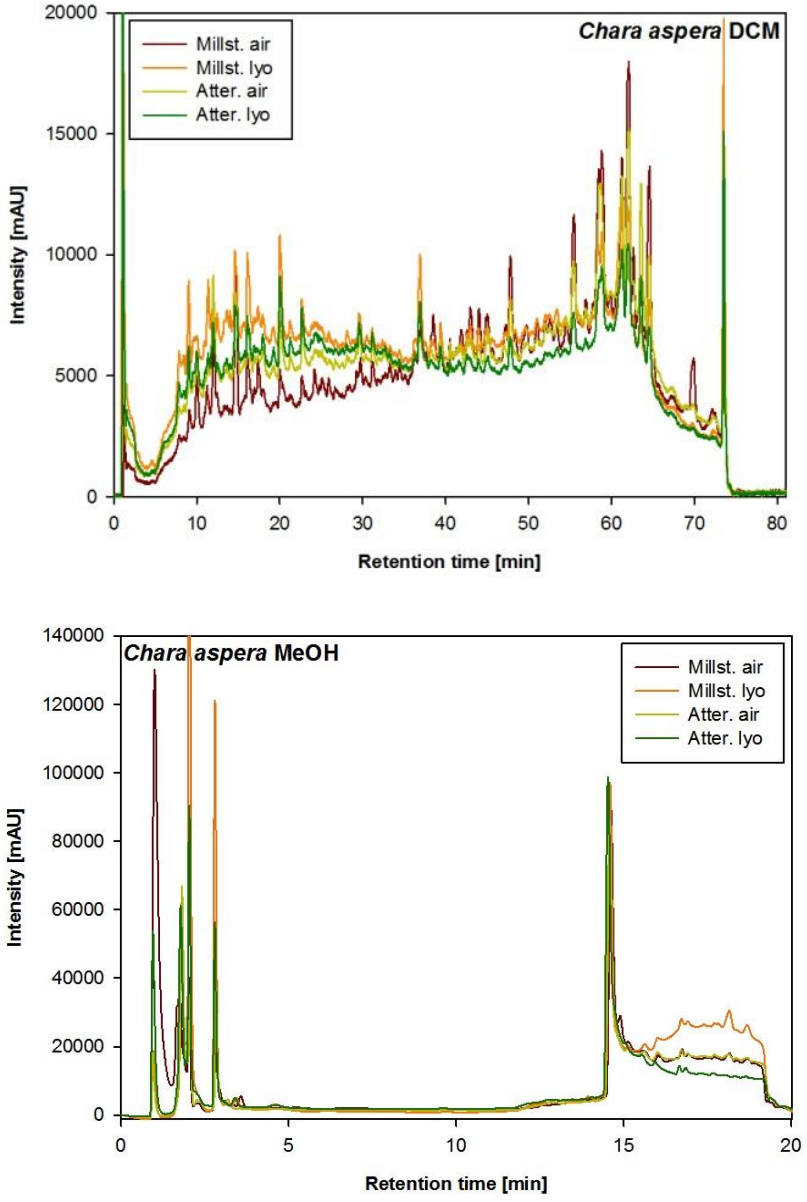

Fig. 22: HPLC chromatograms of *Chara aspera* extracts (all sampling sites and drying methods) at 254 nm. Upper: chromatogram of the DCM extracts; lower: MeOH extracts. DCM = dichloromethane, MeOH = methanol, Millst. air = air dried algae from Lake Millstättersee, Millst. lyo = lyophilised algae from Lake Millstättersee, Atter. air = air dried algae from Lake Attersee, Atter. lyo = lyophilised algae from Lake Attersee.

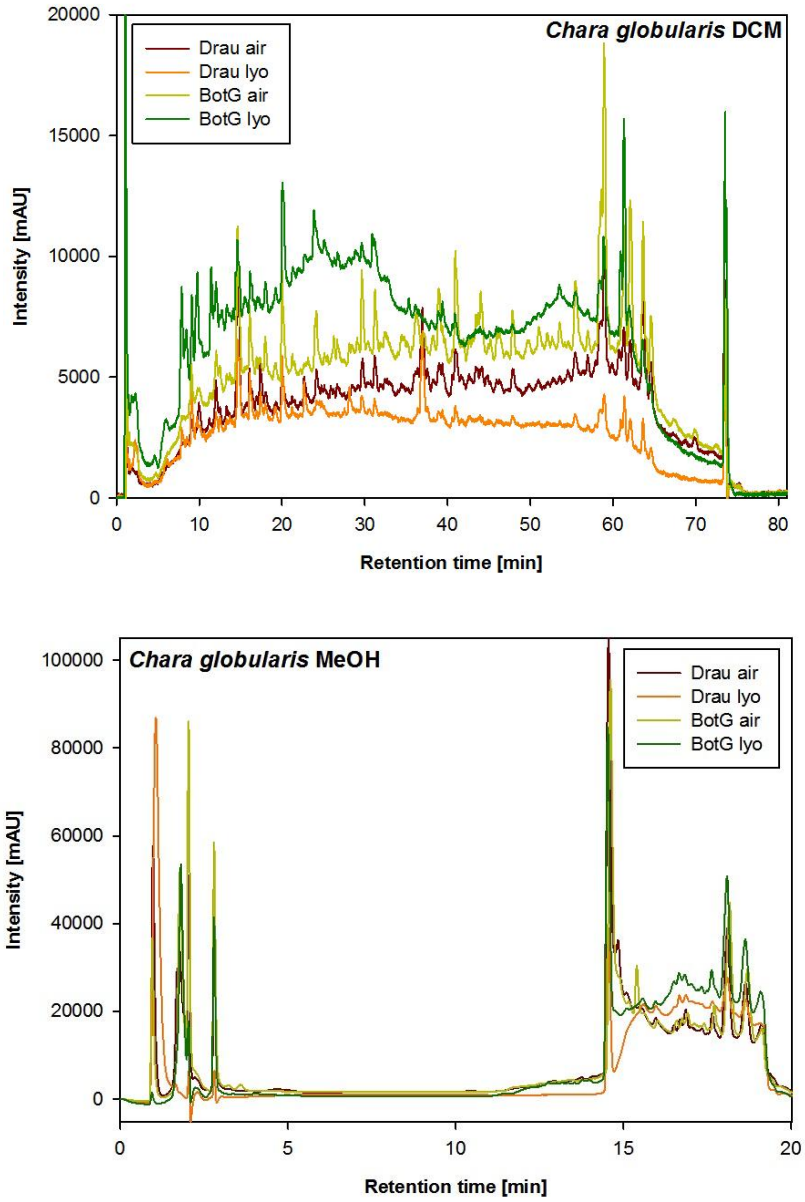

Fig. 23: HPLC chromatograms of *Chara globularis* extracts (all sampling sites and drying methods) at 254 nm. Upper: chromatogram of the DCM extracts; lower: MeOH extracts. DCM = dichloromethane, MeOH = methanol, Drau air = air dried algae from Obere Drau, Drau lyo = lyophilised algae from Obere Drau, BotG air = air dried algae from Botanischer Garten, BotG lyo = lyophilised algae from Botanischer Garten.

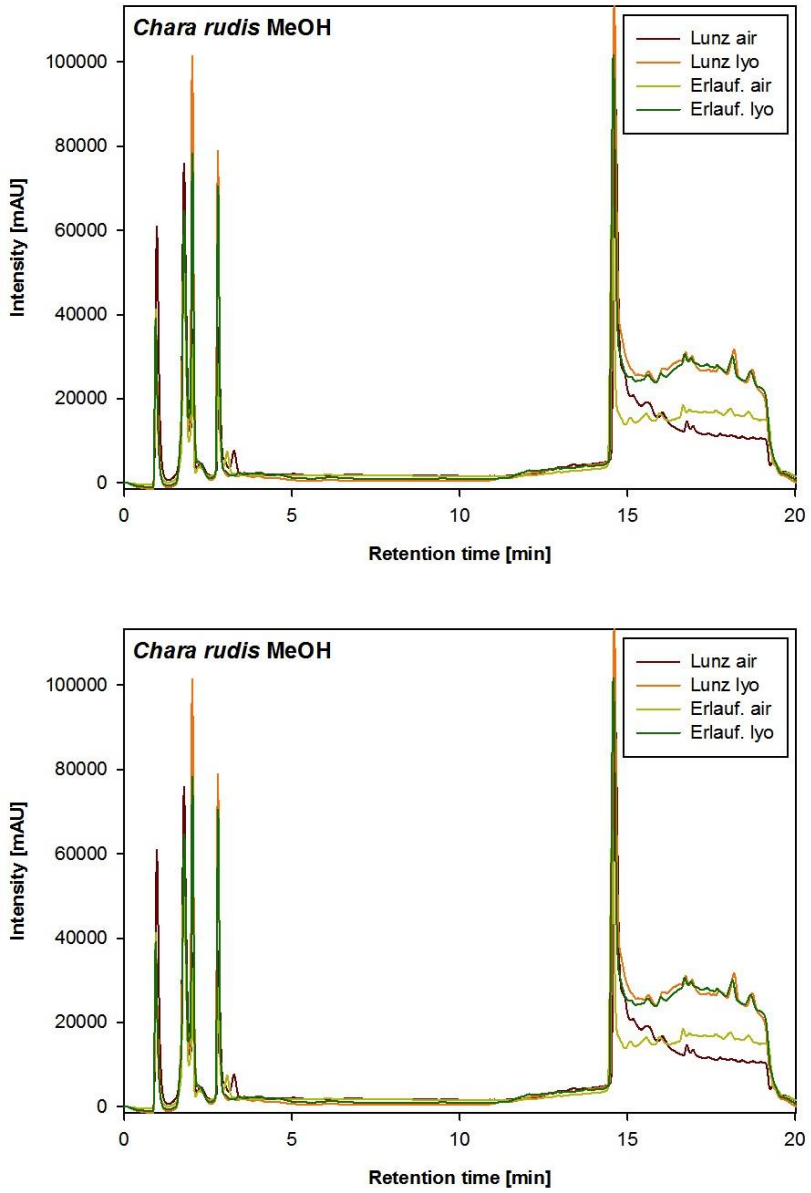

Fig. 24: HPLC chromatograms of *Chara rudis* extracts (all sampling sites and drying methods) at 254 nm. Upper: chromatogram of the DCM extracts; lower: MeOH extracts. DCM = dichloromethane, MeOH = methanol, Lunz air = air dried algae from Lake Lunzer Untersee, Lunz lyo = lyophilised algae from Lake Lunzer Untersee, Erlauf. air = air dried algae from Lake Erlaufsee, Erlauf. lyo = lyophilised algae from Lake Erlaufsee.

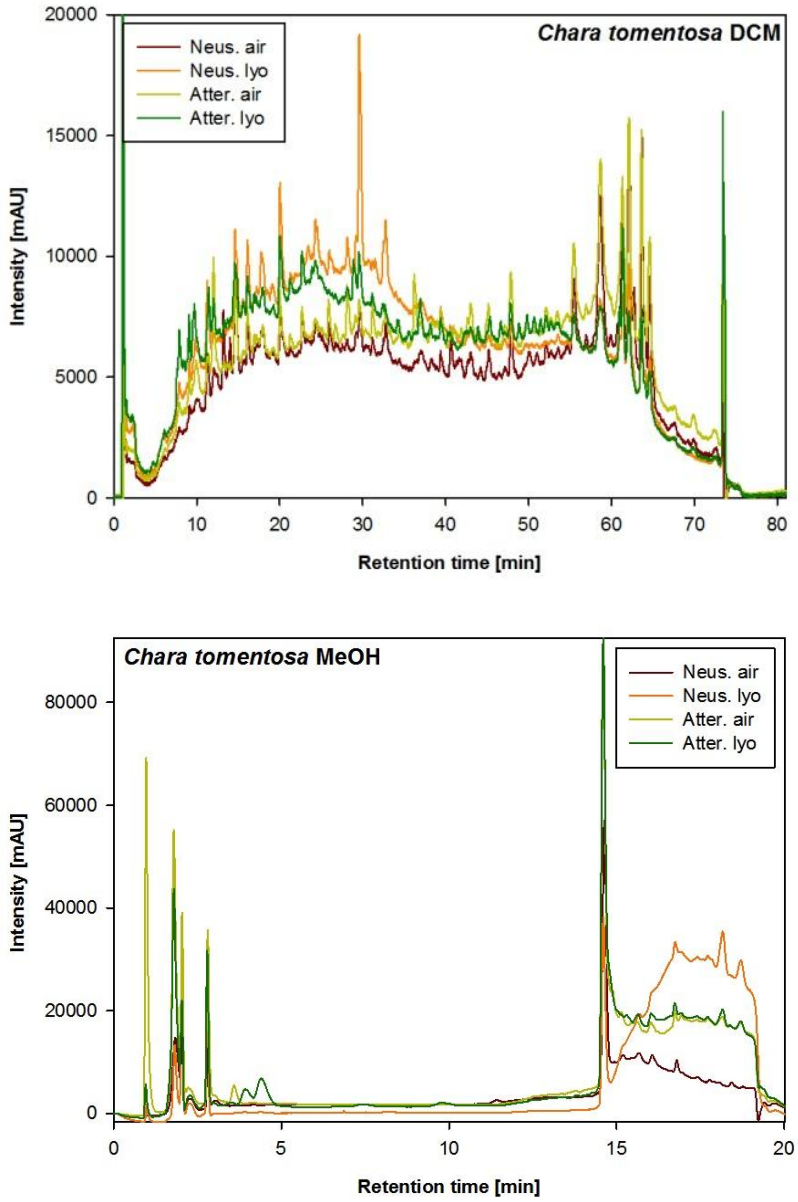

Fig. 25: HPLC chromatograms of *Chara tomentosa* extracts (all sampling sites and drying methods) at 254 nm. Upper: chromatogram of the DCM extracts; lower: MeOH extracts. DCM = dichloromethane, MeOH = methanol, Neus. air = air dried algae from Lake Neusiedlersee, Neus. lyo = lyophilised algae from Lake Neusiedlersee, Atter. air = air dried algae from Lake Attersee, Atter. lyo = lyophilised algae from Lake Attersee.

According to the HPLC chromatograms in Figures 22-25 all *Chara* species showed compounds with similar retention times. The absorption spectra at 254 nm were used for comparing the similarity of the peaks between the extracts, not for determining the substance classes. Moreover, no difference between the two drying methods, air drying and lyophilisation, could be revealed.

5.5. GC-MS chromatograms

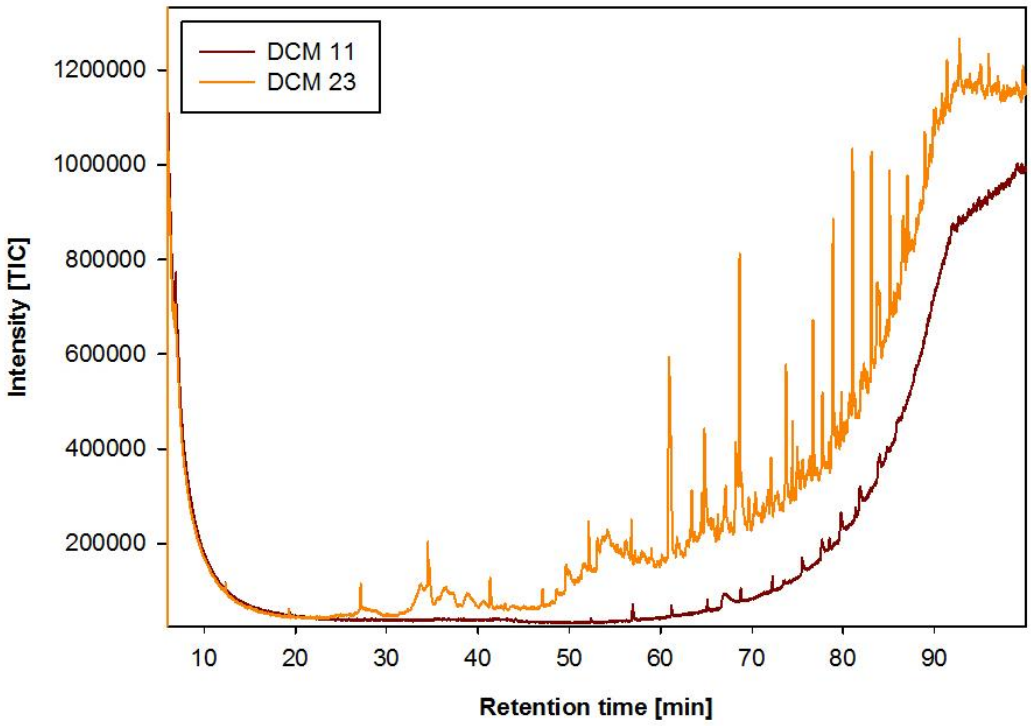

Fig. 26: GC-MS chromatogram of the allelopathically active DCM 23-extract (*Chara rudis* Lake Erlaufsee DCM ice extract; inhibition of ++) and inactive DCM 11-extract (*Chara globularis* Botanischer Garten DCM ice extract) on photoautotrophic target organisms; TIC = total ion current.

The GC-MS chromatograms indicated that only the DCM ice extract of *Chara rudis* (DCM 23) was allelopathically active; this non-polar extract contained several unidentified compounds (Fig. 26), which could not be matched in the available mass spectra database (WILEY229.LIB, NIST147.LIB). The chromatograms of the N-Butanol ice extracts are not shown.

5.6. Composition of Chara material

The organic content was rather similar for all stonewort species whereas the proportion of inorganic compounds was highly variable with the exception of *Chara rudis* (Fig. 27). *Chara tomentosa* from the Lake Neusiedlersee had the lowest amount of inorganic material.

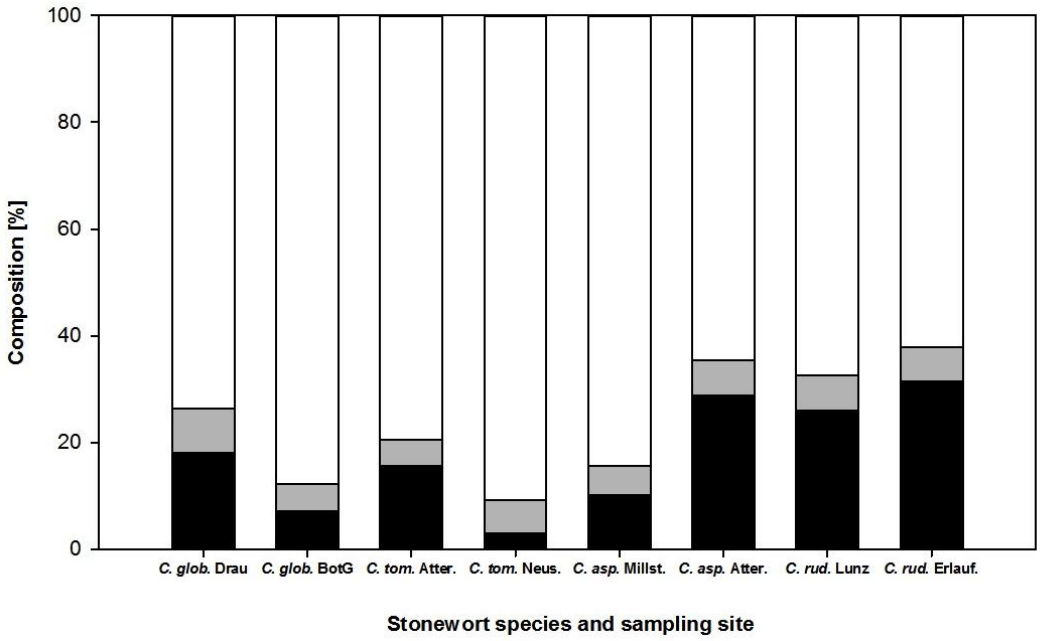

Fig. 27: Composition of *Chara*. Black = inorganic content, light grey = organic content, white = water content, *C*. *glob*. Drau = *Chara globularis* Obere Drau, *C*. *glob*. BotG = *Chara globularis* Botanischer Garten, *C*. *tom*. Atter. = *Chara tomentosa* Lake Attersee, *C*. *tom*. Neus. = *Chara tomentosa* Lake Neusiedlersee, *C*. *asp*. Millst. = *Chara aspera* Lake Millstättersee, *C*. *asp*. Attersee = *Chara aspera* Lake Attersee, *C*. *rud*. Lunz = *Chara rudis* Lake Lunzer Untersee, *C*. *rud*. Erlauf. = *Chara rudis* Lake Erlaufsee.

6.) Discussion

This study provided intra- and interspecific comparisons of four *Chara* species concerning their allelopathic effect on different prokaryotic and eukaryotic target organisms.

All fresh *Chara* material had a characteristic pungent, sulphuric smell; *Chara tomentosa´*s aroma resembled to garlic. According to thin-layer chromatography and HPLC-analyses, no intraspecific differences (sampling sites) in the compound composition were observed, which indicates that the allelochemical pattern is independent of environmental conditions. We however did not study the influence of environmental factors on the amount of synthesized allelochemicals. It was already proven that abiotic factors such as light, nutrient availability and the presence of certain phytoplankton taxa influences the amount of exuded allelopathic compounds (Reigosa et al. 1999, Van Donk & Van de Bund 2002, Berger & Schagerl 2004, Kurashov et al. 2014). For excluding any growth inhibition of the target organisms by nutrient depletion, culture media with a sufficient amount of nutrients were used. According to Van Donk & Van de Bund (2002) and Gross (2003a), seasonal differences might also have an

effect on the strength of allelopathy (stronger in summer, lower in autumn), which however can be neglected in this study because the sampling period took place from May to July.
Only Cyanobacteria were inhibited by young *Chara*-shoots, which suggests antibiotic activity of their exudates. Also other studies showed inhibitions of different Cyanobacteria by young *Chara* shoots tested in similar bioassays (Berger & Schagerl 2003, 2004). According to Keating (1978) cited in Berger & Schagerl (2003), (2004), Pakdel et al. (2013), the presence of microbes and other contamination of non-axenic *Chara* material is negligible in the bioassay approach due to their low concentration. No intra- and interspecific difference within the other *Chara* species was observable in the tests with living material. In our study, we found no increase of the inhibition zone with time, but the zone became more clearly visible; this was also shown by Pakdel et al. (2013).

Similar to the assays with living material, also extracts resulted in increased inhibitions of Cyanobacteria compared to tested eukaryotes. This was also reported in some previous studies (Berger & Schagerl 2003, Berger & Schagerl 2004). It is commonly known, that Cyanobacteria as well as stoneworts are able to cope with low light conditions (Schwarz et al. 1996, Havens et al. 1998, Mur et al. 1999). By colonizing a similar light niche, competition for light between these two groups might explain the major inhibition of Cyanobacteria. Differences in cell wall components between pro- and eukaryotes might play a major role in the much more sensitive reaction of Cyanobacteria (Gross 1999, Berger & Schagerl 2004).
Extracts of dried material did not affect *Chlorella vulgaris* and *Scenedesmus acuminatus*. Also Mulderij et al. (2003) did not find any inhibition of *Scenedesmus obliquus* (neither by *Chara globularis* nor by *Chara contraria*). Bioassays of Pakdel et al. (2013) also showed no inhibitions on *Scenedesmus quadricauda*, whereas Lürling et al. (2006) found inhibitions on *Scenedesmus obliquus* by *Chara globularis*. This contradictory results might be explained by species specificity and different methodological approaches.
The non-polar DCM extracts of dried *Chara* material generally did not show any inhibition of the photoautotrophic target organisms, which indicated the hydrophilic/polar character of allelochemicals, as polar MeOH-extracts caused inhibitions. In contrary to most other studies, which commonly extracted in MeOH and subsequently divided into moderately lipophilic and hydrophilic fractions (Bankova et al. 2001, Berger & Schagerl 2003, 2004, Pakdel et al. 2013), we extracted dried material with both non-polar and polar solvents (first DCM and then MeOH).

Vibrio fischeri showed a different inhibition pattern, because application of both DCM and MeOH extracts resulted in a strong decrease of bioluminescence. This bioluminescent marine bacterium is not naturally present in freshwater systems and probably more sensitive than the other freshwater target organisms. Heterotrophic bacteria might possess some other nutrient uptake-mechanisms than photoautotrophic bacteria and it is possible that different membrane structures and easily passing low molecular weight (volatile) substances caused the increased sensitivity (Gross 1999 cited in Berger & Schagerl 2003, Gross et al. 2007).

N-butanol ice extracts caused strong inhibitions of *Scenedesmus acuminatus*, *Synechococcus elongatus* and *Synechococcus leopoliensis*, which indicate the presence of volatile allelochemicals. Some other studies also suggested that low molecular weight substances could be allelopathically active (Anthoni et al. 1980, Bankova et al. 2001, Van Donk & Van de Bund 2002, Lürling et al. 2006, Kurashov et al. 2014). Bankova et al. (2001) identified some volatile compounds but did not test them separately for possible inhibitory effects. *Chlorella vulgaris* was not affected by any of the extracts.

Despite *Scenedesmus acuminatus* showed no inhibition in the bioassays with living *Chara* material, it was inhibited by the N-butanol ice extracts. This effect could be caused by higher concentrations of active compounds in the extract experiments after re-dissolving them in MeOH than it would occur *in situ*. It is however a challenge to measure *in situ* concentrations due to the dilution in natural water bodies (Gross et al. 2007, Kurashov et al. 2014). Since laboratory experiments can hardly simulate the abiotic and biotic *in situ* parameters, there is an urgent need for additional field studies for testing allelopathic effects. Some methods were discussed by Gross et al. (2007).

Concerning the application of the two different drying methods, only small differences were observed in the tests of MeOH extracts on photoautotrophic organisms, although a strong fishy smell was recognized during air drying at 30 °C. Lyophilised material also showed a characteristically smell, but this was much “fresher”. It has to be mentioned that stoneworts during both drying methods lost the sulphuric smell. The fishy smell could be caused by dead, attached *Dreissena polymorpha* mussels present at some sampling sites; the mussels were removed after drying before extraction to avoid any bias.

HPLC-analyses showed no difference in the extract composition. Extracts of lyophilised material resulted in a higher number of inhibitions in the bioassays than the air dried material, which might indicate a better conservation of algal compounds at -100 °C. DCM extracts of dry material never inhibited any photoautotrophic target organism.

As several studies suggested that allelochemicals have negative effects on the photosynthesis of target organisms (Wium-Andersen et al. 1982, Gross 2003a), we included tests of the overall activity of the PSII with one extract, which was proven to be allelopathically active in the bioassays. We found a steep decline of the photoautotrophic target organism's maximum quantum dark yield, but also a recovery after 30 and 60 min, respectively. This regeneration might be explained by degradation of allelochemicals by the target organisms. Our findings are comparable to allelopathic effects caused by *Chara globularis* reported by Lürling et al. (2006). In the present experiments, the two green algae *Chlorella vulgaris* and *Scenedesmus acuminatus* regenerated faster than the two Cyanobacteria species. As mentioned before, *C. vulgaris* was never affected in the bioassays, which indicates, that PAM might be a more sensitive measurement tool concerning short-time bioassays. Similar to the PAM measurements also the *V. fischeri* assays tested the immediate inhibition by *Chara* extracts.

HPLC-analyses revealed only little intra- and interspecific differences. Furthermore the comparison with flavonoid markers in the TLC-chromatograms showed that flavonoids were not present; these substances are known as defence against grazing in higher plants. These findings fit perfectly to the results of Bankova et al. (2001).

The GC-MS libraries (WILEY229.LIB, NIST147.LIB) and the Kovats retention index of possibly active volatile substances extracted from the ice did not provide reliable correlations for the identification of single compounds. This would need a higher amount of extracts for a fractionation, a better separation of the peaks in chromatography and additional purification steps.

Although two strongly and two weakly inhibiting *Chara* species were chosen (Berger & Schagerl 2004), we were not able to clearly distinguish between "strong" and "weak" allelopathic activity. According to Berger & Schagerl (2004), *Chara tomentosa* and *Chara rudis* had lower allelopathic activities than *Chara aspera* and *Chara globularis*, but they used other photoautotrophic target organisms. Thus species specificity, as mentioned in several prior studies, may play an important role for better understanding of allelopathic interactions (Berger & Schagerl 2003, Mulderij et al. 2003, Berger & Schagerl 2004, Pakdel et al. 2013). Such studies need to consider target organisms and stoneworts from the same water body. Furthermore, *in situ* investigations are still scarce, but they would be essential for providing new and more detailed insights into the complex mechanisms of allelopathy in aquatic environments. According to Berger & Schagerl (2003), (2004), more than one compound causes allelopathic activity. Gross et al. (2007) claimed that several combined methods (e.g. *in situ* dialysis bags and coexistence experiments) should be used for detecting allelopathic

effects. Allelochemicals released by *Chara* seem to be mostly antibiotic, which was also shown in this study for both heterotrophic and photoautotrophic prokaryotes. Bankova et al. (2001) observed antibiotic effects by *Chara globularis* in bioassays on the human pathogen *Staphylococcus aureus*. This antibiotic effect might be of interest for in-depth pharmaceutical studies as well. Besides that management of algal-blooms, which are mostly formed by dominant Cyanobacteria (Paerl et al. 2001, Hu & Hong 2008), may be overcome by using the allelopathic activity of stoneworts.

7.) Conclusion

Summing up, species specificity seems to play a major role in allelopathic interactions among aquatic algae. Only little differences between air drying and lyophilisation of the algae were observed. A hint for volatile substances, which are allelopathically active, was provided by the results of the ice extracts after lyophilisation. Concerning intra- and interspecific comparison, no big differences were obtained. Moreover, allelochemicals of stoneworts had an antibiotic effect on both heterotrophic and photoautotrophic bacteria. The highest amount of photoautotrophic target organism inhibition was caused by polar MeOH and N-butanol of the ice extracts indicating the polar nature of these substances.

8.) Acknowledgements

I thank my two supervisors Prof. Michael Schagerl (Department of Limnology and Bio-Oceanography), Prof. Liselotte Krenn (Department of Pharmacognosy) and all my colleagues. Special thanks also to Andrea Lubich for helping me with the HPLC-analyses. I am very grateful to the members of the Kärntner Institut für Seenforschung Klagenfurt for providing stonewort field maps and Prof. Ines Fritz as well as Michaela Bauer from the IFA Tulln for the possibility of using their lab for the luminescence measurements. Last but not least, I thank my boyfriend Jan Steger and my family for the immense support during my studies.

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10.) Zusammenfassung

Armleuchteralgen (Characeen) sind für ihren geringen Epiphytenbewuchs bekannt, jedoch existieren bisher nur wenige Studien, die sich mit potentiellen allelopathischen Effekten befassen. In dieser Studie wurden vier Armleuchteralgenarten im Zeitraum von Mai bis Juli 2014 an verschiedensten österreichischen Gewässern (jeweils zwei Standorte pro Art) gesammelt und getestet. Ein Teil der gesammelten Pflanzen wurde für Versuche mit Lebendmaterial (Sprosse) verwendet, ein weiterer Teil für die Extraktionen mit Dichlormethan und Methanol vorbereitet.

Um etwaige Wachstumshemmungen von Mikroalgen durch *Chara* zu testen, wurden jeweils zwei Arten photoautotropher Grünalgen beziehungsweise Cyanobakterien und ein Ansatz mit heterotrophen Proteobakerien als Zielorganismen verwendet. Im Gegensatz zu den Cyanobakterien zeigten Grünalgen eine weitaus größere Resistenz gegenüber Allelochemikalien aus *Chara*. Im Vergleich zu den photoautotrophen Organismen, welche hauptsächlich durch polare Methanolextrakte gehemmt wurden, zeigten die heterotrophen Bakterien ein sehr unterschiedliches Hemmungsmuster. Sie wurden auch von den apolaren Dichlormethan-Extrakten gehemmt, die bei den photoautotrophen Organismen keine Wirkung zeigten. Es konnten weder inner- (Standorte), noch zwischenartliche Unterschiede festgestellt werden. Weiters wurden die Extrakte mittels HPLC (Hochleistungsflüssigkeits-Chromatographie) und GC-MS (Gaschromatographie und Massenspektrometrie) analysiert. Die Ergebnisse der HPLC zeigten keine inner- und zwischenartlichen Unterschiede. Bei der GC-MS konnten einige Komponenten eines allelopathisch aktiven Dichlormethan-Eisextraktes (*Chara rudis* Erlaufsee) detektiert werden.

Zusätzlich wurden zwei Trocknungsmethoden für das Frischmaterial, Lufttrocknung bei 30° und Gefriertrocknung bei -100 °C, verglichen. Hierbei zeigten sich nur geringe Unterschiede in den Hemmungen durch *Chara*-Extrakte. Die anschließenden HPLC-Analysen wiesen ähnliche Ergebnisse auf. Zusätzlich wurde das kondensierte Eis der Lyophilisation gesammelt und mit Dichlormethan und N-Butanol extrahiert, um flüchtige Substanzen auf allelopathische Aktivität zu testen. Interessanterweise zeigte sich hierbei bei einer der Grünalgenarten und allen Cyanobakterien eine starke Hemmung durch die polaren N-Butanol Eis-Extrakte. Diese wirksamen Extrakte wurden mittels GC-MS analysiert, es konnte jedoch keine der Verbindungen identifiziert werden. Hierzu wären zusätzliche Aufreinigungsschritte notwendig.

Das Methanolextrakt der gefriergetrockneten *Chara tomentosa* des Standortes Attersee mit starker Hemmwirkung, wurde mit pulse amplitude modulated fluorescence (PAM) getestet, wobei eindeutig eine Verringerung der Photosyntheseaktivität gezeigt werden konnte.

11.) Summary

Stoneworts (Characeae) are commonly known for their almost epiphyte free appearance, nevertheless studies dealing with their potential of allelopathic activity are scarce.

In this study four stonewort species, two sampling sites each, were collected between May and July 2014 from different aquatic habitats in Austria and tested. Some shoots of the collected stonewort material were used for experiments with living *Chara* and the other material was prepared for the extractions with dichloromethane and methanol. For detecting growth-inhibiting effects caused by *Chara* species on microalgae, two photoautotrophic green algae species and two Cyanobacteria as well as one heterotrophic Proteobacterium were chosen as target organisms. In contrast to Cyanobacteria, the green algae were much more resistant to allelochemicals. Compared to the photoautotrophic organisms, which were mainly affected by polar methanol extracts, the heterotrophic Proteobacterium showed a very variable inhibition pattern. Additionally, this Proteobacterium was inhibited by the non-polar dichloromethane extract, which did not show any inhibition of the photoautotrophic target organisms.

Neither intra- (sampling sites) nor interspecific differences were observable. Furthermore the extracts were analysed by means of HPLC (high performance liquid chromatography) and GC-MS-analyses (gas chromatography and mass spectrometry).

The HPLC results indicated no intra- or interspecific differences. The GC-MS-analyses showed several compounds of an allelopathically active dichloromethane extract (*Chara rudis* Lake Erlaufsee).

Additionally two drying methods, air drying at 30 °C and lyophilisation at -100 °C, were compared. Only small differences between the inhibitory activity of these *Chara* extracts were observable. HPLC-analyses provided similar results.

Besides that, the condensed ice of the lyophilisation was collected and extracted with dichloromethane and N-butanol to test the allelopathic activity of volatile substances. Interestingly, a strong inhibition of one green algal species and all Cyanobacteria by polar N-butanol ice extracts occurred. These active extracts were analysed via GC-MS (gas chromatography and mass spectrometry), however no specific substances could be identified. Therefore several purification steps would be needed.

The methanol extract of the lyophilised *Chara tomentosa* from Lake Attersee, which showed inhibitions in the experiments, was measured by using pulse amplitude modulated fluorescence (PAM) where a decrease of the photosynthetic activity was observed.

12.) Appendix

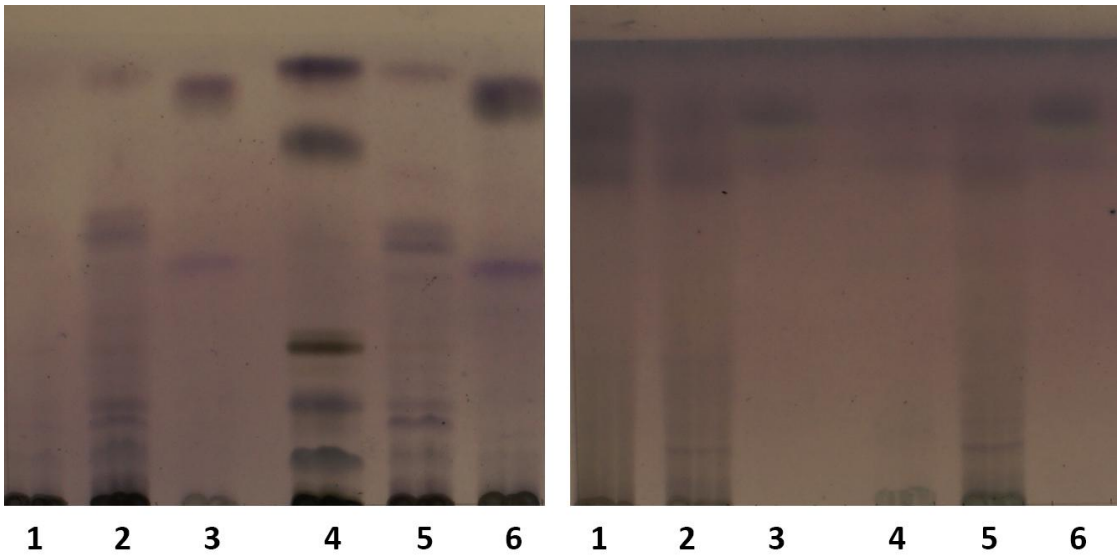

Fig. 28: Thin-layer chromatograms of *Chara globularis* extracts under visible light. Detection: anisaldehyde-sulphuric acid reagent and heating at 100 °C for 5-10 min. Left: DCM extracts in system B (Table 6); right: MeOH and N-butanol extracts in system A (Table 6). 1 = MeOH extracts of lyophilised algae from Obere Drau, 2 = MeOH extract of air dried algae from Obere Drau, 3 = N-butanol ice extract of lyophilised algae from Obere Drau, 4 = MeOH extracts of lyophilised algae from Botanischer Garten, 5 = MeOH extract of air dried algae from Botanischer Garten, 6 = N-butanol ice extract of lyophilised algae from Botanischer Garten.

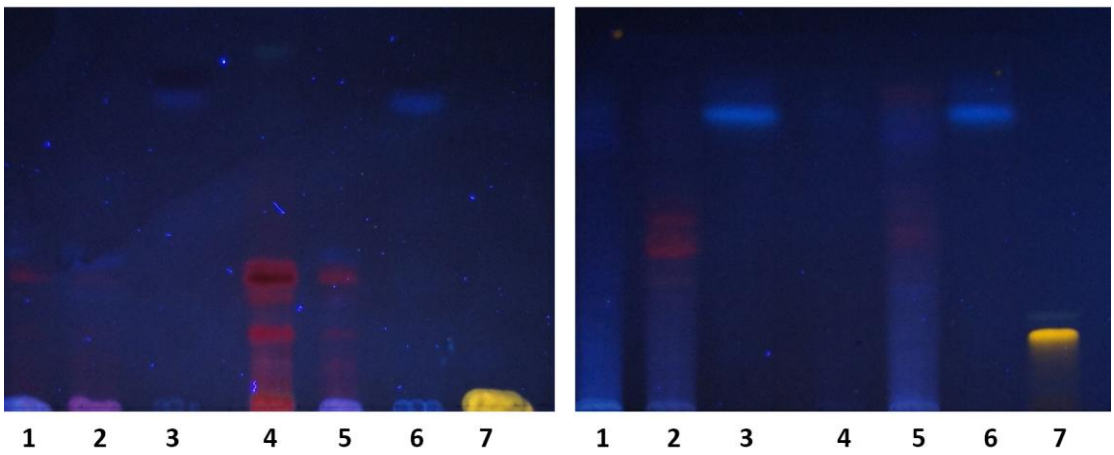

Fig. 29: Thin-layer chromatograms of *Chara globularis* extracts under UV_{366nm}. Detection: natural product reagent and polyethyleneglycol. Left: DCM extracts in system B (Table 6); right: MeOH and N-butanol extracts in system A (Table 6). 1 = MeOH extracts of lyophilised algae from Obere Drau, 2 = MeOH extract of air dried algae from Obere Drau, 3 = N-butanol ice extract of lyophilised algae from Obere Drau, 4 = MeOH extracts of lyophilised algae from Botanischer Garten, 5 = MeOH extract of air dried algae from Botanischer Garten, 6 = N-butanol ice extract of lyophilised algae from Botanischer Garten, 7 = flavonoid markers (rutin in system A and quercetin in system B).

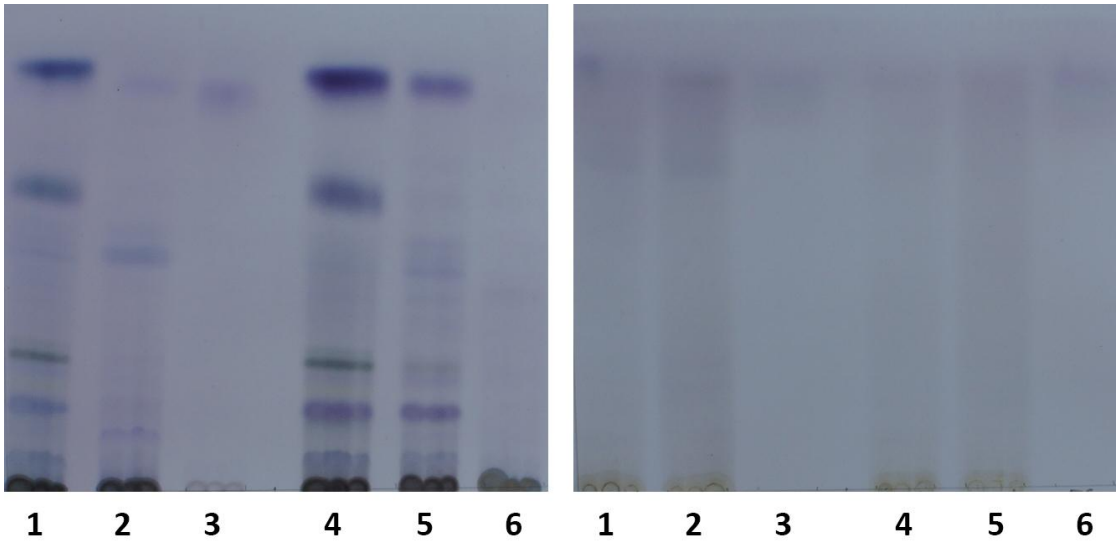

Fig. 30: Thin-layer chromatograms of *Chara rudis* extracts under visible light. Detection: anisaldehyde-sulphuric acid reagent and heating at 100 °C for 5-10 min. Left: DCM extracts in system B (Table 6); right: MeOH and N-butanol extracts in system A (Table 6). 1 = MeOH extracts of lyophilised algae from Lake Lunzer Untersee, 2 = MeOH extract of air dried algae from Lake Lunzer Untersee, 3 = N-butanol ice extract of lyophilised algae from Lake Lunzer Untersee, 4 = MeOH extracts of lyophilised algae from Lake Erlaufsee, 5 = MeOH extract of air dried algae from Lake Erlaufsee, 6 = N-butanol ice extract of lyophilised algae from Lake Erlaufsee.

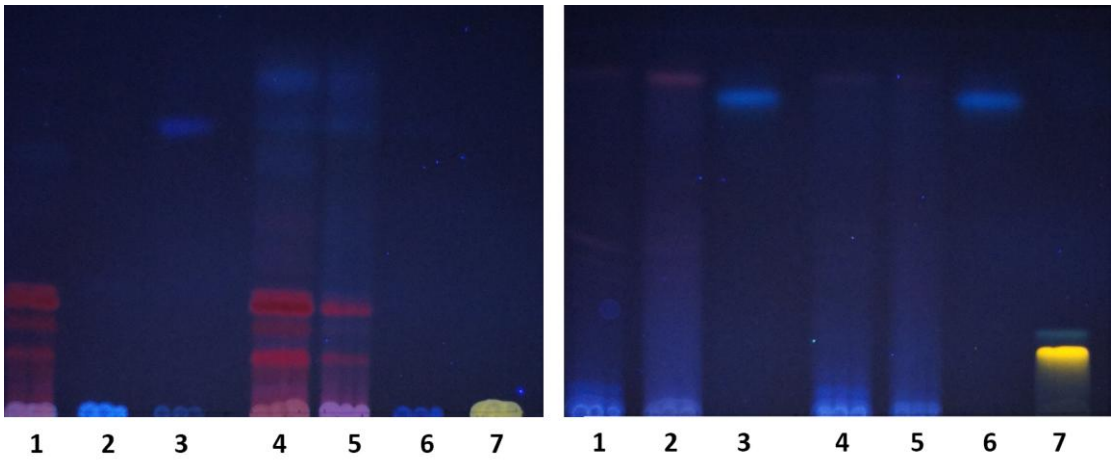

Fig. 31: Thin-layer chromatograms of *Chara rudis* extracts under UV_{366nm}. Detection: natural product reagent and polyethyleneglycol. Left: DCM extracts in system B (Table 6); right: MeOH and N-butanol extracts in system A (Table 6). 1 = MeOH extracts of lyophilised algae from Lake Lunzer Untersee, 2 = MeOH extract of air dried algae from Lake Lunzer Untersee, 3 = N-butanol ice extract of lyophilised algae from Lake Lunzer Untersee, 4 = MeOH extracts of lyophilised algae from Lake Erlaufsee, 5 = MeOH extract of air dried algae from Lake Erlaufsee, 6 = N-butanol ice extract of lyophilised algae from Lake Erlaufsee, 7 = flavonoid markers (rutin in system A and quercetin in system B).

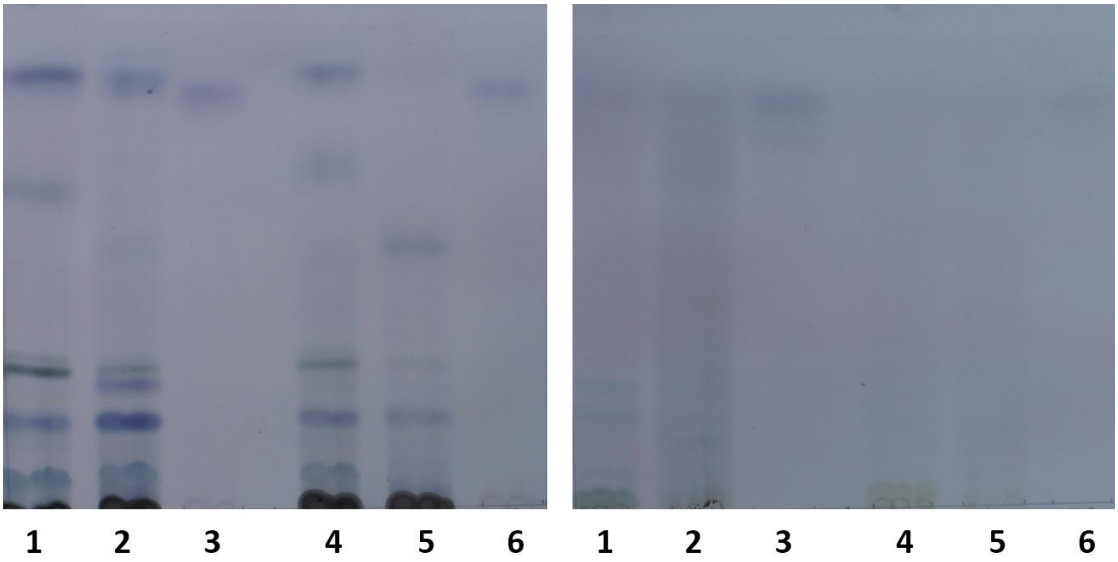

Fig. 32: Thin-layer chromatograms of *Chara tomentosa* extracts under visible light. Detection: anisaldehyde-sulphuric acid reagent and heating at 100 °C for 5-10 min. Left: DCM extracts in system B (Table 6); right: MeOH and N-butanol extracts in system A (Table 6). 1 = MeOH extracts of lyophilised algae from Lake Attersee, 2 = MeOH extract of air dried algae from Lake Attersee, 3 = N-butanol ice extract of lyophilised algae from Lake Attersee, 4 = MeOH extracts of lyophilised algae from Lake Neusiedlersee, 5 = MeOH extract of air dried algae from Lake Neusiedlersee, 6 = N-butanol ice extract of lyophilised algae from Lake Neusiedlersee.

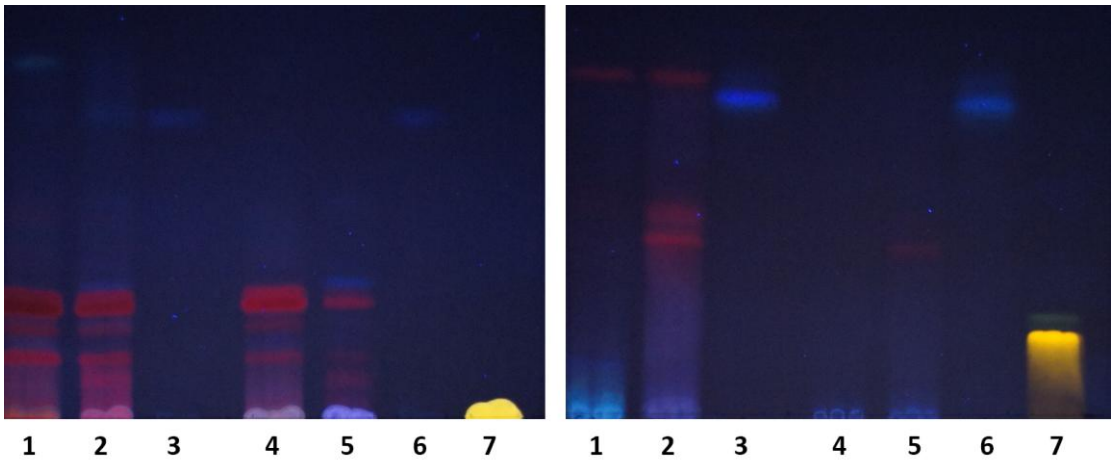

Fig. 33: Thin-layer chromatograms of *Chara tomentosa* extracts under UV_{366nm}. Detection: natural product reagent and polyethyleneglycol. Left: DCM extracts in system B (Table 6); right: MeOH and N-butanol extracts in system A (Table 6). 1 = MeOH extracts of lyophilised algae from Lake Attersee, 2 = MeOH extract of air dried algae from Lake Attersee, 3 = N-butanol ice extract of lyophilised algae from Lake Attersee, 4 = MeOH extracts of lyophilised algae from Lake Neusiedlersee, 5 = MeOH extract of air dried algae from Lake Neusiedlersee, 6 = N-butanol ice extract of lyophilised algae from Lake Neusiedlersee, 7 = flavonoid markers (rutin in system A and quercetin in system B).

13.) *Curriculum vitae*

Barbara Mähnert

Education

2013 – 2015:	Master program Ecology with focus on limnology and phycology (MSc degree). Master thesis: "Allelopathic activity of stoneworts" (Supervisors: Prof. Michael Schagerl and Prof. Liselotte Krenn)
2009 – 2013:	Bachelor program Biology with focus on ecology (BSc degree). Bachelor thesis: "Desiccation and regeneration of the intertidal brown seaweed *Fucus serratus*" (Supervisor: Prof. Michael Schagerl)
2002 – 2009:	Herta Reich grammar school Mürzzuschlag (Styria)
2000 – 2002:	Konrad Lorenz grammar school Gänserndorf (Lower Austria)
1998 – 2000:	Elementary school Gänserndorf (Lower Austria)
1996 – 1998:	Elementary school Ada Christen-Gasse (Vienna)

Work experience

2009:	University of Vienna: Astaxanthin analyses for BDI (BioEnergy International AG)
2015:	Tutor at University of Vienna: Course "Aquatische mikrobielle Ökologie" (summer term 2015)
2014 – 2015:	University of Vienna: Student assistant of Prof. M. Schagerl

Volunteer activities

2013:	Geotag Nationalpark Kalkalpen
2014:	Geotag Nationalpark Gesäuse
2014:	Tag der Artenvielfalt Wienerwald
2015:	Tag der Artenvielfalt Wienerwald

Further skills

Languages:	Englisch, Italian, Latin
IT:	MS Office, Sigma Plot, Endnote
Driver license:	B