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Metabolomics for Toxicity Analysis using the Chlorophyte *Scenedesmus vacuolatus*

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Metabolomics for Toxicity Analysis using the Chlorophyte *Scenedesmus vacuolatus*

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„The real voyage of discovery consists not
in seeking new landscapes, but in having new eyes.“
Marcel Proust

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Contents

Abbreviations

A. thaliana	*Arabidopsis thaliana*
ATP	Adenosine Tri Phosphate
CAS RN	Chemical Abstract Service Registry Number
C. reinhardtii	*Chlamydomonas reinhardtii*
DMSO	Dimethylsulfoxide
EC50	Median effective concentration
ED50	Median effect dilution in Brack *et al.* (1999)
fL	Femto Litre ($1 fL = 10^{-9}$ µL)
GC-MS	Gas chromatography – mass spectrometry
GC-TOF MS	Gas chromatography – time of flight Mass spectrometry
HCl	Hydrochloride
HPLC	High performance liquid chromatography
ISO	International Organization for Standardization
MOX	2% Methoxyamine•HCl in Pyridine
MS	Mass spectrometry
MSTFA	*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide
NMR	Nuclear magnetic resonance spectro.....
OECD	Organization for Economic Co-operation and Development
PC	Principal component
PCA	Principal component analysis
PS II	Photosystem Two
PTFE	Polytetrafluorethylen.
S. vacuolatus	*Scenedesmus vacuolatus*
TIC	Total ion current / total ion chromatogram
TICC	Total ion current chromatogram
UFZ	Umweltforschungszentrum Leipzig – Halle

Summary

Summary

Thousands of chemicals are in widespread use and many of them are potential contaminants in fresh water ecosystems. In ecotoxicology, the assessment of the environmental hazards posed by chemical pollutants is typically resulting from single-species exposure analysis. A model organism, known as a primary producer and applied in ecotoxicological laboratory bioassays for water quality is the fresh water chlorophyte *Scenedesmus vacuolatus*. One major ecotoxicological objective is the detection and classification of chemicals regarding their mechanism of action and their effect characteristics. This objective could be addressed due to the assessment of the impact of a chemical on the metabolite level of an organism. Due to the molecular interaction with the chemical, the metabolism of the organism can be affected firstly, and characteristic metabolic patterns of a disturbance should be detectable. In this context, "Metabolomics" is a promising technique. Metabolomics is the unbiased and comprehensive analysis of all low molecular weight metabolites, e.g. sugars, amino acids and fatty acids, present in a biological system, such as cells and organisms.

In the here presented doctoral thesis, a metabolomics approach was developed using gas chromatography-mass spectrometry (GC-MS), for the analysis of hydrophilic and lipophilic metabolites from *Scenedesmus vacuolatus*. Hence, this study can be seen as a first step into the direction of the above mentioned objective of ecotoxicology. An isolate of the unicellular fresh water chlorophyte was synchronously cultured in a clonal population with a 24-hours life cycle followed by sample harvest, tissue homogenisation, metabolite extraction, phase separation, metabolite derivatisation and the metabolite analysis. The developed metabolomics approach resulted in approximately 600 hydrophilic and 300 lipophilic signals from metabolite extracts of the chlorophyte *S. vacuolatus.* The variability of semi-quantitative data between aliquot-, sample- and experimental-replicates has been studied, to be able to distinguish between metabolite patterns of toxicant exposed algae from references. The variability between replicates of samples and of experiments was approximately 20% with the developed metabolomics approach. This value was lower than that described by the literature. Thus, the synchrony was shown to be advantageous to minimise time- and toxicity-unrelated variability in the metabolic composition of sample replicates and therefore, the suitability of the developed method was shown for toxicological studies.

The mass spectra of hydrophilic components from 14 hours old reference algae were searched against custom libraries and approximately 1/6 of hydrophilic metabolites were assigned to a metabolite structure. The metabolomics results, presented here, were comparable to those described by the literature and thus, the developed approach was considered to be useful for the investigation and identification of hydrophilic and lipophilic metabolites from *S. vacuolatus*.

Within the next steps, the developed metabolomics approach was examined regarding its potential for the analysis of time- and concentration-dependent changes in the metabolome of *S. vacuolatus*, induced by a chemical. Therefore, the chlorophyte was exposed to the well known herbicide prometryn (*s*-triazine), used as a model chemical. Prometryn is known to block the photo system two (PS II) and to inhibit *S. vacuolatus* growth in cell size.

First, the concentration-dependent impact of prometryn was studied during the 14 hours (t_{14}) developmental phase of *S. vacuolatus*, analysing the algal metabolome and cell size (growth) in parallel. A dilution series (1:2) of eight concentrations was prepared ranging from 0.002 to 0.26 µmol L^{-1}. The lowest concentration of prometryn applied in this study (0.002 µmol L^{-1}) was comparable to those measured in natural fresh-water ecosystems (see introduction). After exposure, harvest, sample preparation, GC-MS analysis and data evaluation, the metabolite composition from treated algae was compared to that of reference algae using multivariate statistical principal component analysis (PCA). Concentration-related changes of the hydrophilic metabolite composition were found for 14 hours old algae. Hydrophilic metabolite extracts were responding more clearly than lipophilic at the lowest used prometryn concentrations. The lipophilic metabolite composition and the inhibition of cell growth of the chlorophyte showed effects for the higher used prometryn concentrations. Hence, the developed metabolomics approach was applicable to detect chemical induced changes in the *S. vacuolatus* metabolite pattern, and at even lower prometryn concentrations compared to the measurement of the inhibition of growth in the algal cell size. Thus there is potential of the metabolomics approach to detect low-concentration effects, which could show high sensitivity of the method.

Second, the metabolomics approach was applied to study the metabolome and the effects of prometryn at different time points. The chlorophyte development was studied by measuring the metabolic composition and cell growth within the first 14 hours, for references and one prometryn effect concentration of 0.1 µmol L^{-1}. This probative prometryn concentration was selected causing distinct effects on algal growth and metabolism after 14

hours of treatment as an endpoint, but no cell death. And therefore, a time resolution of the metabolic effect of the chemical was expected to be detectable scrutinising the metabolome as well as algal growth at seven growth stages (t_0, t_4, t_6, t_8, t_{10}, t_{12} and t_{14}) after 0, 4, 6, 8, 10, 12 and 14 hours of exposure. Changes of the metabolic patterns were estimated in comparison to reference samples of similar growth stages. Time dependent changes of the hydrophilic and lipophilic metabolic compositions were found due to metabolic differences for each analysed growth stage. The metabolome of treatment samples also showed a time dependent trend, but were different from that of references. Moreover, the metabolic composition was responding at earlier growth stages (4 hours) than algal growth in cell size. Thus, the developed metabolomics technique was applicable for the identification of time related changes in hydrophilic and lipophilic metabolic compositions, using the multivariate statistical method PCA. The Metabolomics approach was shown to detect responses at early growth stages after 4 hours of *Scenedesmus vacuolatus* exposure to prometryn, which might be suitable for the early detection of effects in ecotoxicology.

Based on pattern recognition, the approach was able to enlighten and to describe the effects of prometryn on the hydrophilic and lipophilic metabolic composition of *S. vacuolatus*.

However, the development of the metabolomics approach led to some continuing questions from the physiological point of view, such as:

(1) Which are the most responding biochemical components to prometryn?
(2) What are the identifications of these components?
(3) What concentration dynamics do these metabolites show in response to the chemical?

Accordingly, an identification and characterisation of the dynamics of important metabolites responding to prometryn could provide further important information. For this reason one demonstrative data set from hydrophilic metabolite extracts from *S. vacuolatus*, obtained from the study of time dependencies, was studied. A data evaluation method was established and approximately 40 identified biochemically important metabolites were selected for further studies, using PCA. The concentration dynamics of these identified metabolites were studied with semi-quantitative peak area information. The dynamics of the abundance of metabolites was interpreted regarding biochemical interactions of the organism with prometryn, such as the disturbance of energy metabolism, which can be related to the impact of prometryn blocking the energy supply from the photosynthesis. The additional application of the concept

"phenotypic anchoring" – the link of phenotypic and metabolomics information – supported the understanding and interpretation of metabolomics results.

In the first part of the discussion, the actual state of this work is critically addressed and perspectives in optimisation of the metabolomics protocol are recommended. In the second part, the compound identification is discussed and in the third, metabolomics results for hydrophilic compounds from 14 hours old reference samples of *S. vacuolatus* are compared to those of two other chlorophytes, described by the literature. The ecotoxicological application of the developed metabolomics approach along with the use of phenotypic anchoring is addressed the fourth part of the discussion.

In conclusion, the developed metabolomics approach can be applied as a screening tool to detect changes in the metabolic composition of the chlorophyte *S. vacuolatus*. Due to time- and concentration-dependent metabolic patterns, found in this study, the demonstrated metabolomics technique can be applicable for classification of effects of chemicals and therefore, suitable for ecotoxicological studies. Furthermore, the potential of metabolomics was shown to reach a deeper biochemical-physiological insight into organism-chemical interactions on the metabolite level. Consequential, the developed metabolomics approach was shown to be helpful to enlighten the mechanism of toxic action of chemicals, due to additional gained information compared to classical ecotoxicological parameters, e.g. mechanistic action of prometryn on the metabolite level of the organism.

Introduction

1 Introduction

Thousands of currently used and new chemicals were found in the environment with specific or unspecific modes of action, such as pesticides and industrial chemicals. All of those chemicals are potentially toxic to the biosphere and many of them are found in the aquatic environment (Schwarzenbach *et al.*, 2006). One major task of ecotoxicology is to provide scientific tools and methods for the understanding and predicting of chemical-induced effects in the biosphere (Newman, 2001).

Ecotoxicological Bioassays

One of the most commonly used techniques in ecotoxicology is the application of the principle of bioassays to assess the efficacy of chemicals. A bioassay is defined as an experiment for estimating the effects of single or mixtures of chemicals or environmental samples on biological parameters, such as mortality, growth or reproduction of single, selected living test species (Eggen and Segner, 2003).

For the assessment of the toxic potential of chemicals using bioassays the application of small organisms has been described as advantageous for several reasons (e.g. by Eggen and Segner, 2003):

(1) The organisms react faster due to a shorter life cycle and thus, a more rapid analysis of effects can be possible.
(2) Cultures can be maintained under highly controlled laboratory conditions.
(3) The reproducibility of the results is increased.
(4) One sample can represent a mean value of many individuals.
(5) Smaller sample volumes are required and allow, therefore, work- and costs-efficient analyses.

Nevertheless, bioassays with single species may lead to an over- or underestimation of the actual effects that occur in the environment. Overestimation or false positive results show a significant effect of toxicity in the laboratory but not in the environment. In contrast, underestimation or false negative results show no significant effect of toxicity in the laboratory test, but in the environment.

Aquatic Toxicology

Aquatic toxicology is defined as the qualitative and quantitative study of the adverse or toxic effects of chemicals and other anthropogenic materials or xenobiotics on aquatic organisms (Rand & Petrocelli, 1985). To assess

the toxic potential of chemicals in the aquatic biosphere with laboratory studies various bioassays are applied studying small plant and non-plant organisms. For example, chlorophytes are relevant test organisms in phytotoxicity assessment for regulatory purposes such as OECD guideline 201 (2006) or ISO 8692 (2002).

Phytotoxicity Assessment with *Scenedesmus vacuolatus*

Chemicals may directly or indirectly affect relevant aquatic ecosystem functions, such as primary production, where the macro- and microalgae occupy the lower trophic levels within food webs. Due to the fact that changes in the diversity and abundance of macro- and microalgae could have an indirect but significant effect on the freshwater community, they are often used in environmental toxicity assessment. Fundamental ecotoxicological research is performed with the chlorophyte *S. vacuolatus*, e.g. studies on mixture toxicity concepts (Altenburger *et al.*, 2004), mode-of-action analysis (Adler *et al.*, 2007; Altenburger *et al.*, 2006 and Neuwoehner *et al.*, 2008) or effect-directed identification of phytotoxicants in environmental samples (Brack *et al.*, 1999). Therefore, the test organism *Scenedesmus vacuolatus* provides good information about currently used observation parameters in ecotoxicology. Moreover, synchronous culturing of the green freshwater alga *S. vacuolatus* in clonal populations, can be of advantage as it keeps variability between sample replicates and experiments low and it can therefore minimise variability of results, which is not related with toxicity

s-Triazines

Faust *et al.* (2001) reported that *s*-triazines are highly toxic to algae and other primary producers in aquatic systems. *s*-Triazines, such as prometryn and atrazine, are widely used as active ingredients of herbicides in weed control. They inhibit the photosynthetic electron transport by binding to the plastochinone QB niche of the D1 protein of the photosystem II (PS II) reaction centre, thus displacing the electron acceptor (Fuerst & Norman, 1991; Wakabayashi & Böger, 2004). However, *s*-triazines have contaminated surface and ground waters (Solomon *et al.*, 1996; Guilliom *et al.*, 1999). For example, Brack *et al*. (1999) reported the detection of prometryn besides other chemicals in the riverine Spittelwasser in the industrial region of Bitterfeld (Germany). *S. vacuolatus* cell reproduction is inhibited by 50% (EC50) when treated with 0.05 - 0.06 µmol L^{-1} (~14 µg L^{-1}) prometryn (Faust *et al.*, 2001; Grimme *et al.*, 1998). The federal ministry for the environment, nature conservation and nuclear safety (BMU, 2004) reported a limit value of 0.5 µg L^{-1} prometryn for a good

quality of surface waters. Nevertheless in 2007, Rotter (2008) detected a mean prometryn concentration of approximately 0.75 µg L^{-1} river water in the region of Bitterfeld. The author described that prometryn reached its highest value during the same year in May and June with approximately 1.2 and 1.7 µg per litre river water. Rotter (2008) applied the PICT concept (pollution induced community tolerance) to microalgal communities and detected effects after exposure to 0.02 µg L^{-1} prometryn during long-time field studies. Therefore, amongst other *s*-triazines, prometryn can be applied as a reference substance on chlorophytes with a clearly understood mode of toxic action and real environmental exposure (Schmitt-Jansen & Altenburger, 2005).

Ecotoxicological Parameters of Effect

In ecotoxicological bioassays with chlorophytes, growth of the cell, for example in cell size and biomass of an organism, is one of the most important effect parameters studied because it is representing algal health. Eggen and Segner (2003) report that classic measurement of endpoints, such as death or retarded growth, were developed in the 1970s and -80s along with the development of ecotoxicological methodologies for the effect assessment on acute exposure to high concentrations. The authors describe that these descriptive and relatively crude parameters do not enable mechanistic conclusions. The concentrations of many chemicals in the environment decreased in the last decades and new difficulties appeared, such as the presence of low doses, multiple effects of the same compound and chemical mixtures. Therefore, often the effect can not be predicted from chemical analytics anymore and new bioanalytical approaches are necessary, supporting the assessment and extrapolation of possible sublethal effects.

Among others, the parameter growth is a well-defined physiological endpoint of a cascade of biological (biochemical, physiological or even morphological) responses to a chemical. Thus, these responses can result from the reaction of the biological system to the binding of chemicals to its primary target (Anderson & Conning, 1992). Knowledge on the molecular interactions could be useful to classify the mechanism of action of a chemical and hence allow extrapolation or generalisation for specific classes of chemicals (Escher & Hermens, 2002). For such an extrapolation of the mode of action of chemicals, there is a need for a more detailed understanding of the molecular mechanisms and molecular processes, such as the response of the biochemical metabolism in *S. vacuolatus* to toxicity. However, in most cases molecular interactions of chemicals are widely unknown. Therefore, in addition to previously-mentioned objectives,

ecotoxicology aims to identify the underlying molecular events that lead from initial exposure to the chemical to the ultimate manifestation of toxic injury in an organism, which is called effect translation. Thus, the question to address is 'What are the downstream metabolic processes of toxic exposure in an organism and how does the organism deal with the injury?'.

Metabolites, Metabolome and Metabolomics

Metabolites

Metabolites are small biochemical molecules. These molecules provide starting material, intermediates and products of the metabolism and are required for normal functioning such as maintenance, growth, development and reproduction of a cell or organism (Dettmer *et al.*, 2007). In addition, those molecules are classified as primary metabolites. Secondary metabolites are not directly involved in these processes, but usually have important functions, e.g. alkaloids to protect the plant against predators.

In contrast to genes and proteins, which are based on sequence or structural similarity, metabolites are constructed from a large diversity of functional chemical groups. Hence, they show up in a complex mixture of chemicals with a large range of chemical diversity, such as amino acids, sugars, nucleotides, organic acids, organic phosphates, aromatic acids, (poly)amines, fatty acid esters, fatty alcohols, sterols and terpenoids (Fiehn *et al.*, 2000 a; Sumner *et al.*, 2007).

Metabolome

The overall metabolic composition present in a biological system is defined as the "metabolome" or the metabolic profile (Fiehn *et al.*, 2000 a). Thus, the metabolome can be the metabolic compositions of cells, organisms or even of a community containing more than one individual or species (meta-metabolome).

The number of metabolites in an organism is species-specific. For example, the yeast metabolome has been estimated to contain approximately 600 metabolites (Förster *et al.*, 2003), but the metabolome of higher organisms is reported to be more complex. In the metabolome of plants 15,000 metabolites were estimated within one species (Sumner *et al.*, 2007) and over 200,000 structures of metabolites are estimated to occur within the plant kingdom (Fiehn *et al.*, 2001 and Fiehn, 2002). Plants and microbes are also known to contain more than 200,000 secondary metabolites (Hartman *et al.*, 2005). Sumner *et al.* (2007) conclude that plants could

contain the most complex metabolome of all organisms due to the high diversity of their secondary metabolites.

Moreover, the metabolome has a temporal component as some metabolites may reflect an immediate response to an environmental stimulant over time, while others represent effects from longer time periods, such as minutes, hours and days, respectively. Therefore, accounting for high time-resolution of the analysis of the metabolic profile can provide functional information of a biological system and biochemical pathways (fluxomics).

Metabolomics

Metabolomics is defined as the unbiased and non-hypothesis-driven approach focussing on the simultaneous analysis of large groups of biomolecules (e.g. Sumner *et al.,* 2007 and Dunn *et al.,* 2005). The metabolomics approach is a powerful approach for the comprehensive qualitative and quantitative analysis of metabolites in a biological system. A global view on *in vivo* dynamics from metabolites is achieved with metabolomics (Glinski and Weckwerth, 2006) and the range of this approach is what it differentiates it from the other approaches, such as metabolic profiling or metabolic fingerprinting, which are described and differenciated, for example by Sumner *et al.* (2007). Metabolite profiling or metabolic profiling is a targeted approach for the identification and quantification of a limited number of metabolites related through similar chemical structures or metabolic pathways (e.g. Sumner *et al.,* 2007 and Dunn *et al.,* 2005). Metabolic fingerprinting is an unbiased, cumulative approach for the classification and screening of samples. Identification and quantification is not necessarily performed (e.g. Sumner *et al.,* 2007 and Dunn *et al.,* 2005). Metabolomics is applied as a non-targeted technique to gain valuable qualitative and quantitative information about endogenous metabolites of a biological system.

Among other analytical platforms for the metabolomics approach, gas chromatography-mass spectrometry (GC-MS) is applied. The GC-MS system captures a low detection limit of 1 ng (Donzé *et al.,* 1998) and is highly sensitive for the analysis of metabolites (Dunn *et al.,* 2005). The analysis results in a metabolic composition usually containing both known and unknown analytes useful for the characterisation of the metabolome at a specific time point and under defined conditions. Approximately 300-500 low-molecular weight metabolites can be detected and approximately 100 hydrophilic and 100 lipophilic components can be identified with current custom libraries (Sumner *et al.,* 2007). A GC-MS system has an upper mass range of 1000/ 1500 Dalton. For example, the unified atomic mass unit (u) of amino acids ranges between 75 (Gly) and 204.23 (Trp). Many secondary

metabolites also have atomic masses less than 1000. Hence, GC-MS analysis enables the multi-parallel analysis of a large number and diversity of hydrophilic and lipophilic low molecular weight metabolites (primary and secondary). Therefore, the application of a GC-MS-based metabolomics approach can enable detection of metabolites and dynamics in the metabolome responsible or involved into responses to chemicals on the majority of primary metabolites. Hence, this approach can provide a deeper insight into metabolic states and dynamics useful to understand and enlighten molecular and cellular processes in response to chemicals.

Environmental Metabolomics

Lin *et al*. (2006) and Viant (2007) review applications of metabolomics approaches to several non-plant organisms under biotic or abiotic stress conditions, but environmental plant metabolomics studies are also published. For example, Bölling & Fiehn (2005) developed a metabolite profiling procedure for the unicellular green alga *Chlamydomonas reinhardtii* under nutrient deprivation and gained more than 100 low molecular weight hydrophilic compounds. As a result, the authors recommended precisely controlled growth conditions for the alga and homogenous sample material to study complex metabolic changes. Furthermore, e.g. Kaplan *et al.* (2004) studied the tolerance mechanisms of tale cress (*Arabidopsis thaliana*) in response to temperature stress. Roessner *et al.* (2006) investigated tolerance mechanisms of barley (*Hordeum vulgare)* against exposure to toxic boron concentrations. Cadmium stress on *A. thaliana* was determined by Sarry *et al.* (2006) and on bladder campion (*Silene cucubalus*) by Bailey *et al.* (2003). Johnson *et al.* (2003) studied salt stressed tomatoes. In the final example, Desbrosses *et al.* (2008) studied the metabolome of the legume (*Lotus japonicus*) during the nitrogen fixation symbiosis that takes place during the interaction of the plant with rhizo-bacteria (*Rhizobiaceae*). To date, no plant metabolomics study has been found compiled under the aspects of aquatic ecotoxicology and microalgae. Moreover, metabolomics approaches in ecotoxicology are rare (see below).

In summary, the authors publishing in the field of environmental metabolomics recommend the metabolomics approach as appropriate to detect alterations from metabolites in response to environmental stimuli. As the biochemical regulation of the metabolism of an organism is extremely sensitive to exogenous stimulation, e.g. due to enzymatic activity, the metabolome can alter within seconds (Morgenthal *et al.,* 2006; Lisec *et al.,* 2006). Hence, multi-parallel metabolite analysis may discover previously unknown metabolites and unknown regulatory functions of metabolites in

metabolic pathways (Keurentjes *et al.,* 2006). Furthermore, metabolomics is a promising approach to support ecotoxicological investigations on the molecular level due to the high complexity of the metabolome. Thus, the information about alterations and disturbances in the metabolic composition, e.g. of an organism, induced by a toxicant, could be correlated to those of the phenotype, such as traditional observation parameters of toxicity (e.g. growth or reproduction) and therefore support the revelation of the mechanism of toxic action of the chemical.

Viant *et al.* (2006) studied toxic actions of dinoseb in medaka fish embryos (*Oryzias latipes*) and suggested that not only alterations of metabolic concentrations can be seen in the targeted system, but also effects on the different biochemical pathways, due to primary and secondary effects. The authors identified several potentially affected metabolic pathways after exposure to those chemicals, causing effects on the health of the organism, which was comparable to Sarry *et al.* (2006), who studied the effect of cadmium exposure in *A. thaliana* cells. Thus, metabolomics could be used, to define at-risk plant populations or communities (Schnackenberg & Beger, 2007). A risk is defined as the probability that an undesired event or effect may occur (Addiscott and Smith, 1998). Certain impacts on the organisms health, in response to chemicals, are species specific (Henning, 2007) and each species may contain its own chemotypical expression pattern (Keurentjes *et al.,* 2006). Using a range of various model species in ecotoxicological studies, should enable to cover as many levels of sensitivity as possible, which can support a more substantiated risk assessment.

Linking the Metabolome with the Phenotype

Organisms exposed to a chemical may show phenotypic restrictions expressing their compromised health state, e.g. growth retardation and reproduction inhibition. Molecular changes can be demarcated as effects of toxicity by linking them to phenotypic outcomes in the biological system. Hence, Tennant (2002), Waters & Fostel (2004), Ankley *et al.* (2006) and Viant (2007) called for phenotypic anchoring. Phenotypic anchoring is defined as a process of determining the relationship between a particular expression profile and the toxicological phenotype of the organism for a particular exposure or dose and at a particular time (Tennant, 2002). The authors recommended the application of a proof-of-principle concept prior to characterising molecular responses to environmental stressors, to minimise unrelated signals and therefore demarcate toxicant-induced changes of compounds from algae in the metabolomics data set. Applying this concept, authors like Ankley *et al.* (2006) see the use of time and

concentration-dependent molecular data in toxicological risk assessment and the suitability of this information for regulatory decision making for biomarkers of toxic injury. This fact has been underlined by Waters & Fostel (2004), who report the use of phenotypic anchoring in conjunction with lower doses of the toxicant to classify agents and to explore molecular mechanisms of toxicity that occur prior to phenotypic outcomes.

Aims of this Work

The aim of this work was to develop a metabolomics approach suitable for toxicity analysis in a microalgal population. To enable good repeatability and broad applicability of the approach a standardised protocol was developed. The multistep protocol included: (1) Synchronous culturing of the chlorophyte *S. vacuolatus*, (2) standardised harvest of algal biomass, (3) sample storage, (4) tissue homogenisation and metabolite extraction, (4) preparation of extracts for the analysis with a GC-MS system, (5) analysis of hydrophilic and lipophilic, low molecular weight plant metabolites and (6) quantitative and qualitative multivariate data analysis.

Synchronous culturing of clonal populations of the unicellular green alga *Scenedesmus vacuolatus* was used to achieve low variability between the analytical metabolomics results. In addition, efforts were made to ensure reproducibility of the results from different experimental approaches. A further objective was the development of a procedure providing demarcation of compounds from the chlorophyte and disregarding contaminant compounds (e.g. from the sample preparation procedure). As a means of ecotoxicological research the validated metabolomics approach should be applicable for the analysis of effects of toxicity in the metabolome. Therefore, concentration- and time-dependencies of the algal metabolome were investigated using the model chemical prometryn. The data evaluation required the establishment of a protocol for the qualitative and quantitative analysis of the multivariate data matrices of a number of samples.

From the physiological point of view, the analysis of the methodological potential was studied to gain deeper information about the metabolites responding to prometryn. Hence, a data evaluation procedure was developed to identify biochemically interesting metabolites and to investigate the dynamics in the metabolite concentration. With this additional information metabolic results were interpreted and primary and secondary responses were elucidated. The concept of phenotypic anchoring was used with the purpose of linking changes in the metabolome to distinct developmental stages of the chlorophyte and to toxicological relevant responses to the exposure to prometryn.

Material and Methods

2 Material and Methods

The description of the Material and Methods part of this work is divided into six chapters describing (1) the solvents and chemicals used, (2) the culturing, harvest and storage of *S. vacuolatus* clonal populations, (3) the applied bioassay to study effects of toxicity, (4 + 5) the sample preparation and metabolite analysis, and (6) the method of qualitative and quantitative data analysis.

2.1 Solvents and Reagents

Chemicals, such as methanol and chloroform, were purchased from Merck (Darmstadt, Germany) in the highest purity grade available. 2% Methoxyamine•HCl in Pyridine (MOX) and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) were acquired from Pierce (Rockford, USA). Hydrochloric acid in methanol (methanolic HCl) was purchased from Supelco (Bellefonte, USA). 99.7% pure prometryn (2-methylthio-4.6-bisisopropyl-amino-1. 3. 5-triazine, CAS RN 7287-19-6) was available from Riedel-de-Häen (Seelze, Germany) and trifluoroacetic acid (99.5%) from Fluka (Buchs, Switzerland). Retention index standards for GC, consisting of a mixture of aliphatic hydrocarbons in hexane, was supplied by Sigma (Saint Louis, USA).

2.2 Test Organism, Cultural Maintenance and Sampling

This method chapter describes the work flow to cultivate, harvest and store samples of the green alga *Scenedesmus vacuolatus* in preparation for the metabolite analysis.

2.2.1 Test Organism and Cultural Maintenance

The unicellular fresh water green alga (*Scenedesmus vacuolatus* Shih. *et* Krauss strain 211-15) was acquired from the culture collection Pringsheim (SAG Göttingen, Germany). Populations were grown in sterile, inorganic nutrient, liquid medium (pH 6.4) modified after Grimme and Boardmann (1972) as given in Faust *et al.* (1992). During a 24 hours cell cycle (Figure 1), algae were cultured under synchronous conditions, as described by Faust *et al*. (2001) and Altenburger *et al*. (1990). Thus, *S. vacuolatus* was sustained by cultivating the alga each day in fresh medium inoculated with 10^6 cells per mL from a previously grown *S. vacuolatus* population. The following culture parameters were used: illumination with saturated white light at an intensity of approximately 400 µmol photons s^{-1} m^{-2} in a

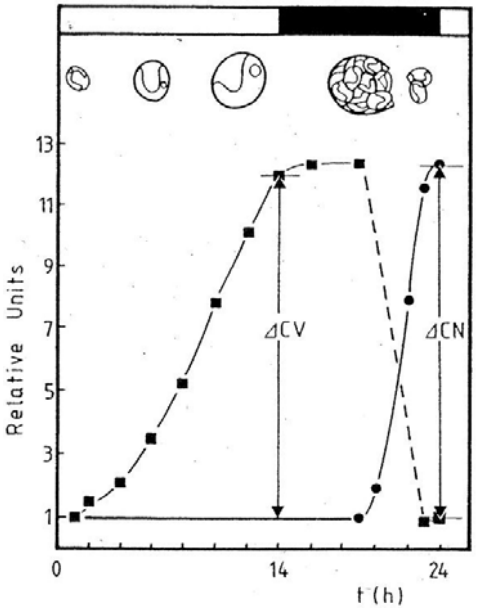


Figure 1: The 24 hours life cycle of synchronised *Scenedesmus vacuolatus* cells under standardised conditions with a 14 hours light and a 10 hours dark phase (copy from Altenburger *et al.* 1990). The parameters cell volume (CV; ■) and cell number (CN, (●) were used to quantify biomass-volume for harvest and toxicity of prometryn on algae during the 14 hours light phase. At the end of the dark phase cells divide and release autospores.

14:10-h light:dark cycle, bubbling regime with compressed air and 1.5% CO_2 and 28 °C medium temperature. Furthermore, cultural synchronisation was monitored daily according to Altenburger *et al*. (2004).

2.2.2 Determination of Cell Density and Algal Growth

Parameters of algal growth and cell density were determined using an electronic cell analyser (CASY®1, Schärfe System GmbH, Reutlingen, Germany). Algal cell number, cell diameter in µm and cell volume in fL (femto litre; 1 fL = 10^{-9} µL) were measured during algal growth phase and quantified by applying a protocol presented by Altenburger *et al.* (2008).

2.2.3 Harvest

Standardised 10 µL biomass-volume of algae cells were harvested per sample from approximately 50 to over 600 mL of cultural medium, in order to compare algal extract composition from samples containing similar amount of biomass. Thus, for the harvest the total volume of cultural medium (HV_{tot}) was calculated according to Equation 1. The required biomass-volume (BM_V), in this study 10 µL, was divided by the product of the number of cells per mL (CN) and the mean volume of cells (CV) measured in femto-litre (fL) which equals 10^{-9} µL. CN and CV were measured with an electronic cell analyser CASY (®1, Schärfe System GmbH, Reutlingen, Germany) as described above.

$$HV_{tot} = \frac{BM_V}{(CN * CV)} \quad (1)$$

Cells were harvested using the vacuum driven AS 310/3 three-place filtration system (Schleicher & Schuell GmbH – Whatman Group, Dassel, Germany) with polycarbonate Isopore membrane filters (0.4 µm pore size; Millipore S.A.S., Molsheim, France) due to better time and work efficiency with many samples of large volume compared with other techniques

available. In addition, a purpose built 2.5-litre bottle was installed for the collection and disposure of large amounts of filtrate (Figure 2), between the vacuum pump and the filtration system.

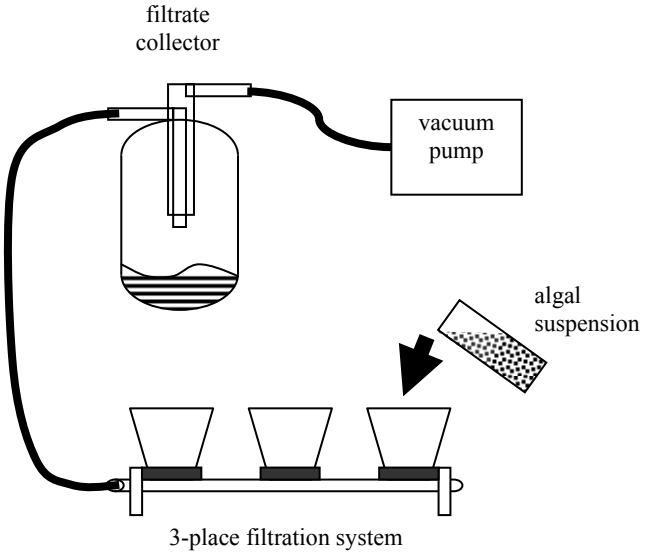


Figure 2: Experimental design with a a vacuum driven 3-place multi filtration system to harvest 100 to 600 mL of the cell suspension from the chlorophyte *Scenedesmus vacuolatus*. After filtering the algal suspension the filtrate is collected in a 2.5 L bottle, which is placed between the multi-filtration system and the vacuum pump.

During filtering, algal cells were washed twice with 20 mM NaCl in bi-distilled water, which corresponds to the molarity of salts in standard cultural medium. Subsequently, the biomass was washed from the filter with 20 mM NaCl into an 80 mL tube and centrifuged at 3300 x g for 5 min at 22 °C. The resulting pellet was transferred into a 2 mL tube with screw cap and the supernatant was discarded. After further centrifugation at 4000 x g for 10 min at 22 °C, the supernatant was removed and the remaining pellet with algal cells was quick-frozen in liquid nitrogen. In general harvest from filtration to freezing of samples in liquid nitrogen took approximately 20 minutes for three samples and approximately two hours for more, e.g. 12, samples.
In addition, procedure blanks were prepared to detect contaminations of the alga metabolome deriving from the metabolomics procedure up to the sample analysis by GC-MS. The blanks were prepared by filtering the average amount of Grimme and Boardman cultural media as harvested for reference samples. In the following procedure, blanks were treated the same as algal samples.

2.2.4 *S. vacuolatus* Sample Storage and Conservation

After harvest, samples were stored at -80 °C for a maximum of three days until lyophilisation. Samples were freeze dried in their frozen state for

approximately 3 days at -40 °C and 0.2 mbar in a Lyovac GT2 lyophilisator (Finn Aqua GmbH; Hürth, Germany). Afterwards, the dried cells were either directly disrupted for metabolite extraction or stored at -80 °C for a maximum of two months.

2.3 Bioassay to Study Effects of Toxicity

A bioassay was applied to validate the metabolomics approach for the analysis of effects toxicity. Chemical toxicity to a living system depends, among other phenomena, on exposure, which is a function of dose and time (Rozman and Doull, 2000). Additionally, the concept of phenotypic anchoring was recommended, for example by Tennant *et al.* (2002), to define effects of toxicity in gene expression data. Thus, the influence of various concentrations of a model chemical as well as of the experimental exposure time on the metabolite composition and the growth of synchronous grown algae were investigated.

2.3.1 Used Chemical to Study Effects of Toxicity

The reference chemical prometryn with the molecular structure $C_{10}H_{19}N_5S$ (molecular weight = 241.37) was used to study effects of toxicity on *S. vacuolatus* and to validate the demonstrated metabolomics approach due to a link of metabolic effects of toxicity to the algal phenotype, as well as to study the mechanism of action of a toxicant. This *s*-triazine is reported as a PS II-inhibiting herbicide (Wakabayashi and Böger, 2004), which is shown to be toxic to algae and to disturb growth of *S. vacuolatus* (Altenburger *et al.,* 1990). All experiments were carried out using a stock solution containing a concentration of 0.103 mmol prometryn per litre dimethylsulfoxide (DMSO).

2.3.2 Treatment of *S. vacuolatus*

Toxicity assays started with a homogenous population of genetically identical autospores (young algal cells at the beginning of cell cycle). Exposure experiments were performed with an initial density of 10^6 algal cells mL^{-1} in sterilised 600 mL glass cylinders with conical bottom and a cap. Cylinders were filled with algal cell suspension, cultural medium and the test substance dissolved in DMSO to reach a total cultural volume of 300 to 600 mL. In all samples of a toxicity assay the end concentration of DMSO was kept at a constant level of 0.1%, which is known to cause no observable effects on *S. vacuolatus* growth and its metabolites. Algae were exposed during growth phase as the treatment started with the inoculation of cultures with autospores (t_0) and lasted up to 14 hours.

Concentration-Response Investigations

To investigate concentration dependent responses of algae, four cultures, treated with 0.1% DMSO, served as references. Eight cultures with different concentrations of prometryn with a serial dilution step of 1:2 of the stock solution were incubated with the final nominal concentrations in the test tubes ranging from 0.002 to 0.3 µmol L^{-1} prometryn (Table 1). For the investigation of concentration responses of algal metabolites and growth, populations were exposed during under illumination, from 0 to 14 hours of culturing.

Table 1: List of prometryn concentrations used for the exposure of *S. vacuolatus* cultures during the 14 hours developmental phase under light conditions to study concentration-dependent responses in the algal metabolome as well as in the algal cell volume (size).

Prometryn Concentration in:	
µmol L^{-1}	µg L^{-1}
0.002	0.5
0.004	1
0.01	2
0.02	4
0.03	8
0.07	16
0.13	32
0.26	64

Time-Response Investigations

The study of responses to prometryn during algal growth phase, two samples from autospores at t_0 were used as references, representing starting point of the time course. Six further time points of algal growth phase were investigated from growth stage 4 hours, with a two-hour sampling interval. Hence, algae were sampled after 0, 4, 6, 8, 10, 12 and 14 hours of growth during light conditions. Six single samples served as references and two of each algal culture served as treated samples at each time point. Treated populations were exposed to a final concentration of 0.1 µmol L^{-1} (~ 24.75 µg L^{-1}) prometryn and 0.1% DMSO.

2.3.3 Phenotype Investigation

As a means to link metabolic compositions of algae to distinct developmental stages and to demarcate alterations caused by the chemical, the concept of phenotypic anchoring was applied. Algae were grown in a synchronous regime. Algal growth was determined by measuring cell volume and cell diameter (as described in Section 2.1.2). The trend of mean

algal cell volume development during the 14 hours growth phase was visualised using Origin Pro 7.5G SR3 software (edition 7.5853 (B583), 2004; Origin Lab, Southhampton, USA), shown for example in Figures 13 and 14.

Linking Metabolic Data with Information about the Phenotype of *Scenedesmus vacuolatus*

The concept of phenotypic anchoring was applied in this study to link phenotypic data to those of the metabolic composition of *S. vacuolatus*. Therefore, various phenotypic parameters, such as survival, growth or reproduction of the alga, were consulted investigating algal growth in this study as well as studying literature, e.g. from Adler *et al.* (2007), Neuwoehner *et al*. (2008) and Altenburger *et al.* (1990). A list of measured parameters found for *S. vacuolatus* is given in Table 6.

Linking Published Time Points of Effects of Toxicity in *S. vacuolatus* with those Presented in the Metabolomics Approach of this Study

Furthermore, time points of measured effects in the metabolome from *S. vacuolauts*, investigated in this study were compared with those of other observable effects in the alga during treatment with *s*-triazines (Table 6, Section 4.4.1), described by the literature. For example, ATP concentration was studied by Maretzek (1992) or algal photosynthesis by Schmitt-Jansen and Altenburger (2007). The physiological link of metabolomics data was applied to support validation of the metabolomics approach to demonstrate suitability of the data for toxicity analysis as well as to support the understanding of the mechanism of action that can explain toxicity induced variation in metabolite profiles.

2.4 Algal Metabolite Extraction and Separation of Hydrophilic and Lipophilic Metabolite Fractions

In the following chapter, a protocol will be introduced to extract metabolites from *S. vacuolatus* cells and to separate these metabolites into hydrophilic and lipophilic phases for the metabolomics study.

2.4.1 Metabolite Extraction

Metabolites were extracted from the algal cells using a protocol, developed earlier by Laue (personal communication). Each 2 mL micro tube with screw cap and sample material was filled with 1 mL of a 4 °C cold single phase solvent mixture of methanol, chloroform and 0.015% trifluoroacetic acid in water (10:5:4, v/v/v) and 150 mg of washed glass beads (0.5 mm

diameter; Carl Roth GmbH + Co, Karlsruhe, Germany) were added. Glass beads were washed with sulphuric acid and rinsed with bi-distilled water until a neutral pH was reached, before use. Cells were crushed using a cell disruptor (Genie, Milian; Meyrin, Switzerland) at 5 °C for 15 min in the dark. For this procedure, SupraSolv® solvents (Merck) with less than 0.05% of water and solvent stable materials were used, such as glass material as well as tubes and pipette tips from Eppendorf (Wesseling-Berzdorf, Germany) to minimise contaminations of samples during the procedure which can lead to an overlay of metabolite signals from the alga. The success of cell disruption or destruction of cell walls was assessed with a BX60F5 microscope and a 100 x / 1.35 Oil Iris (Olympus Optical Co, LTD, Japan). Furthermore, the extracts showed a green colour which is likely to be due to extracted chlorophylls. The tube was centrifuged for 5 min at 20,000 x g and 5 °C (Sigma 2 K15; Osterode, Germany). For further processing 0.9 mL of the supernatant of all samples were decanted into a 10 ml screw-top glass tube. The residual as well as the pellet containing cell matrix and glass beads were discarded.

2.4.2 Separation of Hydrophilic and Lipophilic Metabolites

In order to separate hydrophilic from lipophilic metabolites and unwanted polymers, such as proteins and RNA, a liquid-liquid or two-phase extraction was performed based on a method by Laue (personal communication). Phases were separated by adding 0.8 mL of water and 1.6 mL of chloroform, shaking the mixture intensively for 20 s and centrifuging for 10 min at 5,000 x g and 5 °C (Hettich; Tuttlingen, Germany). Finally, three phases were separated (Figure 3).

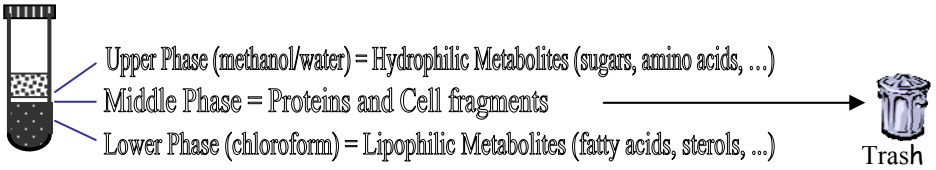


Figure 3: Three extract phases derived from the liquid-liquid extraction of *S. vacuolatus* hydrophilic (upper phase) and lipophilic (lower phase) metabolites. The middle phase, containing proteins and cell fragments was discarded.

Phase extracts, either from the upper 0.9 mL colourless hydrophilic methanol/water phase (methanol comes from the metabolite extraction solvent) or from the lower 1.5 mL green lipophilic chloroform phase, were transferred separately into a 3 mL pre-cleaned reaction vial with screw cap and PTFE/red rubber septa (Supelco; Bellefonte, USA). The white middle phase, containing mostly proteins and RNA (Weckwerth *et al.*, 2004) and the residues of the upper and lower phases were discarded.

The 3 mL reaction glass vials were cleaned with a Mucasol glass cleaner (Brand; Wetheim/Main, Germany) by sonication and washed with acetone and water prior to cleaning an apparatus dishwasher to avoid contamination of samples.

2.5 Analysis of Hydrophilic and Lipophilic Metabolites with a GC-MS system

Complex mixtures of compounds, such as plant metabolite extracts, can be analysed by gas chromatography-mass spectrometry (GC-MS) if they are volatile and thermally stable and if they can be separated by gas chromatography. Identification of compounds requires mass spectrometric detection of undamaged structures within a complex metabolite mixture, while some compounds fulfil these conditions, others do not. In order to improve volatility of hydrophilic and lipophilic compounds with various chemical properties, derivatives of functional groups were made.

2.5.1 Derivatisation of Algal Metabolites

In preparation for the derivatisation, extracts of hydrophilic and lipophilic metabolites, in 3 mL reaction vials, were dried under a stream of nitrogen and at 40 °C (Dri-block DB-3, Techne; Staffordshire, UK). A heating block (Dri-block DB-3; Techne, Staffordshire, UK) was used instead of a water bath for all temperature incubations above room temperature, to avoid increasing the water content of the sample, which could have been disrupted the derivatisation. Drying took a maximum of 3 hours for the hydrophilic phase and 0.5 hours for the lipophilic phase.

The two-step derivatisation protocols for hydrophilic and lipophilic metabolite extracts used in this work were established earlier and provided by Laue (personal communication). Laue modified derivatisation protocols specified earlier by Roessner *et al.* (2000) and Fiehn *et al.* (2000 a). Roessner and colleagues studied the metabolome of potato tubers (*Solanum tuberosum*), while Fiehn and others studied tale cress (*Arabidopsis thaliana*) leaves.

In the first step of the two-step derivatisation procedure, dried residues of hydrophilic algal extracts were incubated in 200 µL of 2% methoxyamine•hydrochloride in pyridine (MOX) for 120 min at 80 °C. During this procedure, keto groups are converted to methoxime derivatives. In addition, this procedure prevents formation of multiple derivatives when enols are present during silylation (product information; Pierce Biotechnology, Rockford, USA; http://www.piercenet.com/products/browse.cfm?fldID=03010305). Dried residues of lipophilic phase compounds were incubated in parallel under similar temperature conditions

with 300 µL methanolic-hydrochloride (HCl) for 240 min. With this reagent, methyl esters of volatile (short chain) fatty acids are formed to yield a better stability during GC-MS analysis (product information, Supelco, Bellefonte, USA). After methoximation or methanolysis reaction, samples were cooled to room temperature and dried for a maximum of 3 hours under stream of nitrogen at 40 °C.

In a second derviatisation step, dried residues of hydrophilic and lipophilic phases were silylated with 100 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) for 20 min at 90 °C. MSTFA replaces labile hydrogens on a wide range of hydrophilic compounds with a $Si(CH_3)_3$ group and is used to prepare volatile and thermally stable derivatives for GC-MS (product information; Pierce Biotechnology, Rockford, USA; http://www.piercenet.com/products/browse.cfm?fldID=03010306). It is reported that by-products and the reagent usually elute with the solvent front and thus contamination of metabolite signals from the alga is minimised.

After silylation in MSTFA, samples were equilibrated to room temperature and subsequently transferred to a 100 µL glass insert of a 2 mL brown glass vial, quickly capped with Teflon/red rubber crimp cap and stored at room temperature for a maximum of 3 days until analysis.

2.5.2 Retention Index Calibration Standard

Retention index (RI) can be used for the identification of GC-MS separated compounds. In this study, retention indices resulting from a homologous series of *n*-alkanes were calculated (Kovat's Retention Index). The RI calibration standard for GC analysis was obtained from Sigma Technical Bulletin (Saint Louis, USA) containing 23 *n*-alkanes in the range from C8 to C30 and the 24th n-alkane C32. The concentration of most components was 1 mg mL^{-1} in hexane. The concentration of *n*-Alkanes, where the number of carbon atoms was a multiple of five, was 2 mg mL^{-1} to allow easy determination of the carbon number of peaks of interest (product information; Sigma Technical Bulletin, Saint Louis, USA).

The standard was prepared as follows: an aliquot of the standard mixture was transferred into a reaction glass vial and concentrated under a stream of nitrogen. Afterwards, the residue was dissolved in MSTFA (see Section 2.5.1) to reach a final concentration of 0.01 to 0.02 mg *n*-alkane per mL. This mixture of *n*-alkanes was incubated in MSTFA for 20 min at 90 °C prior to equilibration to room temperature and then transfer brown glass GC-MS vials, ready for analysis. The silylation procedure was undertaken to obtain *n*-alkanes elution times similar to those that would be achieved if they were analysed as internal standards in algal samples (see Section 2.5.1).

The GC-MS analysis of the standards was performed separately from that of the algal samples to avoid overlap with analyte-peaks. The mixture of *n*-alkanes was analysed with the two GC-MS methods for hydrophilic and lipophilic algal extracts, at the beginning and the end of the GC-MS analysis sequence.

2.5.3 Gas Chromatography–Mass Spectrometry (GC-MS) to Analyse Hydrophilic and Lipophilic Metabolites

Derivatised samples of hydrophilic and lipophilic low molecular weight metabolites were analysed with a gas chromatography-mass spectrometry (GC-MS) system.

GC-MS System and Equipment

All system components originated from Agilent Technologies (Santa Clara, USA), including the 6890N gas chromatograph coupled to a quadrupole 5975 mass spectrometric detector. The system was equipped with electron impact ionisation (EI), an autosampler (7683 series) and an injector (7683B series). The machine was controlled by the instrument's ChemStation software (G1712DA, Rev. 02.00).

A 30 m long DB35ms column of middle polarity with a 0.25 mm internal diameter and a 0.25 µm thick film (Agilent Technologies; Santa Clara, USA) was installed in the GC-MS. This column was a low bleed column and highly inert. Additionally, its suitability for the analysis of hydrophilic compounds reduced system maintenance time. In addition, this type of column showed robustness for silylated samples (Laue, personal communication). To protect the main column against contamination, caused for example by non-volatile compounds, a 10 m deactivated pre-column was installed upstream of the main column.

GC-MS Method for the Analysis of Hydrophilic and Lipophilic Metabolites

Prior to the injection of the sample in MSTFA to the GC-MS system, the injector syringe was cleaned automatically to avoid sample contamination. The syringe was washed two times with fresh lipophilic hexane and secondly with hydrophilic 2-propanol solvents of SupraSolv purity (Merck; Darmstadt, Germany). The injector temperature was set at 280 °C and 1 µL per run was injected into the column, across a focus liner with glass fibre wool.

The split ratio (without pulse) was set to 3:1 for hydrophilic samples. Helium, the carrier gas, was set at an initial flow of 1.2 mL min^{-1}. Separation of hydrophilic metabolites was achieved with an oven temperature program of 1 min isothermal at 100 °C, followed by a

temperature ramp of 5 °C min^{-1} to 260 °C, followed by 20 °C min^{-1} temperature ramp to 350 °C and a final 5 min heating at 350 °C. Interface, quadrupole and ion source temperature were adjusted to 280 °C, 150 °C and 230 °C, respectively. Mass spectra were recorded in scan mode after a solvent delay of 4 min at 2.46 scans per second with a mass scanning range of m/z 50 to 650 and a threshold of 50. Retention time correction was done by using Agilent retention time locking software (RTL) in order to minimise run-to-run errors. The RTL head pressure was adjusted to the retention time of octadecane at 17.379 min and set at constant mode.
Solvents containing lipophilic compounds were analysed by GC-MS using the same parameters as described for hydrophilic phase, with the exception that the injection split ratio (without pulse) was set to 20:1 and the initial flow of helium to 1.5 mL min^{-1}. The initial oven temperature was set to 60 °C (1 min isocratic) followed by a temperature ramping with 5 °C min^{-1} to 320 °C and a final 3 min heating at 320 °C. For RTL, the head pressure was adjusted to eicosane with the retention time of 29.700 min.

2.6 Qualitative and Quantitative Data Analysis

Following the GC-MS analysis of algal metabolite extracts a first overview of the analytical results was made with the reconstructed total ion current or total ion chromatogram (TIC). As shown in Figure 4, a TIC contains three dimensional information, which is the retention time in minutes (x-axis), the total ion intensity or abundance (y-axis) and the mass spectrum for an individual retention time (z-axis). Note that one minute of the retention time consists of 100 time segments, not of 60 segments.

2.6.1 Qualitative Data Analysis

For a first insight into the 'TIC-pattern' of algal metabolite extracts, the instruments software ChemStation (G1712DA, Rev. 02.00; Agilent Technologies; Santa Clara, USA) was used, patterns of samples could be roughly assessed for similarities and differences using this software, by overlaying all compounds of two or more TICs. However, this method is not sufficient for quantitative data analysis, nor for a data set with more than 20 samples containing up to 600 components.

The assignment of metabolites were processed using the Automated Mass Spectral Deconvolution and Identification System software (AMDIS; edition 2.62; U.S. Department of Commerce-National Institute of Standards and Technology; Gaithersburg, USA). AMDIS analysis steps are (1) noise analysis, (2) component perception, (3) spectrum deconvolution and (4) compound identification. For further details about AMDIS see Stein (1999). Deconvolution parameters were set to components width of 12,

high resolution, low sensitivity, and high shape requirements. The following AMDIS analysis was set to RI Calibration and Performance and analyte-spectra were searched against the custom spectrum library from National Institutes of Standards and Technology (NIST; edition 2005) as well as against the "Golm Metabolome Database" for hydrophilic compounds (published by Kopka *et al.*, 2005). Applying the minimum spectrum similarity match factor of 20%, a rough insight into the metabolite composition of the chromatogram was gained. In addition, the mass spectral match factor of 90% was applied to gain biochemically more reliable assignments for the metabolite composition.

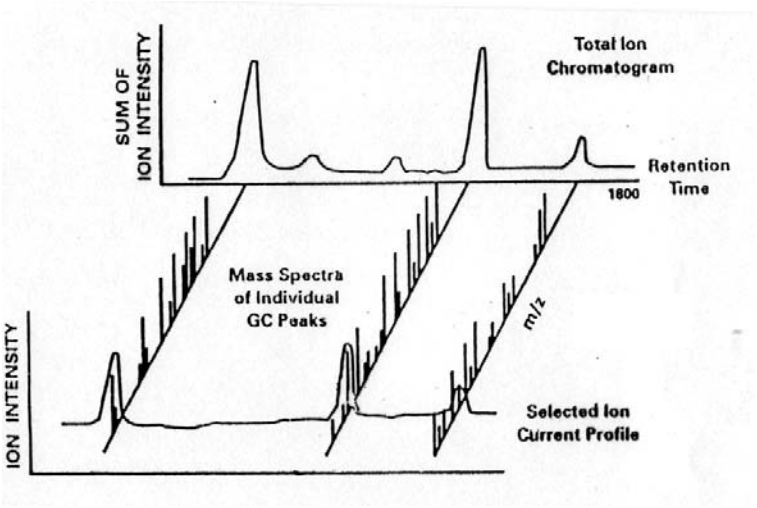


Figure 4: Scheme of a total ion current or chromatogram (TIC) from a GC-MS system to demonstrate the 3-dimensional information of the chromatrographic results in this study. Retention Time (x-axis), Total Ion Intensity (y-axis) and Mass Spectrum of an individual peak (z-axis) are shown.
(Copy from http://chem.tufts.edu/faculty/robbat/research/Research/Ultraf6.gif)

2.6.2 Quantitative Comparison of Chromatographic Results

A protocol for quantitative pattern comparisons was established, including predominantly manual data processing steps, to define a standard composition of metabolites from *S. vacuolatus* and to define changes between algal samples grown under different conditions, such as references and treatments. Values of chromatographic results or quantitative information about eluted compounds can be obtained by integration of peaks.

Applying the instrument's ChemStation software (Agilent, G1712DA, Rev. 02.00), parameters of peak integration were chosen to be suitable for a general application for all samples of one experimental procedure, e.g. all from hydrophilic extracts of an experiment. Finally, due to higher reproducibility of integration results at specific apical retention times, and therefore more simple manual alignment, peak area integration was used

with following parameters shown in Table 2. A three fold signal to noise ratio was applied.

The integration result tables for each chromatogram were exported to MS Excel software (Microsoft, USA), containing retention time- and peak area-values of integrated peaks. Afterwards, peak area data were manually aligned in the order of retention times. The chromatograms and mass spectra were studied in parallel using the ChemStation software (Agilent, Santa Clara, USA). Peak areas of adjacent unresolved signals were summarised in retention time windows. After alignment, format of data enabled quantitative comparison of the area of one and more chromatographic peaks between samples.

On the basis of an aligned data set, peaks originating from the organism *S. vacuolatus* were selected due to comparison of chromatograms with those from the procedure blanks. Contaminant peaks were also identified and those overlaying peaks from algae in an amount of more than 50% were excluded from further data examination. Sporadic peaks were also excluded from further data analyses. Hence, peaks from algae were selected for further data analysis. All remaining signals were accepted as originating from *S. vacuolatus* and data were used for further statistical analyses.

Table 2: Parameters, applied for the automatical integration (GC-MS system's ChemStation software) of the peak areas of chromatographic signals in all total ion chromatograms (TICs) of hydrophilic and lipophilic metabolite extracts from the chlorophyte *Scenedesmus vacuolatus*.

ChemStation Integration Parameters for TICs from Hydrophilic Algal Extracts

Integrator Event Name	Value	Time
Initial Area Reject	1.000	Initial
Initial Peak Width	0.020	Initial
Shoulder Detection	OFF	Initial
Initial Threshold	15.5	Initial

ChemStation Integration Parameters for TICs from Lipophilic Algal Extracts

Integrator Event Name	Value	Time
Initial Area Reject	1.000	Initial
Initial Peak Width	0.020	Initial
Shoulder Detection	OFF	Initial
Initial Threshold	14 (-15)	Initial
Integrator Off		0.001
Integrator On		6.500
Integrator Off		54.050

2.6.3 Analysis of Variability of Analytical GC-MS Data

Variances in the data were determined by calculating the standard deviation and mean coefficient of variation (CV in %) with peak area values of each

integrated signal. Distribution plots and outlier box plots were performed using JMP IN 5.1 software (SAS Institute, Cary, USA) as demonstrated in Figure 6. The coefficient of variation (CV in %) was calculated on the basis of a data set with all integrated and aligned peak areas of the chromatograms derived from 4 sample replicates of *S. vacuolatus* reference cultures, which were grown for 14 hours under light conditions (for details see Section 2.3.2: Concentration-Response Investigations).

Variability between Injections, Extracts & Experiments

The variability between injections, or the technical error during GC-MS analysis, was determined with a data set from a 4-fold analysis of the same calibration standard mixture of *n*-alkanes (Sigma Technical Bulletin; Saint Louis, USA) prepared for the retention index calculation (see Section 2.5.2). The preparative error of the procedure, or the variability between extracts, was analysed with four dependent samples from algae, grown in the same cultural cylinder. The intra-experimental variability, or the variability of results between independently grown cultures from one experiment, was investigated within one experiment using four samples, each derived from an individually grown algal population. Inter-experimental variability, or the variability between experiments, was studied with two independently performed experiments, each with four independently grown samples, grown for 14 hours under light conditions.

2.6.4 Multivariate Statistical Analysis (PCA)

Linear unsupervised principal component analysis (PCA) was used for the multivariate statistical analysis of alterations or differences between various samples, containing several hundred variables, and to identify major metabolites responding to toxicity. PCA was performed using JMP IN 5.1 software (SAS Institute, Cary, USA) to represent low dimensionality of the multivariate data set that captures most of the variance. The data matrix was arranged with samples in rows and peak area values in columns. Missing values were replaced by 1. Before PCA analysis, data were mean centred and, due to the use of a correlation matrix, scaled. PCA score plots show samples in the plane determined by the principal axes. PCA loading plots show variables associated with these samples in the plane of the same principal axes. Positions of PCA scores and loadings in the related plots correlate. The longer the vectors of loadings in the plot, the higher was their influence on the position of correlating samples in the linked scores plot. A circle was used to separate variables with the longest vectors from those with smaller vectors. Thus, major variables influencing the PCA scores plot pattern with a location outside of the circle were used for further analysis of dynamics in metabolite responses. Dynamics of the concentration change of

an individual compound in comparison of such trends between references and treatments were investigated by calculating the factorial difference (MS Excel, Microsoft, USA) and visualisation of trends in diagrams using JMP IN 5.1 software (SAS Institute, Cary, USA).

Results

3 Results

For the analysis of effects of chemicals on hydrophilic and lipophilic metabolites from *Scenedesmus vacuolatus* a metabolomics approach was developed using gas chromatography-mass spectrometry (GC-MS). Validation of the approach for toxicity analysis was performed by applying concentration- and time-dependent investigations of the effect of the herbicide prometryn, which was used as a model chemical. After a summary of results

3.1 Qualitative and Quantitative Analysis of Metabolomics Results for Hydrophilic and Lipophilic Algal Extracts

3.1.1 Qualitative Analysis of TIC's Using the System's ChemStation Software

GC-MS measurement of hydrophilic and lipophilic algal extracts of 14 hours old (t_{14}) algae, grown under reference conditions, resulted in three-dimensional total ion chromatograms (TICs), as demonstrated in Figure 4. Typical two-dimensional total ion chromatograms for hydrophilic and lipophilic metabolite compositions of *S. vacuolatus* are shown in Figure 5. The two-dimensional TICs show the retention time (RT) in minutes on the x-axis (1 min is divided into 100 time segments) and the total ion intensity or abundance of a chromatographic signal on the y-axis. Comparing the chromatographic results from hydrophilic and lipophilic extracts of t_{14} algae with similar scale of total ion intensity (Figure 5), the hydrophilic extract showed a higher density of detected signals.

3.1.2 Qualitative Analysis of TIC's Using the AMDIS Software

Data of the TICs of hydrophilic and lipophilic extracts from 14 hours old algae, as shown in Figure 5, were analysed using the AMDIS software (see Section 2.6.1). This analysis included noise analysis, component perception and spectrum deconvolution. AMDIS analysis of TICs, from 14 hours old reference algae, resulted in 600 hydrophilic and 340 lipophilic compounds.
From the increase in cell size of reference algae during algal growth (Figure 13) alterations in metabolite concentration or number of metabolites in algae during growth were expected. Comparison in the number of compounds in hydrophilic and lipophilic extracts showed differences between the 0 and 14 hours old reference algae. The number of compounds found in extracts of autospores (t_0) resulted in 560 hydrophilic

and 210 lipophilic compounds, which was lower than that of t_{14} reference algae, shown above.

3.1.3 Identification of *S. vacuolatus* Metabolites Using Custom Libraries (Qualitative Analysis)

Identification of compounds was useful for the biological interpretation of alterations in the algal metabolome, regarding growth or the effect of prometryn.

Identification with the custom NIST library (edition 2005) and the use of a mass spectral match factor of $\geq 20\%$ resulted in the assignment of 77 chemical structures in hydrophilic algal extracts (t_{14}). These compounds belong to the chemical classes: amino acids, sugars, organic acids, nucleic acids, fatty acids, phosphorous compounds and other compounds (see Appendix). In the hydrophilic extracts from *S. vacuolatus* reference samples at growth stage 14 hours (t_{14}): 13 amino acids, 2 amino acid derivatives, 13 sugars, 6 sugar derivatives, 5 fatty acids, 24 organic acids and 15 other compounds were defined. The most prominent peaks in the total ion chromatogram of hydrophilic extracts were a number of unidentified analytes, but also proline, malic acid, benzopyrane-2-carboxylic acid and octadecanoic acid. However, a metabolite of the algal extract was assigned reliably, if this spectrum matched to that of the library with a factor larger than 90%. Of the 77 structures identified, 41 hydrophilic algal metabolite structures were assigned reliably and they are listed in Figure 8 and 9. Data obtained during the analysis of hydrophilic metabolites from *S. vacuolatus* were used for a further development of the data evaluation protocol.

The lipophilic metabolite composition from *S. vacuolatus* (t_{14}) was also identified and resulted in 10 metabolites, assigned with a spectral match factor of at least 90%. These metabolites belong to the chemical classes fatty acids, amino acids, sugars and phosphorous compounds. Typically, the most prominent peaks in the TIC of lipophilic extracts were octadecatrienoic acid, hexadecanoic acid, galactopyranoside and glycerol. A number of unidentified lipophilic compounds also produced prominent peaks in the TIC. In this study, lipophilic data sets were analysed to ensure reproducibility of the metabolomics approach, but not to establish a protocol for data evaluation. Nevertheless, the results for the lipophilic metabolite fraction are also interesting to characterise the mechanism(s) of toxicity of prometryn in the chlorophyte *Scenedesmus vacuolatus* and will be an objective in the adjacent work.

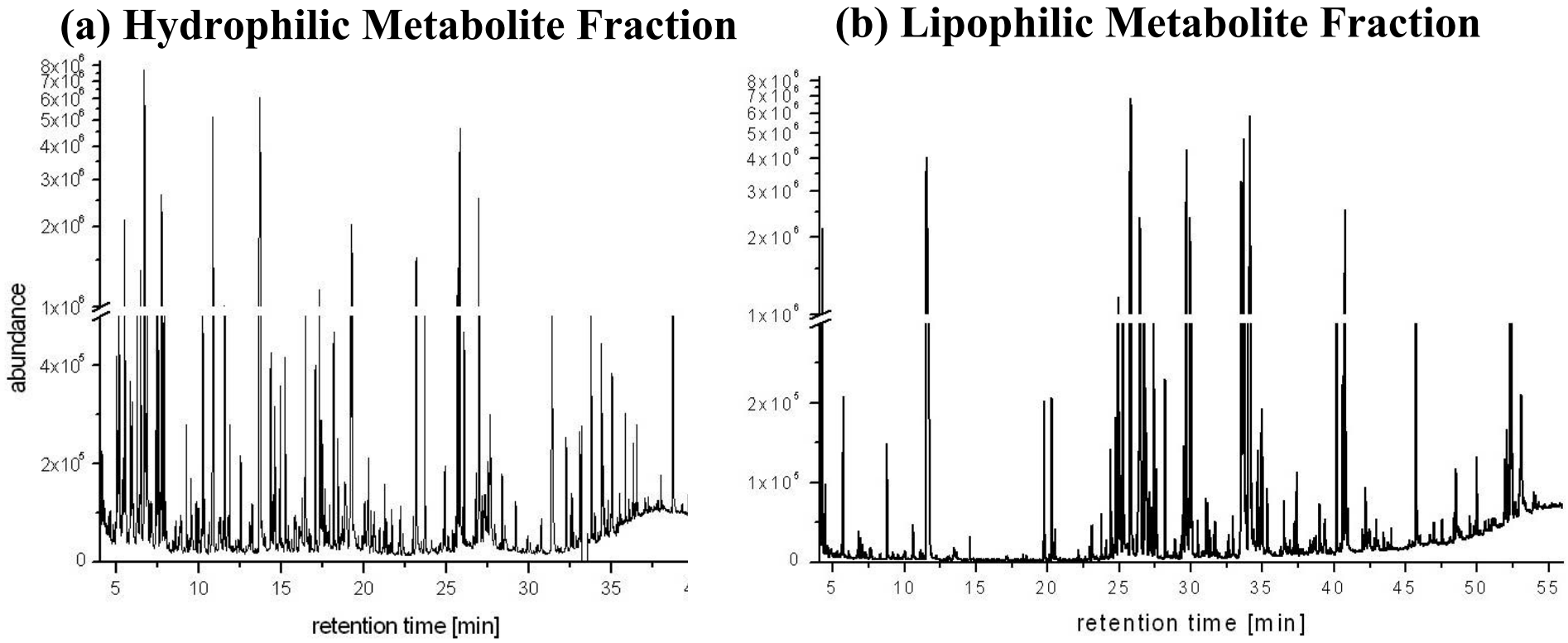


Figure 5: Total Ion Currents or Chromatograms (TICs) from (a) hydrophilic and (b) lipophilic metabolite extracts from 14 hours old populations of *S. vacuolatus*, grown under reference conditions. Note the interruption of the y-axis followed by a logarithmic scale to demonstrate most and less prominent signals in the chromatograms.

3.1.4 Selection of Quantitative Data from *S. vacuolatus* and Reproducibility of the Analytical Results

Using the GC-MS system's ChemStation software (as described in Section 2.6.2) the resulting integration report contained information about the specific retention times of peaks as well as the corresponding peak area values. Typically the integration of peaks of various chromatograms resulted in different numbers of quantified peak areas each time. These differences between the integration results of a number of signals in the chromatograms meant that the data matrices were not directly comparable and that manual alignment of retention times and peak area values was necessary. This manual process took approximately two or three days for one experimental data set and was, therefore, very much labour intensive and time consuming. After manual processing of the metabolomics data from hydrophilic and lipophilic algal extracts, only peaks that were found to be specific for the chlorophyte were used for further quantitative analysis. Peaks from the chlorophyte were selected by comparing data matrices from the alga samples with those from procedural blanks. Finally, a specific number of reliable peaks from the alga were found for each data set analysed. 125 hydrophilic and 109 lipophilic peaks were selected from the concentration series. Within the time course experiment, 113 hydrophilic and 170 lipophilic peaks were found to be from the chlorophyte.

Reproducibility of Analytical Results

The reproducibility of the *S. vacuolatus* metabolomics results using the developed approach was investigated concentrating on the intra- and inter-experimental variability as well as on the preparative error and the technical error. Coefficients of variation (CV) of peak area values of hydrophilic or lipophilic compounds showed right skewed distributions. To describe the distribution of CVs between 4 sample replicates, the median was chosen as most representative for the distribution, compared to the mean. The coefficient of variance (CV) describing the intra-experimental variability had a median of 15%. The related frequency distribution of all CV values from one data set is shown in Figure 6. Within this distribution, 50% of the data varied in their CV in a range from 9 to 30%. Approximately 94% of the data varied by less than a CV of 50%. The inter-experimental variability had a median of approximately 21% (results not shown). The preparative error had a median of approximately 22% (results not shown). In summary, determined median CV values for these three types of variability were found to be of similar order of magnitude, between 15 and 22%. But, to gain CV values of this study, comparable with the literature, e.g. from

Fiehn *et al.* (2000 a) and Weckwerth *et al.* (2004), the mean of CV values was also calculated and found in the range from 20 to 24% CV.

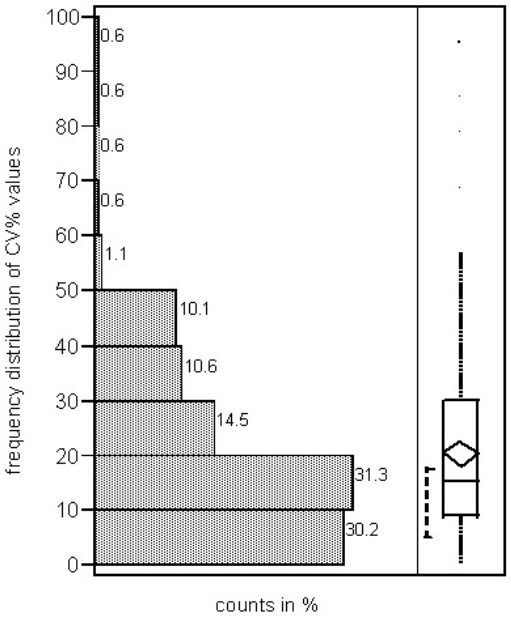


Figure 6: Intra-experimental variability (CV%) in metabolomicss data from four independently grown *S. vacuolatus* samples (n = 4) in one experiment at growth stage 14 hours, presented in a frequency distribution (left) of the CV% values for 179 hydrophilic signals and an outlier box plot (right). Labels on the right side of columns stand for counts of CV%-values in percent for each of the ten classes from 0 to 100%.

In addition, all three types of variability described above include the technical error. Following the repeated analysis of a standard mixture of *n*-alkanes the technical error was estimated. The technical error had a median and a mean of approximately 4% and 5%, respectively, and the apical retention time values of chromatographic peaks varied by approximately 0.01% CV (results not shown).

3.2 Application of Metabolomics for the Qualitative and Quantitative Analysis of *S. vacuolatus* Metabolites in Response to Time and Prometryn Toxicity

Hydrophilic and lipophilic metabolite extracts of the chlorophyte *S. vacuolatus,* grown under reference and/or exposure conditions with the algal toxic herbicide prometryn were analysed. Application of metabolomics with *S. vacuolatus* in relation to exposure-time and chemical concentration supported the validation of the developed approach for the detection of changes of the algal metabolome in response to prometryn. Concentration- and time-responses of the algal metabolites are shown, each with the score plots of hydrophilic and lipophilic metabolite fractions in Figure 7 and 10. To define one method of data evaluation regarding analysis of effects of toxicity in the chlorophyte metabolome, the hydrophilic data set from the time-response investigations was used exemplarily. However,

concentration-related PCA (principal component analysis) results are also shown.

3.2.1 Concentration – Responses of the Algal Metabolites

It was possible to monitor large changes but not necessarily slight changes directly in total ion chromatograms (TIC), which contained hundreds of compounds. Thus, differences between these multivariate chromatographic data were evaluated and visualised on the basis of peak area values, using principal component analysis (PCA).
PCA score plots for *S. vacuolatus* samples, from 125 hydrophilic and 109 lipophilic metabolites, are shown in Figure 7 a & b. In each plot, one prometryn-treated sample is missing (Figure 7 a, concentration 1; Figure 7 b, concentration 3). These samples were excluded from further data analysis due to a clearly abnormal *S. vacuolatus* TIC shape.

In each plot, the two principal components (PC) 1 and 2 on the x- and y-axis, respectively, explain together approximately 70% of variability in each of the two data sets. Reference samples were located at negative values from PC 1 in each plot. In Figure 7 a & b, PCA resulted in a separation of references and prometryn treatments, while treatment scores showed a concentration related shift from positive to negative values of PC 2, highlighted by arrows. In addition, PCA resulted in specific differences between the hydrophilic and the lipophilic chlorophyte extracts. Firstly, hydrophilic extracts from reference samples were differently but more strongly separated from treatment samples on the PC 1-axis, than that of lipophilic extracts. Secondly, PCA analysis of the metabolic compositions of hydrophilic extracts resulted in different concentration-dependent trend of exposed algae and in a differing strong separation from references. Hydrophilic and lipophilic metabolite compositions of references differed from those of treatments after 14 hours of algal exposure to a prometryn concentration of 0.004 µmol L^{-1} (~ 1 µg L^{-1}) and 0.002 µmol L^{-1} (~ 0.5 µg L^{-1}), respectively. In addition, large distance is visible between the metabolic composition of two samples treated with 0.07 µmol L^{-1} (concentration 6) and 0.13 µmol L^{-1} (concentration 7) prometryn (Figure 7 a and b). The EC50 value of algal reproduction for prometryn is ~ 0.05 µmol L^{-1} and should therefore be located between concentration 6 and 7.

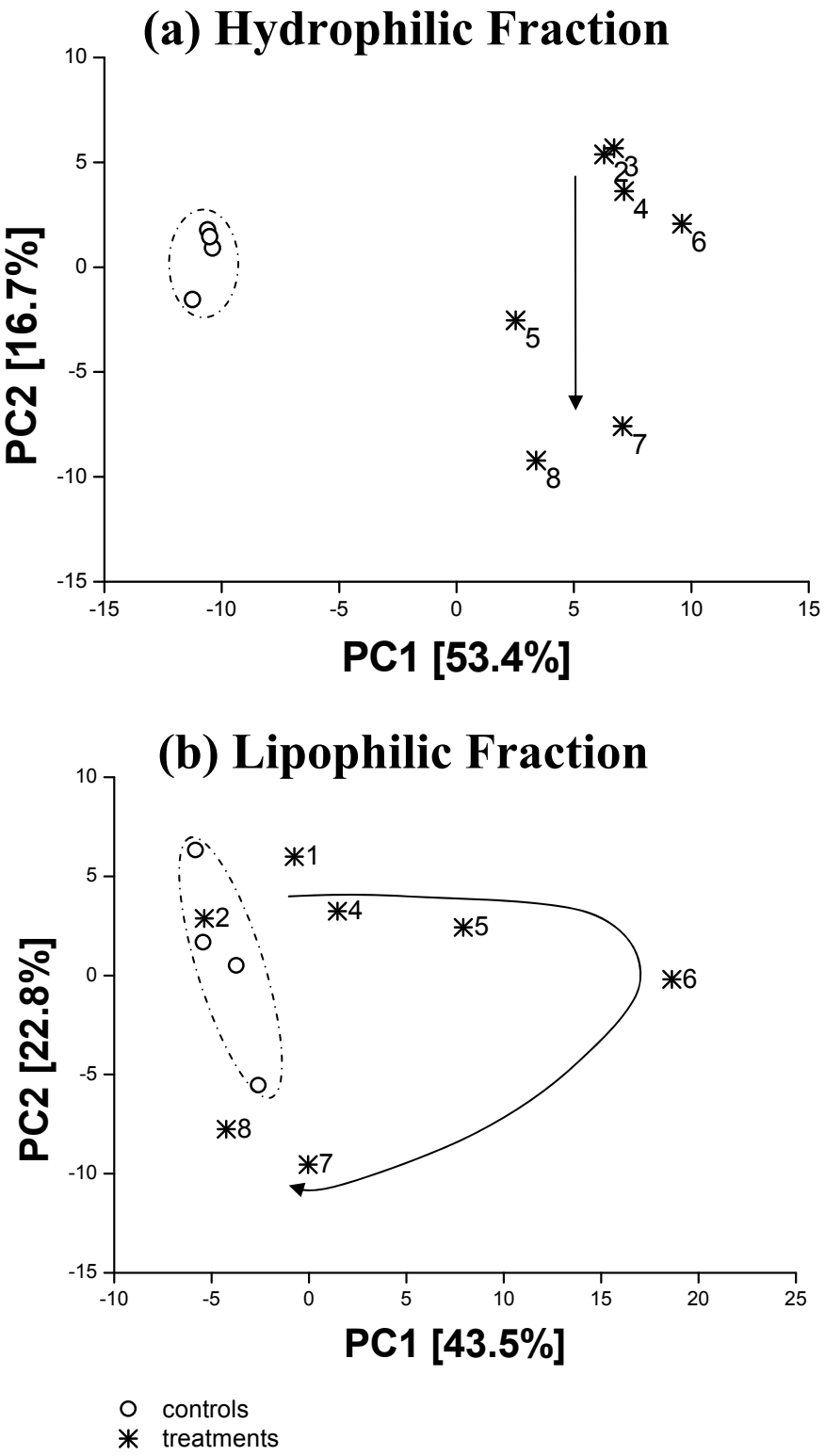


Figure 7: PCA score plots with first and second principal components (PCs) from (a) 125 hydrophilic and (b) 109 lipophilic *S. vacuolatus* metabolites derived from the concentration series experiment. Algae were grown for 14 hours under (o) reference or (*) exposure conditions with different concentrations of prometryn in µmol L^{-1}: 0.002 (1); 0.004 (2); 0.01 (3); 0.02 (4); 0.03 (5); 0.07 (6); 0.13 (7); and 0.26 (8). A concentration dependent trend of sample scores is indicated by arrows. In Figure 7 a prometryn concentration 1 and Figure 7 b prometryn concentration 3 is missing.

3.2.2 Time – Responses during Algal Growth

After the investigation of various concentrations of prometryn and their effects on the metabolome of *Scenedesmus vacuolatus*, consistency of effects during exposure with 0.1 µmol L^{-1} prometryn were studied during the algal growth phase at growth stages 0, 4, 6, 8, 10, 12, and 14 hours (t_0, t_4, t_6, t_8, t_{10}, t_{12}, t_{14}). All results demonstrated for the study of time-responses of the algal metabolome are shown for the data set of hydrophilic metabolic fractions from *Scenedesmus vacuolatus*, with the purpose of validating the developed procedure for data evaluation.

Characterisation of Concentration Dynamics of all Metabolites in Response to Time and Prometryn Exposure, Assigned with a Mass Spectral Match Quality of ≥ 90%

The response ratios of all hydrophilic metabolites assigned with ≥ 90% spectral match quality factor in total ion chromatograms (TICs) were examined. A graphic form similar to Fait *et al.* (2006) was used to improve the visibility of changes in the concentration of several metabolites from *S. vacuolatus*, at growth stages 4 and 14 hours, as shown in Figure 8 and 9, respectively. The concentration of 41 hydrophilic metabolites, assigned with a spectral match quality factor of ≥ 90%, from *Scenedesmus vacuolatus* are shown to demonstrate responses under reference and exposure (0.1 µmol L^{-1} prometryn) conditions.

Comparing peak area values of metabolites from 4 hours old algae with those of the autospores (t_0), it is clear from Figure 8 that changes in metabolite concentrations occurred within the first 4 hours of growth in both references and treatments, but differences between treatments and references are also visible. After 4 hours of growth (t_4) under reference conditions, most of the assigned metabolites were detected with a higher concentration level compared with the autospores. Few metabolite concentrations decreased. In the following paragraph, responses of algal metabolites under reference and treatment conditions will be compared. An extreme decrease in many metabolite concentrations of treatments was found after 4 hours of growth, while few of the assigned metabolites in treatments increased in concentration. One of the greatest disparities observed in metabolite concentration levels between references and treatments, was found for a compound assigned as glutamine (match quality of 97%). Glutamine concentration increased approximately 40-fold during 4 hours under reference conditions, but decreased under exposure to prometryn (0.1 µmol L^{-1}) drastically.

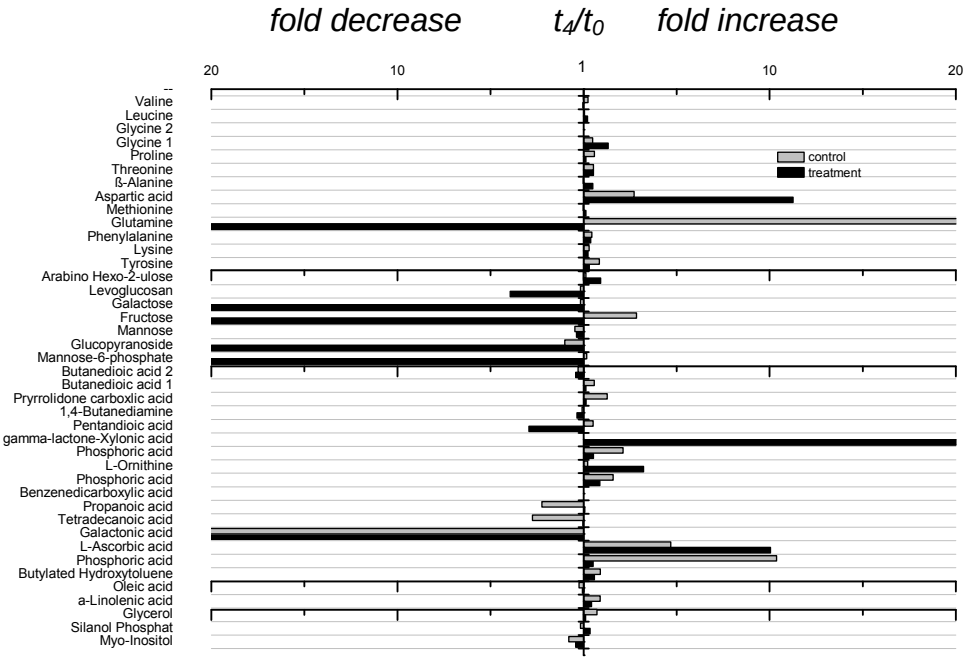


Figure 8: Response ratios of peak area values for 4 hours (t_4) old *S. vacuolatus* treatments (black columns) and references (grey columns) compared with the metabolic status of autospores (0 hours = t_0). Treated algae were exposed for 4 hours to 0.1 µmol L^{-1} prometryn in 0.1% DMSO. The response of all 41 hydrophilic metabolites from *S. vacuolatus*, assigned with a spectral match quality of ≥ 90% (see Section 2.6.1) is shown.

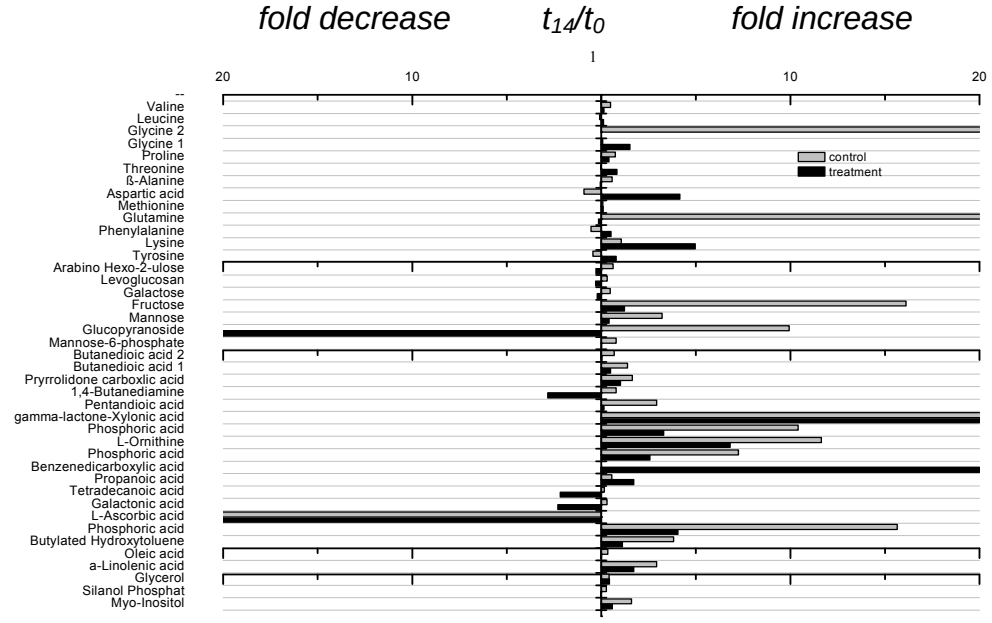


Figure 9: Response ratios of peak area values for 14 hours old *S. vacuolatus* treatments (black columns) and references (grey columns) compared with the metabolic status of autospores (0 hours = t_0). Treated algae were exposed for 14 hours to 0.1 µmol L^{-1} prometryn in 0.1% DMSO. The response of the same 41 hydrophilic metabolites from *S. vacuolatus* as demonstrated in Figure 8 is shown.

Further remarkable differences were found between references and treatments for aspartic acid, sugars and organic acids at growth stage 4 hours (Figure 8). Five of seven sugars showed a great decrease in concentration during 4 hours of exposure, while the concentration in references remained stable, increased or showed a much lower decrease for the same time frame. After 4 hours of growth, galactonic acid concentration in references and in treatments was great decreased compared to autospores (t_0). Concentration of glycine 1 and benzenedicarboxylic acid did not change during four hours of growth either under reference or under treated conditions. At growth stage 4 hours, aspartic acid, ascorbic acid, ornithine, propanoic acid and tetradecanoic acid were expressed higher in treatments than in references.

The response ratio results found for metabolites from 14 hours old algae (t_{14}) were comparable, where most assigned metabolites were found to occur at a higher concentration level compared with the autospores (Figure 9). During growth a positive trend of changes in metabolites from treatments and references was found. Compared to the autospores, some metabolites from 14 hours old reference samples showed an increase in concentration levels (e.g. glycine 2 and glutamine). The concentration of the amino acids aspartic acid and lysine was clearly stronger increased after 14 hours of exposure than under references conditions. Seven further metabolites showed also a decrease in the concentration, but less strong (galactonic acid, 1.4-butanediamine, glutamine, glucopyranoside, galactose, levoglucosan, arabino-hexo-2-ulose and tetradecanoic acid) for the same time frame. In general, concentration responses of sugars increased more under reference conditions than under treated conditions. Glucopyranoside increased under reference conditions during 14 hours of growth, but was greatly decreased in treatments. Similar to sugars, most organic acids responded stronger in references than in treatments. The presence of ascorbic acid was much less expressed in references and treatments after 14 hours, compared to autospores. Another organic acid, here assigned as benzenedicarboxylic acid, stayed stable under control conditions and increased greatly in treatments. Furthermore after 14 hours, abundances of linolenic acid and myo-inositol showed an increase which was higher in references than in treatments. In addition, concentration of ornithine was higher in references than in treatments for the same time frame.

In summary, the investigation of metabolite concentration response ratios, with column graphs (Figure 8 and 9) for all assigned hydrophilic metabolites, enabled a fast grasp of the dimension of change at a specific growth stage of the chlorophyte. Because column graphs are more figurative than tables, for example shown in Table 3, they can be a tool to

summarise the information about metabolic changes. But, for a better overview on the trend of the metabolite concentration over time, single plots of the concentration of individual metabolites, as shown in Figure 12, were preferred and for the selection of biochemically interesting compounds, within the whole metabolic composition of *Scenedesmus vacuolatus*, a multivariate analysis method was demanded.

Biochemically Interesting Metabolites of *Scenedesmus vacuolatus* Responding to Time and Exposure

The metabolome of references and treatments was studied over time and was compared using the multivariate "principal component analysis" (PCA). PCA score plots of *S. vacuolatus* samples, from 113 hydrophilic and 170 lipophilic metabolites, are shown in Figure 10 a & b. In both PCA plots, the two principal components (PC) 1 and 2 stand for the x- and y-axis, respectively. Approximately 45% and 69% of the total variability of each data set are described in Figure 10 a and b, respectively. A clear time-related separation of references and prometryn treatments was found in the same figures. The first time-related effects on the hydrophilic and lipophilic metabolic composition of *S. vacuolatus* reference samples were found after 4 hours of growth.

While PCA scores of hydrophilic and lipophilic extracts of reference samples showed a time-related trend into the direction of positive PC1 values, treatments showed a dissimilar trend (Figure 10 a & b). T_0 reference samples (autospores) were located at negative values of PC 1 in PCAs from hydrophilic and lipophilic chlorophyte extracts. In Figure 10 a and b, from t_0 references on, time related alterations in the composition of hydrophilic and lipophilic extracts were shown for references and treatments, marked by arrows. At growth stage t_{12} hours, the metabolic composition in the hydrophilic fraction of references was found to be close to that of references at growth stage 6 hours, but at t_{14} it was found to perpetuate the trend away from t_0 references. Time trend of treatments was followed by an aggregation of the metabolic composition of t_{14} algae to that of undisturbed algae at t_4 and t_6. It was supposed that this PCA result could be comparable with the column graphs in Figure 8 and 9. But as shown in the column graphs (Figure 8 and 9), treated t_{14} and reference t_4 cells show dissimilarities, especially in the group of organic acids. Hence, it can also be useful to study metabolic dynamics of non-PCA-influential metabolites to gain another perspective on the data. The perspective, gained for column graphs of all assigned metabolites of the hydrophilic metabolite fraction, demonstrates the additional information compared with PCA.

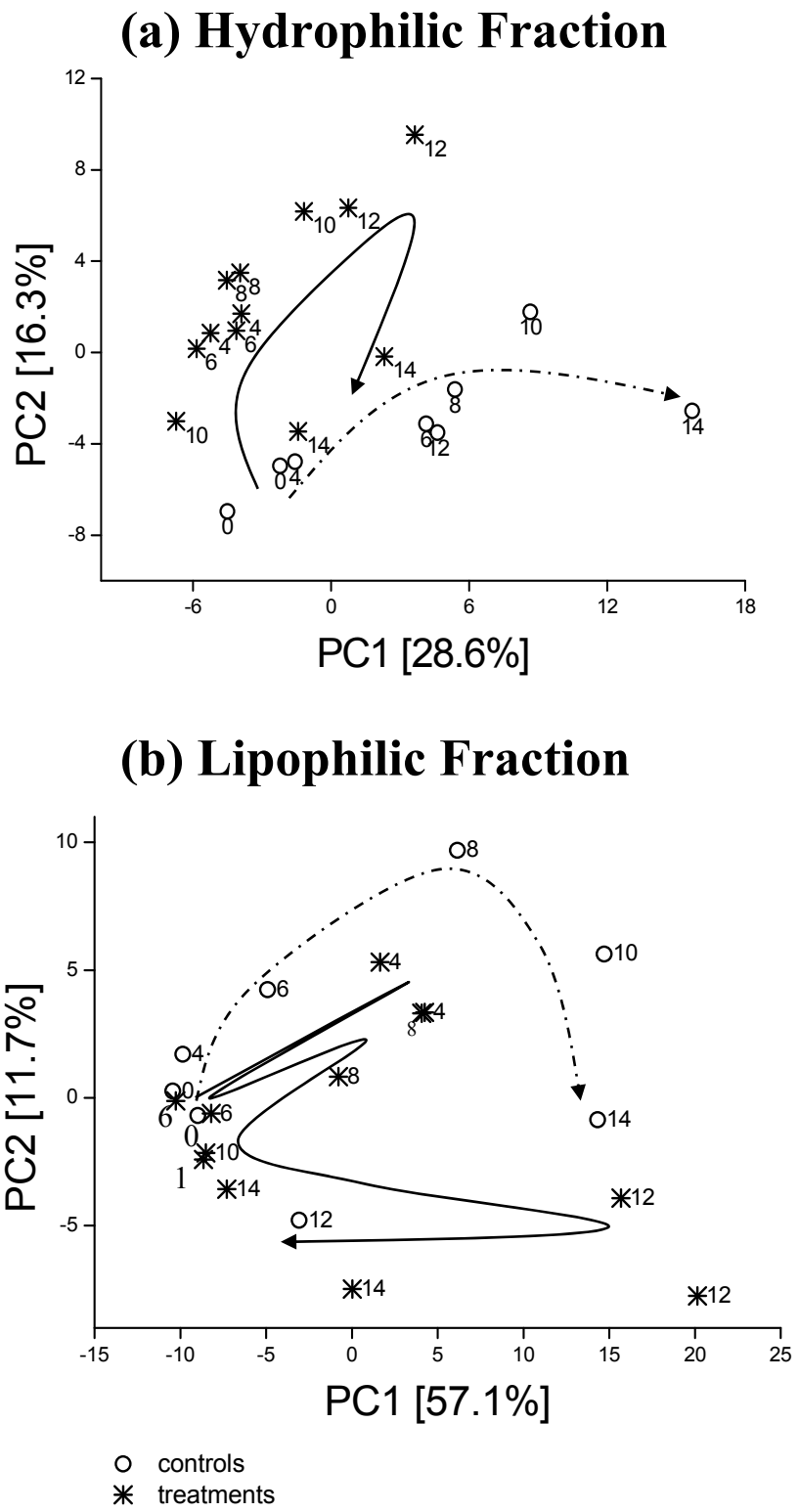


Figure 10: PCA score plots with first and second principal components (PCs) from (a) 113 hydrophilic and (b) 170 lipophilic *S. vacuolatus* extracts derived from the time course experiment (growth stage 0-14 hours). Sample scores are shown in 2 hour steps for (o) reference algae and (*) cultures treated with 0.1 µmol L^{-1} prometryn. Time trend for the sum of metabolite responses from references and treatments is highlighted by arrows, with dashed (references) and solid (treatment) lines, respectively.

Lipophilic metabolic compositions from treatments (Figure 10 b) showed a back and forth trend of score values from treated samples with time, which started at growth stage 4 hours and appeared every second hour.

In summary for the study of concentration and time dependencies of the metabolites of the chlorophyte, references and treatments were separated more clearly by PCA for hydrophilic extracts than for lipophilic extracts. At all analysed growth stages, exposure to 0.1 µmol L^{-1} prometryn caused considerable changes in the composition of hydrophilic and lipophilic algal extracts.

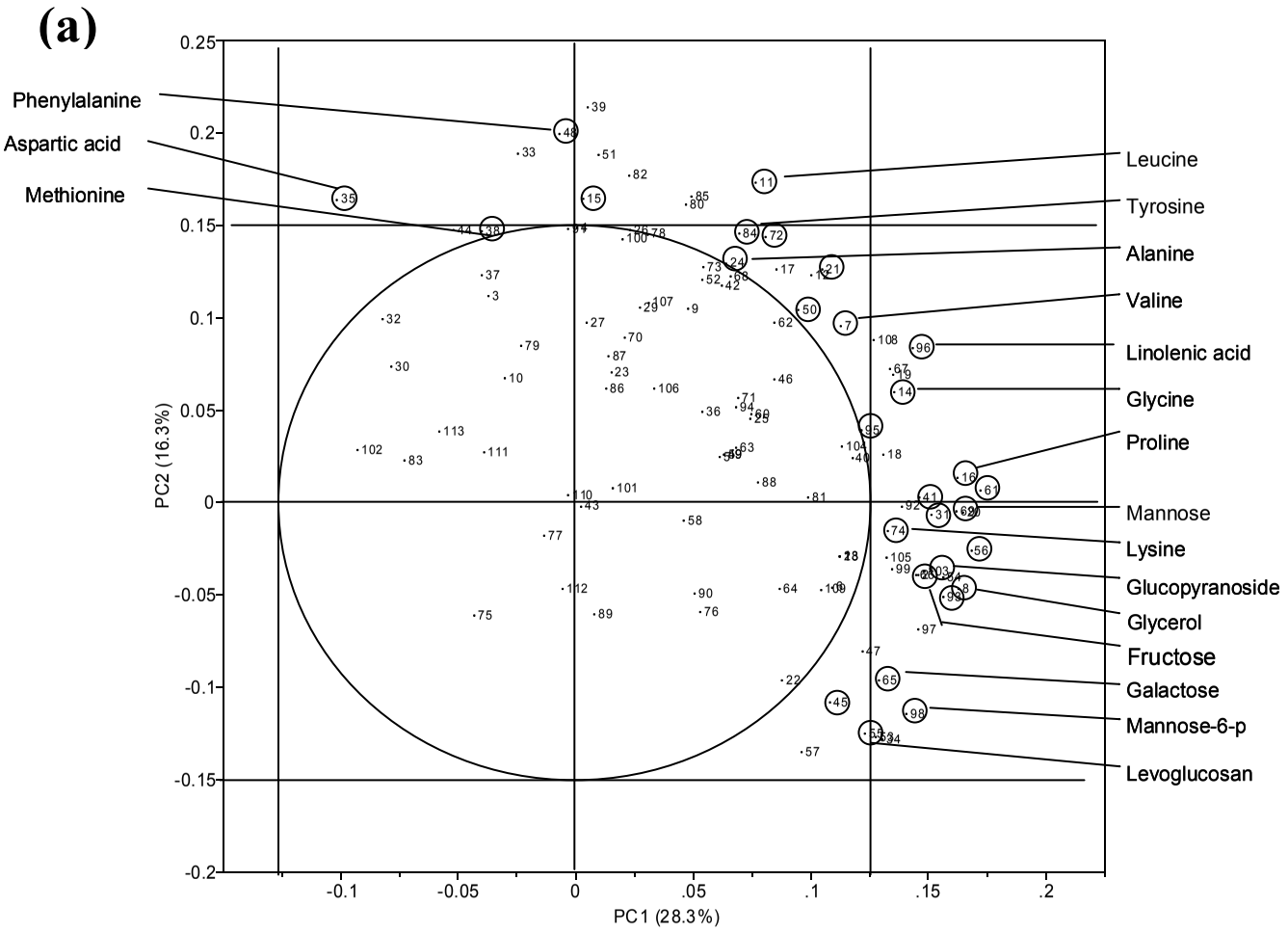
3.2.3 Characterisation of the Algal Metabolome in Response to Time and Prometryn Toxicity

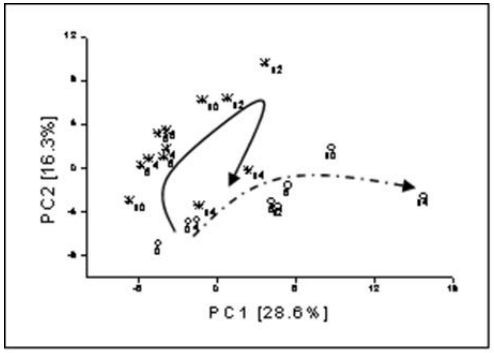
The characterisation of metabolites regarding their responses to time and prometryn exposure are exemplified in this chapter with the data set of hydrophilic metabolites, obtained in the time course experiment. For this data set, a loading plot of 113 hydrophilic algal compounds is shown in Figure 11 a. The x- and y-axis are described by the principal components PC 1 and PC 2, respectively, explaining in their sum 54% of the total variability in the data set. The loadings of this PCA plot were evaluated regarding their influence on the corresponding scores plot pattern, shown in Figure 11 b or 10 a. Each loading or variable of the plot stands for a compound of *S. vacuolatus*.
The most influential compounds were selected by their distance from the origin of the loadings plot. The closer the positions of the variables in the plot the more similar was their characteristic trend of abundance over time. 55 variables lying outside of the circle from the PCA loadings plot (Figure 11 a), were selected as biochemically interesting components. These 55 components were identified, using custom mass spectral libraries and a mass spectral match quality of at least 90%. 29 of these 55 variables, highlighted in Figure 11 a, were assigned a chemical structure and these 29 metabolites were further investigated regarding concentration dynamics (Table 3).

In Table 3 a & b, x-fold changes in peak area values are demonstrated for references and treatments over time compared with that of autospores. The x-fold difference between references and treatments at each analysed time point is demonstrated in Table 3 c. Butanedioic acid and phosphoric compounds were identified more than once in a chromatogram, which is not untypical for a GC-MS chromatogram due to derivatisation and molecule fragmentation. Assigned metabolites, such as sugars, amino acids, organic acids, phosphoric compounds and fatty acids, are

Figure 11: PCA loading plot (a) and PCA score plot (b) pattern of the hydrophilic fraction of metabolites from the chlorophyte *Scenedesmus vacuolatus* over the time course from 0 to 14 hours of algal development; The metabolome of references and prometryn treatments (0.1 µmol L^{-1}) were studied with 113 hydrophilic signals.

(A) 55 compounds outward of the circle were found to be most influential for the PCA score plot result in Figure 11 b. 29 of these 55 compounds were assigned a chemical structure (match quality ≥ 90%) and highlighted. Assigned sugars and amino acids are labeled. Related response ratios of metabolite concentration levels are shown in Table 3; Numbers stand for the order of signals regarding their chromatographic elution time in the GC-MS total ion current. **(B)** PCA score plot of hydrophilic metabolites as shown in Figure 10 a.





highlighted in Figure 11 a. Sugars and amino acids are also labelled in the same plot. Concentration dynamics or changes in peak area values of metabolites were also investigated and the concentration dynamics of six metabolites during growth are shown in Figure 12.

General Description of Variables in the PCA Loading Plot

Generally, variables with positive values of PC 1 (Figure 11 a) showed a continuous increase in concentration under reference conditions during the time course from 0 to 14 hours. This increase of metabolite concentration is shown in Figure 12 b-f for two amino acids (lysine and glycine) and three sugars (fructose, mannose and mannose-6-p). In addition, these three metabolites were also found to be less prominent in treatments than in references. Typically, the concentration of fructose, mannose and mannose-6-p increased at later developmental algal stages (t_{12} and t_{14}), as shown in the same figure. For example, growth of *S. vacuolatus* autospores (t_0) for 12 and 14 hours resulted in a 14- or 17-fold increase of the peak area abundance of lysine and fructose under reference conditions (Table 3 a). Under exposure to 0.1 µmol L^{-1} prometryn, the peak area abundance of lysine and fructose increased approximately 4 to 6-fold for the same time frame. Hence, after 14 hours of growth, concentration of both metabolites was twice the amount in reference algae compared with the concentration in treatments.

To describe the dynamics of metabolites with positive values of PC 1 in a second example, the sugars fructose and mannose as well as the amino acid lysine were selected. While the peak area of these three compounds increased continuously under reference conditions (Figure 12 b, d & e), this trend showed a short-term decrease at growth stage 12 hours for fructose and mannose (Figure 12 d & e, respectively), the peak area of these three compounds was mostly lower under exposure conditions. After 14 hours of *S. vacuolatus* growth under reference conditions, the peak areas of fructose, mannose and lysine were found to be larger compared to those at earlier developmental stages.

A group of variables with positive values for PC 1 and negative values for PC 2 was found, including for example levoglucosan, mannose-6-phosphate and galactose (Figure 11 a and Table 3). The peak area of these metabolites increased slightly and consistently approximately 2-fold under reference conditions, during the 14 hours of *S. vacuolatus* growth. In contrast, these three metabolites were barely found in treatments until growth stage t_{10}, followed by an increase of metabolite concentrations until t_{14} (Table 3). Final peak area abundance of the 14 hours old treatments were similar to that of references at earlier growth stages.

In comparison with the PCA scores plot (Figure 10 a), the concentration of these metabolites correlated positively with latest growth stages of undisturbed algae and negatively with those exposed to prometryn.

Loadings with negative PC 1 and positive PC 2 values showed opposite characteristics to those with positive PC 1 and negative PC 2 values. At early growth stages, these compounds were more prominent in treatments than in references as shown for aspartic acid (Figure 12 a). Concentration of aspartic acid increased 3.7-fold under reference conditions after 4 hours of growth, but 12.3-fold under treated conditions (Table 3). At later algal growth stages, the concentration level of treatments decreased to that level of reference samples. The concentration level of the amino acids phenylalanine and methionine (Figure 11 a) was similar to that of aspartic acid over time, e.g. higher than references, but showed less distinctive differences between references and treatments. Response ratios of these two variables under reference and exposure conditions are shown in Table 3.

Analysis of Metabolite Dynamics in References and Treatments in Response to Time

Following the selection of important biochemical compounds from the PCA score plot pattern and characterisation of their concentration dynamics in response to prometryn, interpreting molecular processes and assessing the potential of the presented approach for its support in toxicity analysis in plants was performed. In Table 3, x-fold changes in peak area values are demonstrated for references and treatments over time in comparison to that of autospores as well as between references and treatments at each analysed time point.

Peak area abundances of 29 of the assigned biochemically interesting metabolites from algae grown under **reference conditions**, were studied in comparison with autospores. During the first 4 hours of *S. vacuolatus* growth under reference conditions concentration of 45% of the 29 compounds were increased at least 2-fold, while two (7%) decreased at least 2-fold (glucopyranoside and mannose) (Table 3 a). During 14 hours of growth under reference conditions, approximately 90% of metabolites increased at least 2-fold (6 amino acids, 2 sugars, 1 fatty acid and 8 other compounds). A prominent increase, ranging from 10- to 17-fold, was found for fructose, lysine, glucopyranoside and two phosphoric compounds. Another phosphoric compound increased approximately 8-fold, glycine approximately 5-fold as well as proline, pentanedioic acid, mannose and linolenic acid approximately 4-fold for the same time frame.

The 29 metabolites described in the previous chapter for reference algae were also studied under **exposure to prometryn** (Table 3 b), compared

with autospores. 4 hours of exposure to 0.1 µmol L^{-1} prometryn, resulted in 24% of the compounds increasing at least 2-fold, such as the amino acids glycine, alanine and aspartic acid. But aspartic acid increased prominently approximately 12-fold and at t_6 approximately 14-fold (Table 3 b). Sugars, such as fructose, galactose, mannose-6-phosphate and levoglucosan as well as a few other compounds (17%) decreased at least 2-fold compared to references. After 14 hours of exposure to 0.1 µmol L^{-1} prometryn, approximately 50% of the metabolites, mostly amino acids and other compounds, increased at least 2-fold compared to autospores. Glucopyranoside and propanoic acid decreased approximately 2-fold.

Comparing the abundances of metabolites in references and treatments (Table 3 c) at growth stage 4 hours, the three amino acids alanine, aspartic acid and glycine, together 10% of the 29 compounds, were expressed approximately 2-fold higher in treatments than in references. At t_4 and t_6, aspartic acid was about 3-fold and 8-fold, respectively, higher expressed in treatments than in references (Table 3 c). 24% of the metabolites were expressed at least 2-fold less in treatments than in references (3 sugars and 4 other compounds). The peak area abundance of treatments at growth stage 14 hours was expressed approximately 2-fold higher in aspartic acid and propanoic acid (7% of the 29 compounds (Table 3 c). Around 45% of the compounds were expressed 2-fold lower in treatments than in references (4 amino acids, 2 sugars and 7 other compounds).

In summary, investigating time-dependencies of metabolite dynamics during the chlorophyte exposure to 0.1 µmol L^{-1}, the concentration of the amino acid aspartic acid was increasing, while that of other amino acids, sugars and organic acids was decreasing compared to references at the same growth stages. In general from algal growth stage 4 hours on, algal exposure to prometryn resulted in a continuously lower or higher concentration level of amino acids and sugars compared to references, followed by a further change of the concentrations of those metabolites at growth stages 12 and 14 hours to the level of references.

Table 3: Time- and prometryn (0.1 µmol L^{-1})-dependent change of the abundance (peak area) of biochemically important hydrophilic metabolites from the chlorophyte *Scenedesmus vacuolatus* over 14 hours of growth. The peak area of 29 hydrophilic metabolites is shown for autospores (t_0) as well as the metabolite abundance response ratios of references and treatments at the developmental stages 4, 6, 8, 10, 12 and 14 hours of algal growth (t_4, t_6, t_8, t_{10}, t_{12}, t_{14}). The biochemical importance of these metabolites was estimated using principal component analysis (PCA), as shown in Figure 11. Peak area dynamics of metabolites labeled with asterisks, are exemplarily shown in Figure 12.

(A) Time-response ratio of metabolites from reference algae at a specific growth stage ($c\text{-}t_x$) in comparison with the peak area of autospores at growth stage 0 hours ($c\text{-}t_0$).

(B) Time-response ratio of metabolites from treatment algae at a specific growth stage ($t\text{-}t_x$) in comparison with the peak area of autospores at growth stage 0 hours ($c\text{-}t_0$).

(C) Treatment Response ratio of metabolites from treatment algae at a specific growth stage (t-tx) compared with reference algae at the same growth stage (c-tx).

Table 3 A	**peak area at t_0 average (n=2)**	**Metabolite time-response ratio: references at a specific growth stage ($c\text{-}t_x$) compared with autospores ($c\text{-}t_0$)**					
Metabolites		**$c\text{-}t_4/c\text{-}t_0$**	**$c\text{-}t_6/c\text{-}t_0$**	**$c\text{-}t_8/c\text{-}t_0$**	**$c\text{-}t_{10}/c\text{-}t_0$**	**$c\text{-}t_{12}/c\text{-}t_0$**	**$c\text{-}t_{14}/c\text{-}t_0$**
Valine	5035253.5	1.0	1.0	2.0	2.0	2.0	2.0
Glycerol	16782784.5	2.0	2.0	2.0	2.0	2.0	3.0
Leucine	6572905.0	1.0	2.0	2.0	2.0	2.0	2.0
Glycine *	5819335.5	2.0	2.0	3.0	4.0	5.0	5.0
Proline	5544661.5	2.0	2.0	2.0	2.0	3.0	4.0
Butanedioic acid1	37673072.0	1.0	1.0	1.0	1.0	1.0	2.0
Threonine	7878849.0	2.0	2.0	2.0	2.0	2.0	2.0
Alanine	887229.5	1.0	1.0	1.0	1.0	1.0	2.0
Butanedioic acid2	60912327.5	2.0	2.0	1.0	2.0	2.0	2.0
Aspartic acid *	12720058.5	4.0	2.0	2.0	4.0	4.0	2.0
Methionine	1340104.0	1.0	1.0	0.0	0.5	1.0	1.0
Pyrrolidone Carboxylic acid	76099932.5	2.0	3.0	3.0	4.0	3.0	3.0
Butanediamine	1883351.0	1.0	2.0	2.0	2.0	1.0	2.0
Pentanedioic acid	1925229.0	2.0	3.0	4.0	0.0	3.0	4.0
Phenylalanine	3059629.0	1.0	1.0	1.0	1.0	2.0	1.0
Levoglucosan	2975558.5	1.0	1.0	1.0	1.0	1.0	1.0
Phosphoric compound1	1173421.0	3.0	6.0	6.0	9.0	9.0	11.0
Phosphoric compound2	3854696.5	3.0	4.0	5.0	6.0	6.0	8.0
Galactose	1545863.0	1.0	1.0	1.0	1.0	1.0	2.0
Fructose *	180721.5	4.0	12.0	11.0	14.0	4.0	17.0
Mannose *	1813924.0	0.5	3.0	3.0	3.0	1.0	4.0
Propanoic acid	7954498.0	1.0	2.0	2.0	4.0	1.0	2.0
Lysine *	4842802.5	1.0	2.0	2.0	2.0	7.0	14.0
Tyrosine	2436530.0	2.0	2.0	2.0	2.0	2.0	2.0
Phosphoric compound3	2103992.5	11.0	11.0	9.0	12.0	13.0	17.0
Oleic acid	3819074.0	1.0	1.0	1.0	1.0	1.0	1.0
Linolenic acid	1047257.5	2.0	2.0	2.0	3.0	3.0	4.0
Mannose-6-p *	8790022.0	1.0	2.0	2.0	2.0	2.0	2.0
Glucopyranoside	2226475.5	0.0	4.0	6.0	3.0	2.0	11.0

Table 3 B	**peak area at t0 average (n=2)**	**Metabolite time-response ratio: treatments (0.1 µmol L^{-1} prometryn) at a specific growth stage ($t\text{-}t_x$) compared with autospores ($c\text{-}t_0$)**					
Metabolites		**$t\text{-}t_4/c\text{-}t_0$**	**$t\text{-}t_6/c\text{-}t_0$**	**$t\text{-}t_8/c\text{-}t_0$**	**$t\text{-}t_{10}/c\text{-}t_0$**	**$t\text{-}t_{12}/c\text{-}t_0$**	**$t\text{-}t_{14}/c\text{-}t_0$**
Valine	5035253.5	1.0	1.0	1.0	1.0	2.0	1.0
Glycerol	16782784.5	1.0	1.0	1.0	1.0	1.0	1.0
Leucine	6572905.0	1.0	1.0	2.0	1.0	2.0	1.0
Glycine *	5819335.5	2.0	2.0	2.0	3.0	3.0	3.0
Proline	5544661.5	1.0	1.0	1.0	2.0	2.0	1.0
Butanedioic acid1	37673072.0	1.0	1.0	1.0	1.0	1.0	1.0
Threonine	7878849.0	2.0	2.0	2.0	2.0	2.0	2.0
Alanine	887229.5	2.0	1.0	1.0	1.0	2.0	1.0
Butanedioic acid2	60912327.5	1.0	1.0	1.0	1.0	2.0	2.0
Aspartic acid *	12720058.5	12.0	14.0	15.0	13.0	12.0	5.0

Table 3 B **Metabolites**	**peak area at t0 average (n=2)**	**Metabolite time-response ratio: treatments (0.1 µmol L^{-1} prometryn) at a specific growth stage ($t\text{-}t_x$) compared with autospores ($c\text{-}t_0$)**					
		$t\text{-}t_4/c\text{-}t_0$	$t\text{-}t_6/c\text{-}t_0$	$t\text{-}t_8/c\text{-}t_0$	$t\text{-}t_{10}/c\text{-}t_0$	$t\text{-}t_{12}/c\text{-}t_0$	$t\text{-}t_{14}/c\text{-}t_0$
Methionine	1340104.0	1.0	1.0	1.0	1.0	2.0	1.0
Pyrrolidone Carboxylic acid	76099932.5	1.0	1.0	1.0	1.0	2.0	2.0
Butanediamine	1883351.0	1.0	0.0	0.0	0.0	0.3	0.5
Pentanedioic acid	1925229.0	0.3	0.2	1.0	0.3	1.0	1.0
Phenylalanine	3059629.0	1.0	2.0	2.0	2.0	3.0	2.0
Levoglucosan	2975558.5	0.2	0.0	0.2	0.0	0.4	1.0
Phosphoric compound1	1173421.0	2.0	1.0	1.0	1.0	4.0	4.0
Phosphoric compound2	3854696.5	2.0	1.0	2.0	1.0	4.0	4.0
Galactose	1545863.0	0.0	0.0	0.0	0.2	1.0	1.0
Fructose *	180721.5	0.0	3.0	0.0	0.0	4.0	2.0
Mannose *	1813924.0	1.0	1.0	1.0	1.0	1.0	1.0
Propanoic acid	7954498.0	1.0	1.0	1.0	2.0	4.0	3.0
Lysine *	4842802.5	1.0	1.0	1.0	2.0	4.0	6.0
Tyrosine	2436530.0	1.0	1.0	2.0	2.0	3.0	2.0
Phosphoric compound3	2103992.5	2.0	1.0	1.0	2.0	5.0	5.0
Oleic acid	3819074.0	1.0	1.0	1.0	1.0	1.0	1.0
Linolenic acid	1047257.5	1.0	1.0	2.0	1.0	3.0	3.0
Mannose-6-p *	8790022.0	0.0	0.0	0.0	0.0	0.4	1.0
Glucopyranoside	2226475.5	0.0	0.0	0.0	1.0	1.0	0.0

Table 3 C **Metabolites**	**peak area at t0 average (n=2)**	**Metabolite treatment-response ratio: treatments (0.1 µmol L^{-1} prometryn) at a specific growth stage ($t\text{-}t_x$) compared with references at the same growth stage ($c\text{-}t_x$)**					
		$t\text{-}t_4/c\text{-}t_4$	$t\text{-}t_6/c\text{-}t_6$	$t\text{-}t_8/c\text{-}t_8$	$t\text{-}t_{10}/c\text{-}t_{10}$	$t\text{-}t_{12}/c\text{-}t_{12}$	$t\text{-}t_{14}/c\text{-}t_{14}$
Valine	5035253.5	1.0	1.0	1.0	1.0	1.0	1.0
Glycerol	16782784.5	1.0	0.4	0.5	0.5	1.0	0.5
Leucine	6572905.0	1.0	1.0	1.0	1.0	1.0	1.0
Glycine *	5819335.5	2.0	1.0	1.0	1.0	1.0	0.5
Proline	5544661.5	1.0	1.0	0.5	1.0	1.0	0.3
Butanedioic acid1	37673072.0	1.0	1.0	1.0	1.0	1.0	1.0
Threonine	7878849.0	1.0	1.0	1.0	1.0	1.0	1.0
Alanine	887229.5	2.0	1.0	1.0	1.0	1.0	0.5
Butanedioic acid2	60912327.5	1.0	1.0	1.0	0.5	1.0	1.0
Aspartic acid *	12720058.5	3.0	8.0	7.0	4.0	3.0	3.0
Methionine	1340104.0	1.0	1.0	-	2.0	3.0	1.4
Pyrrolidone Carboxylic acid	76099932.5	1.0	0.4	0.4	0.3	1.0	1.0
Butanediamine	1883351.0	1.0	0.0	0.0	0.0	0.2	0.3
Pentanedioic acid	1925229.0	0.2	0.1	0.1	-	0.3	0.3
Phenylalanine	3059629.0	1.0	1.0	2.0	1.0	2.0	1.0
Levoglucosan	2975558.5	0.2	0.0	0.3	0.0	0.5	1.0
Phosphoric compound1	1173421.0	0.5	0.2	0.2	0.1	0.4	0.4
Phosphoric compound2	3854696.5	1.0	0.3	0.3	0.2	1.0	0.4
Galactose	1545863.0	0.0	0.0	0.0	0.2	1.0	1.0
Fructose *	180721.5	0.0	0.2	0.0	0.0	1.0	0.1
Mannose *	1813924.0	1.0	0.3	0.3	0.4	1.0	0.3
Propanoic acid	7954498.0	1.0	1.0	1.0	0.4	3.0	2.0
Lysine *	4842802.5	1.0	1.0	1.0	1.0	1.0	0.4
Tyrosine	2436530.0	1.0	1.0	1.0	1.0	1.0	1.0
Phosphoric compound3	2103992.5	0.1	0.1	0.1	0.2	0.4	0.3
Oleic acid	3819074.0	1.0	1.0	1.0	1.0	1.0	1.0
Linolenic acid	1047257.5	1.0	0.5	1.0	0.3	1.0	1.0
Mannose-6-p *	8790022.0	0.0	0.0	0.0	0.0	0.3	1.0
Glucopyranoside	2226475.5	1.0	0.0	0.0	0.3	0.3	0.0

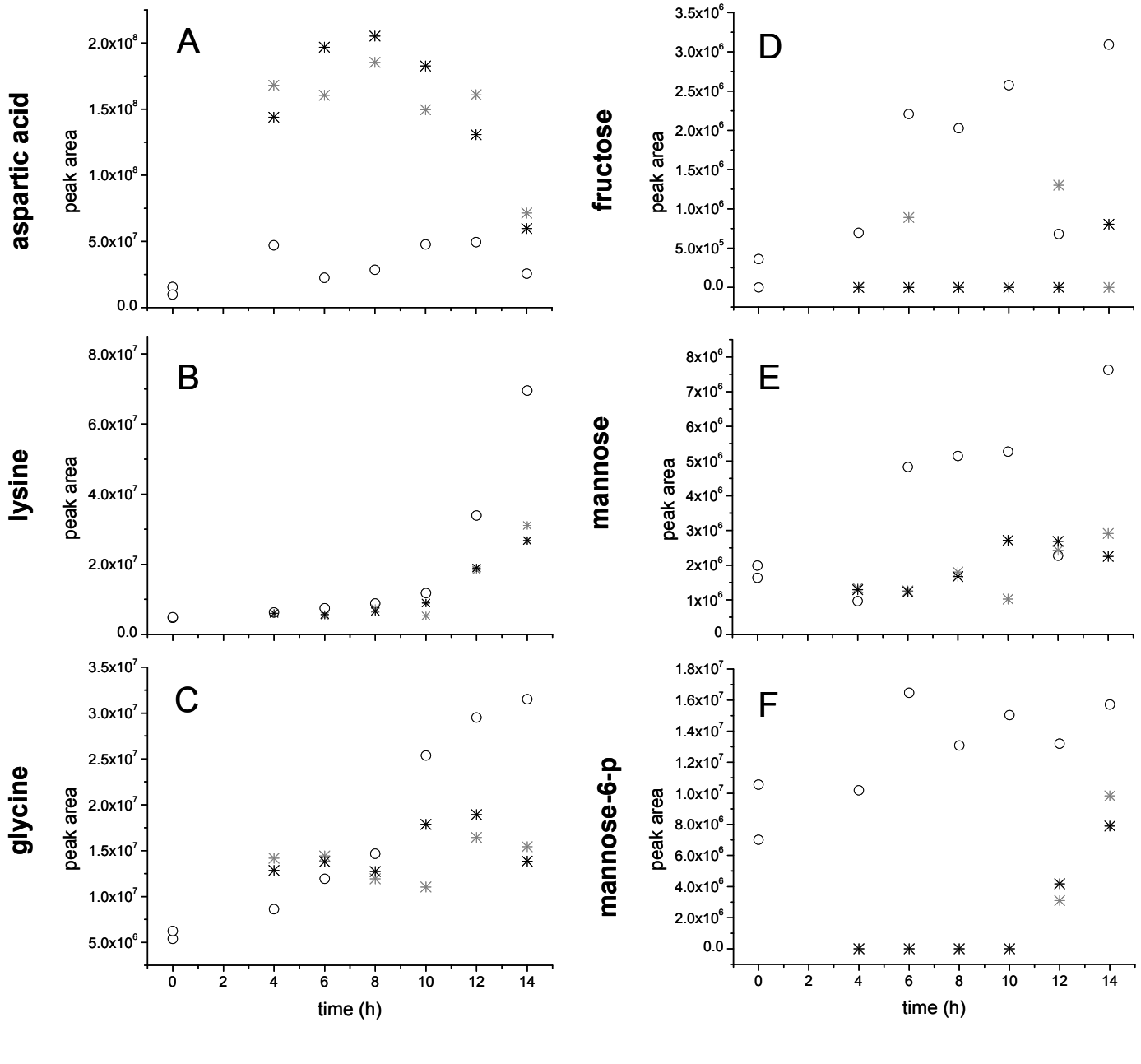


Figure 12: Time- and prometryn (0.1 µmol L^{-1}) dependent dynamics in the peak area abundance of six hydrophilic metabolites (aspartic acid, lysine, glycine, fructose, mannose and mannose-6-phosphate) from the chlorophyte *Scenedesmus vacuolatus*. Peak area values in references (circles) and exposed algae (grey and black asterisks) are shown for 7 growth stages between 0 and 14 hours of the microalgal developmental phase. At growth stage 0 hours, two replicates of references are shown. For each further time point one reference (circle) and two treatment replicates (grey and black asterisks, see legend) are shown.

3.2.4 Characterisation of the Algal Growth in Response to Time and Prometryn Toxicity

Measuring the algal cell size at different experimental time points as well as for reference and treatment conditions (as described in Section 2.2.2) resulted in varying cell volumes for the cultures containing similar cell numbers of approximately 10^6 individuals per mL cultural medium.

Time Response Investigations

In Figure 13, the mean cell volume of *S. vacuolatus* (y-axis) is shown during 14 hours of growth (x-axis) under reference and treatment (0.1 µmol L^{-1} prometryn) conditions.

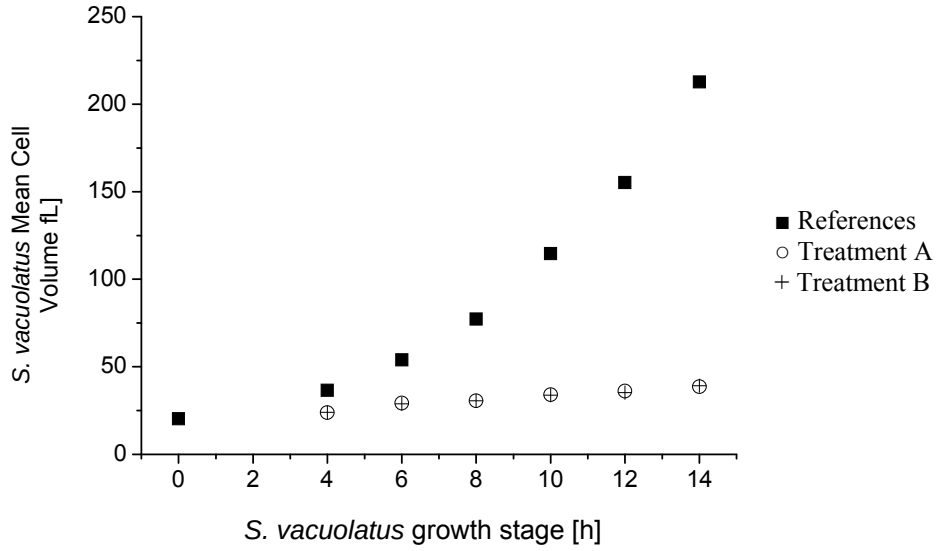


Figure 13: The change of the mean cell volume for references (■) and two treatment replicates (○ and +) of synchronised *Scenedesmus vacuolatus* cells (x-axis) is shown over the developmental phase (growth from 0 to 14 hours) of the chlorophyte (y-axis). After 0, 4, 6, 8, 10, 12 and 14 hours of reference or exposure conditions with 0.1 µmol L^{-1} prometryn, the algal cell volume in fL (femto litre; 1 fL = 10^{-9} µL)) was measured in parallel to the metabolites gain information about phenotypic effects of prometryn.

Under **reference conditions** obtained autospores of *S. vacuolatus* (growth stage 0 hours) showed a mean diameter and a mean cell volume of approximately 3 µm and 20 fL (femto litre) [1 fL = 10^{-9} µL], respectively. After 14 hours of algal growth under reference conditions, cells reached a mean diameter and a mean cell volume of approximately 8 µm and 200 fL, respectively. Hence, during growth phase the mean cell volume of reference chlorophytes increased from ~ 20 up to ~ 200 fL, which is equivalent to a ten-fold increase in cell size.

The cell volumes of chlorophytes **exposed to 0.1 µmol L^{-1} prometryn** were found to be consistently below those of undisturbed algae. After 4 and 6 hours of treatment the cell volume was approximately 2-fold lower than that of reference algae. After 14 hours of growth, treated chlorophyte cells became no larger than 50 fL mean cell volume, which was equivalent to a growth inhibition of 86% or a 5.5-fold lower cell volume compared to that of references. Cell size of treatments was equivalent to a doubling in cell size after 14 hours of growth compared to autospores (t_0).

Concentration-Response Investigations

After 14 hours of exposure with various concentrations of prometryn ranging from 0.002 to 0.3 µmol L^{-1}, mean volumes of cell from treatments and references were compared (Figure 14). Cell sizes for the highest three concentrations of prometryn, ranging from 0.07 to 0.3 µmol L^{-1}, were found to be from 2.5-fold up to 7-fold lower than those of references. Exposure of *S. vacuolatus* to the fifth-highest prometryn concentration (0.03 µmol L^{-1}) resulted in a 1.3-fold lower algal size compared to references.

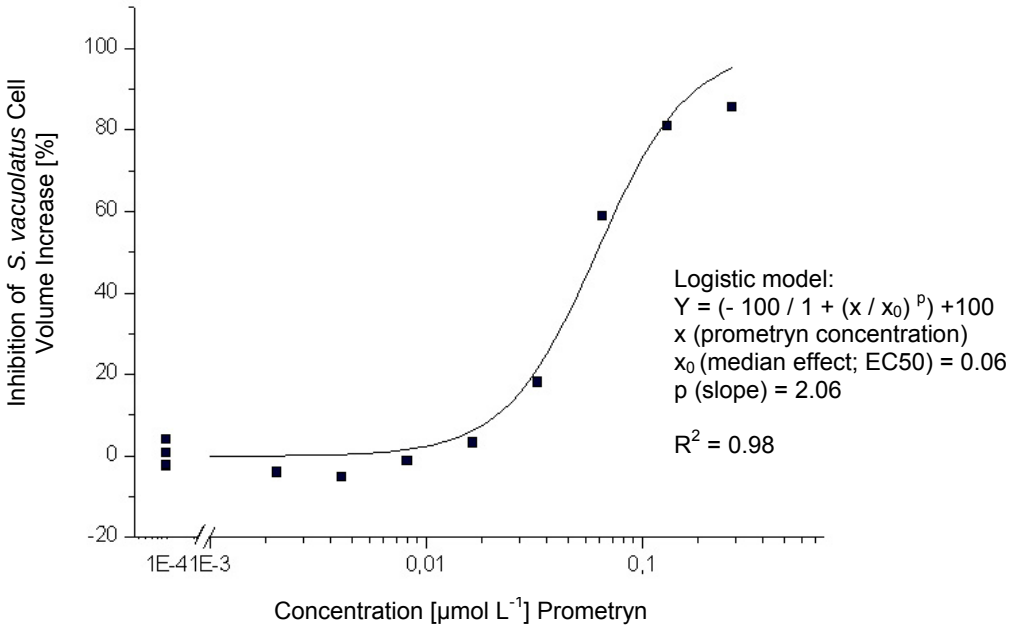


Figure 14: Concentration-response relationship between the inhibition of the cell volume increase of the chlorophyte *Scenedesmus vacuolatus* and 14 hours exposure to 8 prometryn concentrations in µmol L^{-1}:
(1) 0.002; (2) 0.004; (3) 0.01; (4) 0.02; (5) 0.03; (6) 0.07; (7) 0.13 and (8) 0.26. In addition, 4 references are also shown, with two values of them overlaying.

In Figure 14, a sigmoidal concentration effect curve is shown. It describes the inhibition of the increase of the mean cell volume of *S. vacuolatus* (y-axis), 14 hours exposed to various prometryn concentrations (x-axis),

compared to references. The cell volumes of chlorophytes exposed to the four highest prometryn concentrations (0.03, 0.07, 0.13 and 0.26 µmol L^{-1}) increased significantly less than references, which is equivalent to a larger inhibition effect on algal growth. Algal cells treated with the highest prometryn concentration (0.26 µmol L^{-1}) grew up to a mean cell volume of 26 fL. This mean volume corresponded to a growth inhibition effect of approximately 86% and was similar to that mean cell volume of references after 4 and 6 hours of growth. 14 hours of exposure to 0.1 µmol L^{-1} prometryn inhibited growth by ~ 65%.

Varying cell volumes also caused differences in the total biomass in algal cultures, that contained similar numbers of cells. Thus, changes in total algal biomass in the cultures had to be considered during cell harvesting, to ensure harvest of similar amounts of biomass.

Results describing the effects of prometryn on the algal growth (Figure 13 and 14) were compared with results describing those on the metabolic composition of *S. vacuolatus* (Figure 7 and 10). At the metabolite level, clear multiple effects resulted from prometryn exposure from growth stage t_4 on, for example demonstrated by aspartic acid and sugars, such as galactose and mannose-6-p. The concentration dynamics of aspatic acid and mannose-6-p are shown in Figure 12 a and f. The metabolic composition of *S. vacuoluatus* (Figure 7 a & b) responded at lower concentrations of prometryn (0.002 µmol L^{-1}) than the cell size during the concentration series, as shown in Figure 14. At the phenotypic level, prometryn resulted in a 0.6-fold lower cell volume of treatments at growth stage 4 hours compared with references (t_4).

In summary, by comparing the concentration- and time-related impact of prometryn on *Scenedesmus vacuolatus*, the metabolic composition was shown to respond at earlier growth stages and at lower concentrations than the chlorophyte's cell volume or cell size. The hydrophilic metabolic composition was found to show more clear effects regarding prometryn exposure than the lipophilic metabolic composition, in time- and concentration-series.

3.3 Summary of Results

The metabolomics approach for the analysis of hydrophilic and lipophilic metabolites from *Scenedesmus vacuolatus* with a GC-MS system, developed in this study, resulted in approximately 600 hydrophilic and 300 lipophilic signals. The variability between replicates of samples and of experiments was approximately 20% with the developed metabolomics approach (see Section 3.2.1). Components were identified using custom libraries and approximately 1/6 of hydrophilic metabolites were assigned

a biochemical structure (see Appendix). The chromatograms were characterised for reference conditions as well as for concentration- and time-dependencies.

Concentration-related changes of the hydrophilic and lipophilic metabolite composition were found for 14 hours old chlorophytes, while the lipophilic metabolite composition was affected at higher prometryn concentrations (see Section 3.2.1). The algal cell size was also affected at higher prometryn concentrations compared to the lipophilic metabolite composition (see Section 3.2.4).

Time-dependent changes of the hydrophilic and lipophilic metabolic compositions were found (see Section 3.2.2). The hydrophilic and lipophilic metabolite composition of references showed a time dependent trend. The time-dependent trend of hydrophilic and lipophilic metabolite compositions from *S. vacuolatus* treatments was different from that of references, but this difference was more significant in hydrophilic than in lipophilic metabolite compositions. Comparing the time-dependent impact of 0.1 µmol L^{-1} prometryn on the metabolic composition and algal growth in cell size, metabolic composition was affected after 4 hours of exposure and therefore earlier than algal growth in cell size (see Section 3.2.4).

Discussion

4 Discussion

In this study, a metabolomics approach was developed for the analysis of hydrophilic and lipophilic metabolite extracts from the chlorophyte *Scenedesmus vacuolatus*. Because it was the first time this organism was used for metabolomics, a standard protocol was developed for the methodological approach. Thus, in the following chapter the developed metabolomics protocol will be discussed critically in comparison with the literature. Additionally, further optimisation perspectives are outlined to support future studies using this protocol. In the second part of the discussion the protocol as well as the results of the compound identification will be addressed in comparison with the literature. The third chapter focuses on the application of metabolomics for the analysis of effects of toxicity and on the biological interpretation of the metabolomics results from time- and concentration related experiments together with algal growth results (cell size) and in comparison with the literature.

4.1 Metabolomics Approach - Workflow

In this section, the metabolomics approach with *S. vacuolatus* will be discussed based on the structure of the workflow regarding similarities and differences compared with the literature as well as proposals for further optimisation. The workflow can be summarised within the following steps: experimental design including cell harvest, sample preparation, metabolite analysis, data processing and data analysis (Figure 15). This workflow scheme is comparable to the literature, e.g. Rubtsov *et al.* (2007) and Goodacre *et al.* (2007), but details of the selected protocols were developed with respect to the target organism, experimental design and techniques available in the institute. To date there is no standard protocol used for metabolomics and the methods are still under development. Thus, the steps of the workflow (Figure 15) are discussed in detail in the following.

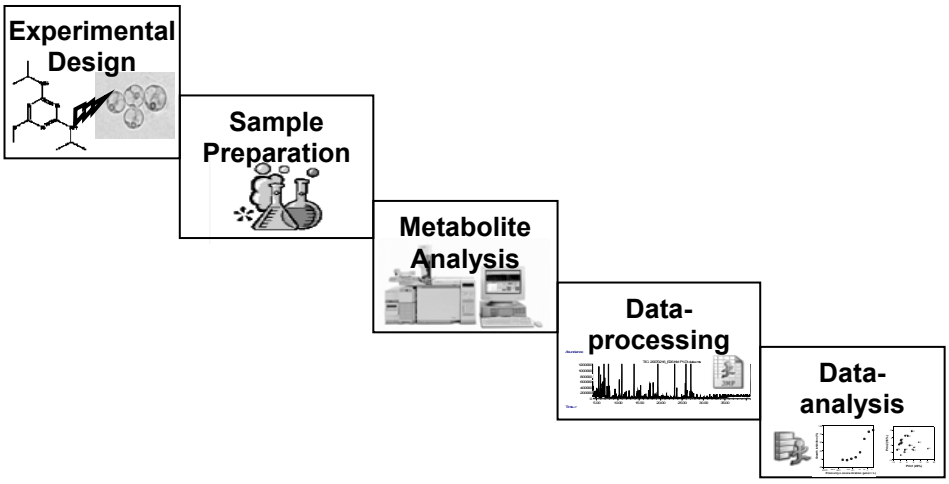


Figure 15: Rough workflow for the metabolomics approach with *S. vacuolatus*, consisting of 5 steps, in order from top to bottom.

4.1.1 Experimental Design and Harvest

The first step of the workflow was the design of the experiment for *S. vacuolatus* with synchronised culturing, exposure of cells to the chemical and cell harvest. In this section, the advantages of synchronisation for the developed metabolomics approach will be addressed. The harvest protocol will also be critically discussed regarding the time consume required and an alternative will be recommended.

Synchronised clonal population of *Scenedesmus vacuolatus*

Synchronous clonal populations of genetically identical cells of the green alga *S. vacuolatus* were used to identify stage-specific differences in control algae and treatments. During the growth phase of 14 hours, cells showed a ten-fold increase in size. The individual cell volume during algal growth corresponded to those described in former studies (Altenburger *et al.*, 1990; Faust *et al.*, 1992). The use of synchronised clonal populations is in contrast to most previous work in the field of plant metabolomics. Plant metabolomics focused on the analysis of higher plant tissues or individuals and has the advantage that observed responses are already averaged for many individuals, thus the variability between data sets, derived from two independently processed experiments, should be low and therefore, the method is suitable for the analysis of metabolic responses.

In this study, the mean of the relative standard deviation among peak area values (CV) of the preparative error as well as the intra- and inter-experimental variability was approximately 20%. Values of the variability for the metabolite extraction of various plants are summarised in Table 4.

Table 4: Comparison of the variation coefficient (CV) of the intra- and inter-experimental variability from this study with CV values reported in four plant metabolomics studies by Fiehn *et al.* (2000 a), Weckwerth *et al.* (2004), Ossipov *et al.* (2008) and Broeckling *et al.* (2005). In addition, the metabolite number, sample number, the tissue and organism are listed which was used to obtain the CV value.

Mean CV [%]	Metabolite number	Sample number	Tissue	Organism	Authors
~ 20%	113	4 replicates from 1 and from 2 experiments	synchronised clonal population	*Scenedesmus vacuolatus* (unicellular Chlorophyte)	This study
~ 40%	11	18 replicates from one experiment	leaves from one individual plant	*Arabidopsis. thaliana* (higher plant)	Fiehn *et al.* (2000 a)
~ 39%	652	28 replicates from one experiment	leaves from one individual plant	*Arabidopsis thaliana* (higher plant)	Weckwerth *et al.* (2004)
~ 40%	283	6 – 8 samples from one experimental group	leaves from one individual plant	*Betula pendula* (higher plant)	Ossipov *et al.* (2008)
~ 40%	120	3 replicates from 4 experiments	cell culture from roots	*Medicago truncatula* (higher plant)	Broeckling *et al.* (2005)

In Table 4, the mean CV value of approximately 20%, obtained in this study, was compared with that described by four plant metabolomics studies. Fiehn *et al.* (2000 a) studied 18 *A. thaliana* plants and the peak areas of the hydrophilic fractions. Weckwerth *et al.* (2004) also worked with *Arabidopsis thaliana* plant leaves. Broeckling *et al.* (2005) studied hydrophilic and lipophilic metabolites from cell cultures of the legume *Medicago truncatula* and Ossipov *et al.* (2008) studied leaves of silver birch trees (*Betula pendula* Roth). All of the authors described a CV of 40%, estimated in a comparable way to the one described in this study. The authors termed this value biological variability and concluded that it accounted for the largest source of variability of their whole metabolomics approach. Therefore, in the present study intra- and inter-experimental variability of ~ 20% was half of the value described in the literature for samples from leaves of individual plants or from cell cultures of legume roots. Hence, metabolite extracts from the unicellular chlorophyte *S. vacuolatus*, derived from $>10^6$ genetically nearly identical

individuals from synchronized clonal populations, reduced the source of variation substantially. Therefore, the metabolomics approach with synchronised clonal populations of *S. vacuolatus*, presented here, is suitable for the analysis of toxic effects on the molecular level.

Harvest

The harvest protocol was developed based on the techniques available at the institute to create a procedure that minimised work and time required. The harvest of a constant amount of biomass was important for the comparison of metabolites at different conditions of the organism to distinguish for example, effects of time or of the toxicant.

Amount of biomass for the metabolite extraction

The harvested amount of biomass, used for the extraction of hydrophilic and lipophilic metabolites, was optimised to ensure a similar amount of algal biomass in each sample. This was important because *S. vacuolatus* cells at different growth stages showed comparable cell density (10^6 cells mL^{-1}) but various cell sizes. For example, the cell size of autospores from *S. vacuolatus* had a 3 µm diameter and 20 fL mean cell volume, whereas 14 hours old algae cells had a cell diameter of 8 µm and a mean cell volume of 200 fL. Thus, concentrations of metabolites in algae cells can vary due to variation in cell size over time (growth) as well as during reference and exposure conditions (e.g. inhibition of metabolic processes). Even if the number of 10^6 cells mL^{-1} would be similar for all samples, differences in the total biomass-volume of cells, representing various treatment conditions, can cause differences in the total concentration of metabolites. Such biomass-dependent alterations in the metabolite composition of algae are unrelated to toxicity and can hide or hinder the identification of toxicity-related effects. Thus, a standardised amount of the total biomass-volume of cells was extracted to prevent changes in the metabolite concentration not related with toxicity. As a consequence, a total biomass-volume of 10 µL of *S. vacuolatus* cells was calculated and harvested for each sample in this work. This biomass-volume of algal cells gave the best results in GC-MS compound separation and peak resolution.

The amount of total biomass-volume harvested, was estimated according to the available resources of culturing and sample preparation techniques as well as to enable a clear separation of signals in the GC-MS total ion chromatogram with the protocol presented above. Due to the shown metabolomics results obtained with similar biomass-volumes for all samples, e.g. quantified and identified metabolites, the usability of the

protocol with *S. vacuolatus* and its suitability for toxicity analysis is revealed.

Harvest Time

With the multistep protocol described above, harvest of three samples to freezing in liquid nitrogen took approximately 20 min. Where more than three samples had to be harvested at the same time point, e.g. 14 samples of the concentration series at growth stage 14 hours, harvest took up to two hours. This increase in time resulted from only three places in the filtration system to harvest large volumes of cultural medium (up to 600 mL). In the institute, centrifugation was available for a maximum volume of 80 mL with 3300 x g and for 8 tubes per run, which was too small for 600 mL of cultural medium. Thus, centrifugation under these conditions would result in much more effort and time for all samples than the three fold multi-filtration. Hence, the multi-filtration vacuum technique, used here, was most time efficient in comparison to other techniques available in the institute and the time for harvest can be reduced by working with two people.

Nevertheless, the time until stop of metabolic activities was too long when compared with the literature, which describes the aim of a metabolomics approach to stop any metabolic activity as fast as possible to prevent alterations of the metabolic signature (Lee and Fiehn, 2008). Thus, further development of this step of the protocol should be necessary to interrupt the metabolic activity, as fast as possible for all samples at the same time point.

Another experimental design could decrease harvest time down to the 20 min as well. For example, an experiment with approximately 21 samples could be designed by inoculating and harvesting three samples at one time point which was approximately 20 minutes. The other cultures can be processed in a cascade of each three cultures at 6 further time points. Thus, all samples of the experiment can be harvested within 20 minutes and therefore in a similar interruption of metabolic processes for all samples which should improve the metabolomics approach with *S. vacuolatus*.

4.1.2 Sample Preparation

The second stage of the metabolomics workflow (Figure 15) is the sample preparation, including metabolite extraction and separation of hydrophilic and lipophilic fractions. In addition, the use of procedure blanks in parallel to algal samples will be addressed.

Tissue Homogenisation and Metabolite Extraction

In this study, after lyophilisation of the 10 µL biomass-volume of algal cells, an ice-cold solvent mixture of methanol, chloroform and 0.015% trifluoroacetic acid in water (10/5/4, v/v/v) as well as glass beads were added and vibrated heavily in a ball mill. Consequently, the tissue was homogenised and the metabolites were extracted. The success in crushing cells and extracting metabolites became evident from the green colour of the extracts (results not shown), indicating a solution of photosynthetic pigments (chlorophyll). The use of a ball mill is in accordance to the protocol described by Lisec *et al.* (2006) and Fiehn *et al.* (2000 a, b). Lee and Fiehn (2008) compared suitability of glass beads with that of steel balls for grinding in a cell mill of 6 x 10^6 lyophilised cells (~0.75 mg dry weight) from the unicellular alga *C. reihnhardtii*. Finally, the authors recommend steel balls, because the supernatant was removed better from the tubes containing steel balls which have lower volumes, than those from glass beads. In addition, the authors show a 30-40% lower efficiency of glass beads grinding compared to that with steel balls, comparing the sum intensity of all identified peaks. Thus, the use of steel balls, as described by Lee and Fiehn (2008), promises an increase of the extraction efficiency of metabolites from *S. vacuolatus* cells.

Besides the use of a cell mill to homogenise tissue, other methods including grounding of leaves from higher plants with pestel and mortar in liquid nitrogen (e.g. Roessner *et al.*, 2006; Kaplan *et al.*, 2004) or quenching of unicellular algal cells (*C. reinhardtii*) in salt (Bölling and Fiehn, 2005; Lee and Fiehn, 2008) are commonly used in metabolomics. Thus, alternative procedures of tissue homogenisation and extraction of metabolites are available and could be less intensive in work and time, but may differ in the concentration of extracted metabolites. Thus, the method using mortar and pestel can be of advantage for the homogenisation of large amounts of tissue, e.g. from higher plants, which is shown by Kaplan *et al.* (2004), but could cause great loss of biomass applying this method for the metabolite extraction of *S. vacuolatus* cells.

Liquid-Liquid Separation of Hydrophilic and Lipophilic Metabolites

Metabolite extracts were separated into hydrophilic and lipophilic fractions, to enable analysis of as many metabolites as possible. This is comparable with the literature by Ossipov *et al.* (2008), Broeckling *et al.* (2004), Huang *et al.* (2008) and Fiehn *et al.* (2000 a), who reported the parallel analysis of hydrophilic and lipophilic metabolic fractions for similar reasons. In contrast, authors like Roessner *et al.* (2006) and Wagner *et al.* (2003) discarded the lipophilic fraction after liquid-liquid

extraction. Discarding the lipophilic fraction can save time for sample preparation and metabolite analysis, but can be accompanied by the loss of information of the lipophilic fraction.
In addition, for example Lee & Fiehn (2008) and Gullberg *et al.* (2004) decided not to include a solvent partitioning step after extraction, to separate lipophilic from hydrophilic compounds. The authors avoid a separation of extracts to analyse the metabolites in a more unspecific way or over a broader range compared with the analysis of specific metabolites. Excluding the solvent preparation step can save time due to a decrease in sample preparation steps and the need of only one GC-MS analysis method. Additionally, Lee and Fiehn (2008) report that the work with crude metabolite extracts limits matrix effects and cross-contamination by semi-volatile breakdown artefacts, but used a Gerstel automatic liner exchange system for the full extract injections set at 50 °C and exchanged the multi-baffled liner after every 10th injection. However, whether they were able to increase the number of quantified metabolites using crude extracts, compared with their former study with *C. reinhardtii* hydrophilic extracts (Bölling and Fiehn, 2005) or not, is unclear in the report from Lee and Fiehn (2008). Hence, although there are alternative protocols published that avoid the additional preparation of lipophilic fractions, besides the time saved, application of such a protocol can be accompanied with fewer and complementary quantified metabolites. As the major aim of this study was not to provide a method for the high-throughput analysis, the alternatives described above are still possible to increase the efficiency of the protocol.

Use of Procedure Blanks

Blanks of the entire experimental procedure, from harvest to analysis, were used to subtract unrelated signals from the *S. vacuolatus* data sets. This is comparable to Wagner *et al.* (2003), who describe the use of non-sample references to check for chemical contamination and in order to detect carryover effects in the course of the GC-MS sequences. Other scientists in the metabolomics field also used this procedure to identify the compounds deriving from the organism.

4.1.3 Metabolite Analysis

The third step of the work flow in Figure 15 is the chemical analysis of metabolite extracts. A quadrupole GC-MS (EI) system was used to analyse metabolites from hydrophilic and lipophilic fractions. Due to the range of chemical functionality, metabolites were derivatised to transform structure into a stable and volatile form suitable for GC-MS analysis. Thus, in the following, the analysis system will be assessed for

advantages, disadvantages and alternatives. Additionally, the derivatisation procedure used in this study will be discussed in comparison with the literature.

Gas Chromatography - Mass Spectrometry System

Metabolites from *S. vacuolatus* were analysed using a gas chromatography-mass spectrometry (GC-MS) system (Agilent, USA). This is comparable to literature, were the GC-MS system is described as a gold standard system in metabolomics. For example, Roessner *et al.* (2000) recommend the metabolomics application with a GC-MS system for highly reproducible and selective analysis of a broad range of compounds with broad chemical composition. High sensitivity and robustness, as well as the possibility for qualitative and quantitative analysis, make the GC-MS system suitable for metabolomics analyses of complex mixtures and low sample amounts. The advantages and disadvantages of MS techniques have been thoroughly reviewed by Dunn & Ellis (2005). The authors reported restrictions of the analysis system, like the target against non-volatile, high molecular weight metabolites. Thus, GC-MS is a common technique used for metabolomics studies but not unbiased, due to methodological restrictions.

Metabolites from hydrophilic and lipophilic fractions were analysed using one GC-MS system, one single capillary column and two different analysing methods in this work. This is comparable with the literature.
Ossipov *et al.* (2008) report the analysis of hydrophilic and lipophilic metabolites from birch tree leaves, also analysing them with one GC-MS system and one capillary column, but two different analysis methods. Also Huang *et al.* (2008) used one GC-MS system to analyse hydrophilic and lipophilic metabolites from barley plants, but the authors studied also free amino acids with a targeted protocol using a liquid chromatography-mass spectrometry (LC-MS) system. Tolstikov and Fiehn (2002) propose the separation and analysis of highly hydrophilic compounds, such as amino sugars, amino acids, sugar nucleotides, oligosaccharides, and glycosides, by liquid chromatography (LC) methods. LC methods can be suitable for the targeted analysis of hydrophilic compounds, but for the application in metabolomics studies further development and effort is required (Glinski and Weckwerth, 2006). The authors describe the advantages of GC-MS and LC-MS techniques in the identification and quantification of individual compounds in a complex mixture.
Further techniques applied in metabolomics studies are reported, e.g. by Glinsky and Weckwerth (2006), Hall (2006) and Dunn and Ellis (2005), such as nuclear magnetic resonance (NMR) spectroscopy, fourier transform infrared (FT-IR) spectroscopy, capillary electrophoresis–mass

spectrometry (CE-MS) and other mass spectrometry (MS) techniques. The use of a gas chromatography-time of flight mass spectrometry (GC TOF-MS) system is recommended by Glinski and Weckwerth (2006) to reach higher mass resolution and accuracy as well as high sensitivity in full scan mode. For further improvement concerning the complexity of metabolite mixtures, the authors recommend the combination GC x GC TOF-MS to handle high molecular weight metabolites, such as tri- or tetrasaccharides but also organic diphosphates and cofactors and to elucidate unknown structures. Other methods of identification and reasons for lack of identification of metabolites are well summarised by Herebian *et al.* (2007). Glinsky and Weckwerth (2006) report that data acquisition using a single technology, such as GC-MS, cannot fulfil requirements of metabolomics approaches, such as comprehensiveness, selectivity, and sensitivity. However, in this study it was not the major task to identify unknown metabolites. In conclusion, a GC-MS (quadrupole) used in this study is sensitive, suitable, robust and relatively easy to handle for studying the metabolic composition of *S. vacuolatus*.

Derivatisation in a Two-Step Procedure

After separation of hydrophilic and lipophilic metabolite fractions, all extracts were dried and subsequently derivatised. This is in accordance to the literature because many metabolites analysed by GC-MS require chemical derivatisation due to the range of chemical functionality of metabolites (Dunn and Ellis, 2005). For example, volatile derivatives of non-volatile substances can be prepared to render them amenable to separation by gas chromatography.

In this study, samples were derivatised in a two-step procedure using either methoximation and silylation for the hydrophilic fraction or methanolysis and silylation for the lipophilic fraction, to increase the number of detected metabolites compared with a one-step derivatisation. The application of a two-step derivatisation procedure is comparable with the literature, e.g. by Roessner *et al.* (2000) and Fiehn *et al.* (2000 b). The authors worked with potato tubers and *Arabidopsis thaliana* leaves, respectively, and employed a two-step derivatisation procedure combining methoximation and silylation of hydrophilic metabolites due to the range of chemical functionality of the metabolite composition. In addition, Fiehn *et al.* (2000 a), Huang *et al.* (2008), Bölling and Fiehn (2005), Ossipov *et al.* (2008) and Broeckling *et al.* (2005) also report a two-step derivatisation procedure of hydrophilic and lipophilic fractions. The two-step derivatisation of hydrophilic and lipophilic compounds, in this study, is reported by the literature and can increase the number of

detected metabolites due to the covering of a wide range of chemical properties by GC-MS analysis of derivatives.

Derivatisation Conditions and Number of Derivatives

In this work, more than one derivative for a single metabolite was detected (see Figure 8 and 9). Hence, the number of detected compounds may not equal the number of metabolites in the extract before derivatisation. This was also reported in the literature by Roessner *et al.* (2000). The number of derivatives for a compound may depend on the conditions of derivatisation incubation, handling of the sample after derivatisation and analysis parameters. The derivatisation protocol from the present work should be studied regarding influences of procedural parameters on the number of detected derivatives after GC-MS analysis.

Dunn and Ellis (2005) report advantages and disadvantages about the presence of water during derivatisation. The authors report that trimethylsilyl (TMS)-esters can break down in the presence of water because esterification is a reversible reaction, while the removal of water prior to derivatisation can improve its efficiency. Additionally, the authors describe the loss of volatile metabolites, such as lactic acid, after extensive sample drying. As shown in the Appendix, lactic acid has not been identified in one of the three chlorophytes listed (*Scenedesmus vacuolatus*, *Chlamydomonas reinhardtii* and three *Chlorella* species). In contrast, lactic acid has been detected in other plant metabolomics studies as described for example in the metabolic composition *A. thaliana* leaves (Kaplan *et al.*, 2004). Whether lactic acid is a common compound in green micro algae remains unclear, but lactic acid is one of the basic compounds of the plant metabolism. Thus, drying of samples may have been too extensive, leading to the absence of lactic acid and therefore there should be need to optimise the drying regime of the derivatisation protocol in this study.

However, the absence of lactic acid (as discussed in Section 4.1.3), but also of other basic metabolites from the plant metabolism, such as arginine, maltose and fructose-6-phosphate, may demonstrate an insufficiency of the present metabolomics approach for the evaluation of biochemically important primary metabolites in the chlorophyte extracts. The harvest protocol used in this metabolomics approach as well as the procedure of metabolite extraction and –derivatisation or the GC-MS analysis method and the data evaluation or identification procedure should be studied to ascertain the reason for the absence of important primary metabolites in the chlorophyte.

4.1.4 Data-Processing

In the present study, the processing of metabolomics data, such as comparison of mass spectra, retention times and peak shapes, definition of time windows, integration of peak area, alignment, exclusion of contaminants, consideration of shifts in retention times and so forth, were done manually to become acquainted with data and required steps for this procedure. The integration of data, quantification of peak areas and deconvolution of components was done automatically. As the method shown here is under development, the data processing steps were studied in detail and have been listed below to support decision-making for criteria of selection for an automatic tool.

Integration and Quantification of Chromatographic Signals

The extraction and analysis of a standardised amount of 10 µL biomass-volume of S. vacuolatus cells, enabled automatic integration and quantification of peaks using the system's ChemStation software. This procedure caused low variability in apical retention times and supported therefore manual data alignment. Although the quantification of peak heights is a common method in GC-MS data evaluation, it caused unwanted variability in the quantity of high narrow and low broad peaks in the data evaluation of metabolomics data. For example, a well separated chromatographic signal is shown in Figure 16. The shape of this peak changed over time as demonstrated with 20 overlaid total ion currents or chromatograms (TICs). The apical retention time showed a right tended shift for the chromatographic signals. The separation of chromatographic signals can be less clear within the total ion chromatograms, due to overlaying peaks, as shown for example in Figure 5 a between RT 15 and 20 min or in Figure 5 b between RT 25 and 35 min. By using the peak height for the integration of peaks from TICs a broad range of apical retention time (RT) values was caused and therefore, difficulty in the manual alignment on the basis of RTs over the whole data set. However, the evaluation of the area of peaks in the TIC is more commonly used for the quantification of chromatographic signals in metabolomics approaches, for example described by Fiehn et al. (2000 a), Maloney (2004) and Bunk et al. (2006). The integration of peak area values for the quantification, as applied in this study, was suitable for the quantification of chromatographic signals after the analysis of complex metabolite extracts and simplified a consistent integration of peak areas as well as the manual alignment of data. However, a permanent examination of additional information from the TICs and the mass spectra was required to gain a reliably aligned data set.

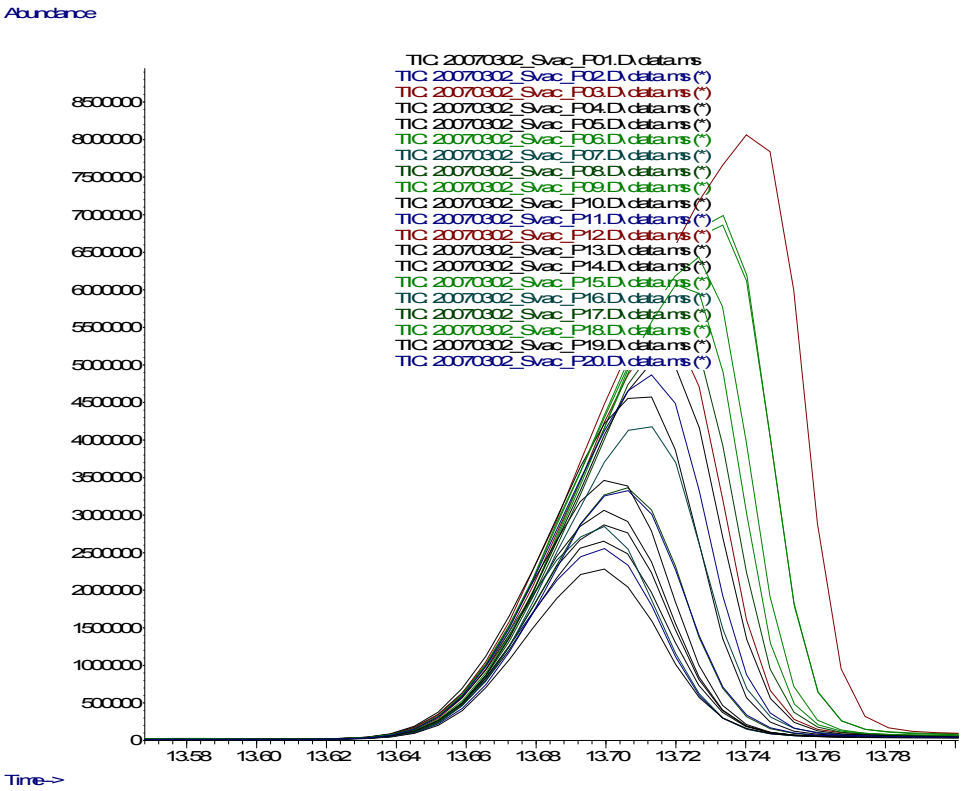


Figure 16: A chromatographic peak at retention time 13.714 min from 20 *S. vacuolatus* hydrophilic fractions from the time course study is shown to demonstrate concentration shift accompanied by a retention time shift to the right of the peak.

Alignment of the Retention Times of Chromatographic Peaks

During manual alignment, single and overlaid total ion chromatograms of samples, mass spectra, library data, and MS Excel spreadsheets were used in parallel.

After manual alignment of retention times from quantified peaks from all samples of an experiment in Excel, approximately 300 compounds were found within all chromatograms of samples from an experiment. From these 300 signals, approximately 150 signals from *S. vacuolatus* were picked by excluding signals of contamination and considering shifts in retention times. Manual alignment required much time and was quite challenging, because of the large data matrix. In the literature, integration and alignment was done automatically using software, such as metAlign (Vorst *et al.*, 2005) or ChromaTOF (Leco Systems, Germany), applied in metabolomics, e.g. by Fiehn *et al.* (2005). Thus, the establishment of an automatic method for the alignment can be helpful, although it was not applied here to assist in understanding the important steps in an automatic tool. The important steps of alignment in this study are listed below. If

these steps can be automated, errors in data handling could be minimised and the process of data analysis could be expedited:

1) Export of quantitative data from GC-MS controlling software (ChemStation)
2) Visual comparison of GC-MS chromatograms
    a. peak shapes or resolution of peaks
    b. retention time
    c. peak abundances
    d. contamination
3) Alignment of peak area values with respect to retention times
4) Exclusion of peaks with contamination greater than 50%
5) Exclusion of peaks which were detected sporadically and which showed unsystematic behaviour, comparing all samples of an experiment

Deconvolution

700 up to 1000 compounds were detected in several hydrophilic and lipophilic extracts from *S. vacuolatus*, depending on the growth stage of the analysed algal cells in this work, using the deconvolution software AMDIS (Automated Mass Spectral Deconvolution and Identification System). Although this is a high number of compounds, not all of them were identified as algal metabolites. The number of detected compounds in this study was comparable to that found by Bölling and Fiehn (2005). The authors used ChromaTOF software and detected approximately 800 metabolites from hydrophilic extracts of the unicellular green alga *Chlamydomonas reinhardtii*. Lu *et al.* (2008) demonstrate that AMDIS has a high rate (70-80%) of false positives in deconvolution compared to other software such as ChromaTOF (10-20%), but propose deconvolution with AMDIS to be the choice for accurate mass spectra. The authors demonstrate also that deconvolution results depend on operator-set software parameters and reveal that there is no efficient data handling system available yet. Lu *et al.* (2008) recommended AMDIS to reach high resolution and mass spectrum purification of GC-MS data and to resolve accurate mass spectra. Thus, AMDIS was used because it is freely available and it was suitable for the deconvolution of compounds in this study.

4.1.5 Data-Analysis

The pre-processed data were used for further statistical analyses including variability, preparative error and experimental variability, to estimate reproducibility of the metabolomics protocol. Reproducibility will be discussed, comparing results with those reported in the literature by Fiehn

et al. (2000 a), Weckwerth *et al.* (2004) and Broeckling *et al.* (2005). In addition, the multivariate statistical analysis method principal component analysis (PCA) was applied to investigate relationships and differences between all samples of one experiment.

Technical Error

The preparative and analytical error as well as intra- and inter-experimental variability were calculated to estimate sources and amounts of variability in order to validate the plant metabolomics protocol with *Scenedesmus vacuolatus* clonal populations. In this work, the technical error of peak size was ~5% in mean for 5 measurements of the same mixture of standard compounds, including 24 aliphatic hydrocarbons. Weckwerth *et al.* (2004) determined approximately 10% (mean CV) for the technical error of signal size after replication of metabolite analysis. Broeckling *et al.* (2005) show the analytical error ranging from CV 5 to 8% for peak area of one internal standard of samples, for the GC-MS system used. Fiehn *et al.* (2000 a) estimated mean relative CVs of approximately 8% for signals from the *Arabidopsis thaliana* leaves, deriving from derivatisation and analysis of 7 aliquots of one sample. Thus, a technical error of 5% of should be acceptable for metabolomics studies.

Comparison of Intra- and Inter-Experimental Variability with Preparative Error

Estimated values for the preparative error as well as the intra- and inter-experimental variability were comparable with a mean CV of 20 and 22%. The variability may have been derived mainly from sample preparation and not from synchronously grown algae and therefore, low variability of shown experimental approaches may contribute to the suitability of *S. vacuolatus* synchronous cultures for the toxicity analysis with metabolomics. In contrast, these similarities reveal that the preparative error can be the largest source of variability in this study. Thus, the sample preparation protocol could be improved as discussed above, including steps such as:

- Harvest
- Sample Preparation
- Derivatisation for GC-MS Metabolite Analysis.

The optimisation of these three protocol steps, while the optimisation of the harvest step is the most promising one, could reduce the preparative error by 20% in this study.

Multivariate Pattern Analysis Using PCA

Following examinations of variability, the multivariate statistical method Principal Component Analysis (PCA) was applied to reduce the variability of information and to investigate alterations in quantity of peak areas between more than hundred metabolites from the chlorophyte *S. vacuolatus*. Groups of samples with similar metabolic compositions, as well as separated samples with metabolic alterations were found. Separated samples described exposure to various concentrations of prometryn and development of algal cells. In addition, the loadings plot of PCA was used to identify biochemically interesting compounds. In the following, PCA preparation, PCA trends of sample scores, identification of interesting biochemical compounds as well as alternatives for non-linear PCA will be discussed.

Missing Values within the Data Matrix

A large number of missing values within the data matrix was evident in metabolomics data from this study. For example, the data set of lipophilic metabolites from the time series experiment was missing approximately 40% of the values. This means that no peak was quantified for specific retention times. The handling of missing values in metabolomics data varies and is still a topic for discussion, as reported by Kaplan *et al.* (2004), Desbrosses *et al.* (2005) and Mounet *et al.* (2007). As an alternative in this protocol for *S. vacuolatus*, the number of missing values could be reduced by using another data processing technique, such as the application of more sensitive peak integration parameters.

Normalisation of the Data for the PCA Analysis

After 'mean centring' (Sumner *et al.*, 2007) of the data, PCA was performed on the correlation matrix, variables in columns (signals) and fixed values in rows (samples), to improve visualisation of data from multivariate analysis and of changes in less-abundant metabolites. Normalisation via log transformation is also common in metabolomics, however the use of only mean centred for the preparation of PCA's is comparable with studies, e.g. conducted by Bailey *et al.* (2003). Desbrosses *et al.* (2005) and Roessner *et al.* (2006), however, applied normalisation or transformation steps in addition to mean centring of data, to reach a sharper separation of samples in the plot by filtering the influence of background noise (Sarry *et al.*, 2006). Bölling and Fiehn (2005) also describe the normalisation of measured data to the amount of sample material and internal standards to enable comparison and evaluation of the metabolic composition. As the analysis in this study was conducted on samples with standardised similar biomass from *S.*

vacuolatus, normalisation to reduce the influence of experimental factors on the data set should no longer be necessary. Thus, the experimental design can improve the applicability of the data for statistical and multivariate data analysis.

Software to Perform PCA

In the present work, PCA was performed using JMP IN 5.1 software (SAS Institute, Cary, USA). This software was also reported by Sumner *et al.* (2007). PCA is usually the first step in evaluating multivariate data sets in a metabolomics approach, because it is effective for reducing data as well as for detecting patterns, trends and groups among samples and it is supporting the identification of relationships between the metabolic profile and the 'biochemical phenotype' (Bhalla *et al.*, 2005). Hence, PCA of metabolite compositions can be an efficient means for the detection and explanation of deviating samples and for the analysis of dynamics in the metabolome.

Reproducibility of PCA Results between Samples in an Experiment

The PCA score plots in this work, showed clearly separated groups of samples, lying close together in the plot, demonstrating low variation for similar metabolic compositions at specific time points (Figure 10). This shows the reproducibility of the results and therefore the suitability of working with synchronously cultured clonal populations of *S. vacuolatus* in time- and concentration-dependent studies of the algal metabolome. In addition, the reproducibility of the experimental design from algal harvest to data analysis can be shown. Bölling & Fiehn (2005) studied environmental effects on the amount of hydrophilic compounds of the green alga *Chlamydomonas reinhardtii*. The authors report clear PCA score plot separation of experimental groups and low variability within these groups and argue therefore the suitability of their metabolite profiling approach for metabolomics. On that basis, the authors claim both validation and high reproducibility of the experimental procedure. Following similar reasoning, the experimental reproducibility is also validated in this study, due to close groupings and clear trends of sample replicates from *S. vacuolatus*.

In contrast to the very close groupings of samples in the PCA score plot, other groupings displaying greater deviation from later growth stages were also found, despite being prepared under similar experimental conditions (Figure 10 a). For example in this figure, two strongly separated replicates of treatments at growth stage 10 hours (t_{10}) are shown. One of the two samples was grouped with those treatments from earlier algal growth stages (t_4 and t_6) hours, displaying similarities with

the earlier metabolic composition, while the other t_{10} treatment sample was positioned as expected within the time trend of treatments between stages t_8 and t_{12}. As a result, the first t_{10} treatment sample which was grouped with the lower growth stages, was considered as an outlier and excluded from further discussion.

The distance between groups of sample replicate treatments (Figure 10 a and b) increased at growth stages t_{12} and t_{14}, although the reason for the larger variations between metabolic compositions of similar samples over time remains unclear. For example, a small time shift during inoculation of cultures, even with the advantage of previously prepared treatment cocktails in this study, could have caused such variation. Algae can live from cellular reserves during the first hours of growth, such that a time shift at earlier growth stages in treatments may have been less evident. However, a time shift during inoculation could have caused a shift in activation of further biochemical processes in genetically uniform populations and can cause therefore, a variation in concentration of metabolites within seconds (Morgenthal *et al.*, 2006 and Lisec *et al.*, 2006). Thus, the inoculation method for the experiments can be of relevance.

Trends of Scores in PCA Plots

Visualisation of PCA (principal component analysis) sample scores regarding time course or increasing concentration of prometryn treatment, showed up in continuous trends in this study. Those trends show a complex systematic change of the compound composition, e.g. as a result of organism growth or health decline. Such trend and stage specificity of sample scores are comparable with the literature, for example Viant *et al.* (2005) and Mounet *et al.* (2007) are discussed further in the Section 4.3.2.

Identification of Interesting Biochemical Compounds

Score and loading plots of the multivariate method PCA were used to determine most influential metabolites for a score plot pattern, in this study. The identification of the most influential metabolites improves the applicability of the method, by identifying those metabolites that respond significantly to the chemical. In this way, a few compounds can be selected and further studied in more detail, for example with respect to individual concentration dynamics or the identification of unknowns. This method was also applied by Bölling and Fiehn (2005), who reported the selection and indication of the five largest absolute loading values within PCA loading plots. Hence, the method of identifying biochemically interesting compounds with a multivariate approach is applied in metabolomics. Similarily, Bröckling *et al.* (2005) and Fiehn (2005) used

the vector length of loadings to differentiate between more or less intense responses of corresponding metabolites in plant samples, for the specific principal component. In the work presented here, the most influential metabolites for PCA scores plots were selected and quantitatively investigated for dynamics in their abundance (Figure 11 & 12). The quantitative investigation of dynamics of the peak area abundance of selected compounds was also performed by Bröckling *et al.*, (2005), Roessner *et al.* (2006), Kaplan *et al.* (2004) and Mounet *et al.* (2007). In the field of metabolomics, PCA is usually the first step in multivariate data analyses of dynamics in the metabolome as well as of relationships between single metabolites and the biochemical phenotype (Bhalla *et al.*, 2005). The analysis of individual metabolites, as shown in Figure 12, is also comparable to literature. Thus, PCA can be used for the identifications of alterations in the metabolome due to the influence of, for example chemical stress and could therefore, be used to screen for biomarkers for effects of toxicity.

Alternative Multivariate Methods to PCA

Besides unsupervised PCA, several complementary unsupervised but also supervised multivariate analysis methods can be applied to reduce the information associated with multivariate variables (quantified compounds) and to simplify the analysis of the similarities and differences between samples of an experiment. Unsupervised methods, such as PCA, work without using class information, with the aim of sample classification, while supervised methods should be used to identify the characteristic biomarkers of a damage or reduced health (Dettmer *et al.*, 2007). Alternative unsupervised methods are for example hierarchal cluster analysis (HCA), self-organizing maps (SOMs) and neural networks (NN), while sample properties are pre-defined in supervised techniques, such as partial least squares (PLS), discriminant analysis (DA) and artificial neural networks (ANN). These methods are summarised for example by Sumner *et al.* (2007), Brown *et al.* (2005), Dettmer *et al.* (2007) and Hall (2006). In one example for *Arabidopsis thaliana* plants, reported by Grata *et al.* (2008), unsupervised PCA is compared with HCA, PLS-DA and PCA for the analysis of variance (ANOVA) or orthogonal signal correction (OSC) filtered data. The authors show different separation results for various groups from different tissue locations and study metabolic wound signals at several time points. While two groups were separated by PCA, three were separated by ANOVA-PCA and PLS-DA, while OCS-PCA separated 5 groups in the data set, which was equivalent to the samples analysed (references at 4 time points). Hence, the advantage of alternative multivariate method

approaches to PCA can be the better separation of groups and therefore the more clear identification of biochemically interesting compounds for each state of the organism.

Combining the Information of the Metabolome with that of the Phenotype from *S. vacuolatus* & Identification of Toxicity-Related Changes in the Metabolome

In a total ion chromatogram, the biochemical composition of *S. vacuolatus* and the metabolic responses from the alga could be hidden by changes in the alga metabolome not related to toxicity or development, for example due to the leakage of cell plasma through broken cell walls or contamination due to solvents and medium or changes resulting from diet, temperature and the physiological status of the test organism. Thus, samples were prepared similarly in a proof-of-principle protocol including reference samples and procedure blanks, to demarcate the metabolic signal from *S. vacuolatus*. These proof-of-principle experimental conditions for the demarcation of effects of toxicity from other effects was called 'Phenotypic Anchoring', e.g. by Tennant (2002) and others.
The treatment of algal cells over 14 hours during growth with the highest used concentration of prometryn (0.3 µmol L^{-1}) caused a growth inhibition of approximately 86% (Figure 14). To ensure that cells are not dead in this state and therefore potentially causing changes not related not related to the toxicity in the metabolome, knowledge about cell activity was necessary. Adler *et al.* (2007) study phytotoxic effects in *S. vacuolatus* exposed to atrazine for 14 hours during the algal growth phase and demonstrated nearly 100% algal cell vitality. Atrazine concentrations in the range between 0.162 and 1.29 µmol L^{-1} inhibited *S. vacuolatus* growth, measured as cell volume, in an effect concentration range between EC_{50} – EC_{100} (Faust *et al.*, 1992). Atrazine, which belongs to the same chemical class as prometryn (*s*-triazine herbicide), can cause analogous effects of toxicity in plants if compared with studies by Küster & Altenburger (2007) as well as by Faust *et al.* (2001). Thus, it is assumed that the treated *S. vacuolatus* cells showing 86% growth inhibition were alive and capable of active metabolism. However, metabolic alterations, such as direct, indirect, or compensatory molecular responses, could still include alterations indicative of both a specific toxic mode of action and more generalised responses to stress (Ankley *et al.*, 2006). Within the metabolomics approach presented in this work, it was attempted to distinguish between both kinds of responses by ensuring an experimental design with reference samples and conducting a literature study about metabolic stress signals relevant to prometryn. Metabolic changes between specific growth stages were assumed to clearly

demonstrate growth related effects. Metabolic alterations between references and treatments, as shown for example in the PCA scores plots, have been assumed to demonstrate the effects caused by the toxicant prometryn, even at specific growth stages of the chlorophyte.

4.2 Compound Identification

In this work, following deconvolution, in total approximately 800 components were found in autospores whereas approximately 950 components were found in total in 14 hours old cells from *S. vacuolatus*. In the metabolite extracts from *S. vacuolatus*, approximately 600 hydrophilic and 340 lipophilic components were detected, respectively. The number of metabolites from *S. vacuolatus* increased over time, e.g. in preparation to cell division. In the following section, the method of compound identification will be addressed.

4.2.1 Method of Compound Identification

The National Institute of Standards and Technology (NIST) database (2005 edition) was used to assign a chemical structure to 41 hydrophilic and lipophilic algal metabolites with a match quality of ≥ 90%. This match factor was selected based on communications with people experienced in identifying compounds analysed by GC-MS (oral communication Brack, W. and Birkemeyer, C.). Although the number of identified compounds increased with lower match factors, the reliability of compound identification decreased in parallel.
For example, accepting a match quality of ≥ 20%, to reach an overview about biochemical compounds in the hydrophilic metabolic composition of *S. vacuolatus*, approximately 77 hydrophilic compounds were identified using Golm DB. The identified metabolites are listed in the Appendix. Identification of the same metabolic composition using the NIST library (2005 edition), additional evidence of identification was gained due to identification replicates. In the metabolomics literature, the match factor was rarely reported. Jones *et al.* (2008) identified 51 metabolites of the earthworm (*Lumbricus rubellus*) using a match factor of ≥ 50% with the NIST library (2002 edition). Bölling and Fiehn (2005) listed 77 identified hydrophilic metabolites from the green alga *Chlamydomonas reinhardtii* (Appendix). Hence, the number of identified compounds was comparable to the literature but may be dependent on the database and the match factor used.

A GC-MS system has an upper mass range of 1000/ 1500 Dalton. The analysis of crude hydrophilic and lipophilic algal extracts from *Scenedesmus vacuolatus* with a GC-MS system in this study avoided

biases against certain compound classes including amino acids and their derivatives, sugars and their derviatives, fatty acids, organic acids and other compounds. The unified atomic mass unit (u) of amino acids ranges between 75 (e.g. glycine) and 204.23 (e.g. tryptophan). Trisaccharides and adenosine tri phosphate (ATP) have an atomic mass of 505 u, while chlorophyll and vitamin B12 (cyanocobalamin) have a larger atomic mass of 906.9 and 1355.38 u, respectively. Many secondary metabolites have atomic masses less than 1000 u. Therefore, the metabolomics approach should enable the analytical detection of the biochemical important metabolites, such as arginine, maltose and fructose-6-p as well as of chlorophyll and ATP in the metabolic extracts of a plant. From this point of view, the metabolomics approach can provide potential to detect, identify and study additional basic primary metabolites and many secondary metabolites in detail. The herbicide prometryn has an atomic mass of 241.36 u but was not identified within *S. vacuolatus* extracts, in this study. If prometryn would have been identified within the algal extracts, the concentration of the chemical within the algal cells could have been studied as well.

4.3 Metabolite Composition in *S. vacuolatus* Extracts

In this work, 77 hydrophilic metabolites were identified with custom libraries on the basis of a mass spectral match quality of 20%. These metabolites were divided into eight classes and are listed in the Appendix. All of these metabolites are likely to be involved in the C-, N-, P- and S-metabolism.

4.3.1 *S. vacuolatus* Metabolites in Comparison with those of *Chlorella*- and *Chlamydomonas*-species

At the time of writing, no metabolomics study has been published on the *Scenedesmus vacuolatus*. Therefore, identified metabolites from this chlorophyte were compared with other species assigned also to the phylum *Chlorophyta* and the class *Chlorophyceae*. The metabolite compositions from *Scenedesmus*-, *Chlorella*- and *Chlamydomonas*-species were assumed to show similarities due to close phylogenetic affiliation, but dissimilarities were also assumed, e.g. for *Chlamydomonas*- species, because they have flagella.

The amino acid, sugar and lipid composition in *Chlorella* species was studied by Dunstan *et al*. (1992) and Brown and Jeffrey (1992), who used targeted analytical approaches. The identified hydrophilic metabolites are listed in the Appendix. The analysis of *S. vacuolatus* metabolites was conducted under a wider range of analytical conditions than that of

Chlorella metabolites and the number of detected metabolites from *S. vacuolatus* could not be compared with those reported for the three *Chlorella* species. However, the compounds detected in the hydrophilic *S. vacuolatus* extracts, such as 10 amino acids, 1 amino acid derivative, 3 sugars and 3 fatty acids, conform to the 21 listed metabolites of the three *Chlorella* species, reported by Dunstan *et al*. (1992) and Brown and Jeffrey (1992). Bölling and Fiehn (2005) developed a metabolomics approach for the GC-TOF (time of flight) MS analysis of hydrophilic metabolites from *Chlamydomonas reinhardtii* under S-, P-, Fe- and N-deprivation. The number of the hydrophilic metabolites could be compared between *S. vacuolatus* and *C. reinhardtii*, due to a more similar range of analytical conditions for the analytical conduction compared to a targeted approach as demonstrated, e.g. by Dunstan *et al.* (1992).

Most prominent hydrophilic metabolites from *S. vacuolatus* were proline, malic acid, galactopyranoside and glycerol as well as octadecanoic acid and hexadecanoic acid. Most prominent metabolites reported for the three *Chlorella* species were aspartic acid, proline, glucose, galactose, mannose, hexadecanoic acid and linolenic acid (Dunstan *et al*., 1992; Brown and Jeffrey, 1992). The two most prominent metabolites of *Scenedesmus* (proline and hexadecanoic acid) were comparable with those from the *Chlorella* species. Most prominent metabolites found in *C. reinhardtii* were aspartic acid, alanine, glutamate, lysine, proline, phenylalanine, myo-inositol, ornithine and glycerol. Proline and glycerol were some of the most prominent components in *C. reinhardtii comparable to S. vacuolatus*.

Bölling and Fiehn (2005) identified amino acids, carbohydrates, phosphorylated intermediates, nucleotides, and organic acids in hydrphilic extracts of the chlorophyte *Chlamydomonas reinhardtii*, using a GC-TOF MS system and custom libraries. The authors also identified 77 metabolites, as listed in the Appendix. The metabolic compositions found for *S. vacuolatus* and described for *C. reinhardtii* were not identical. In total, 28 of the 77 metabolites reported for *S. vacuolatus* were also described for *C. reinhardtii* (see Appendix). While the composition of amino acids was mainly similar in both algae, the composition of the other chemical classes listed in the Appendix was often different.
The differences between the chemical composition of the chlorophytes *Scenedesmus*, *Chlorella* and *Chlamydomonas* could be explained by many factors of the metabolomics approach, such as the differences in the experimental design and in other environmental conditions of the organism, e.g. nutrient deprivation and age, but also phylogenetic differences. The analysis method and systems could also cause differences

in the metabolome. However, the metabolomics approaches for *Scenedesmus vacuolatus* and *Chlamydomonas reinhardtii* seem to demonstrate similar sensitivity due to the similar number of identified metabolites of comparable chemical classes, but a direct comparison of the metabolite composition requires identical metabolomics approaches.

In summary, either similarities and differences have been found in the composition of the assigned metabolites for *Chlamydomonas*, *Chlorella* and *Scenedesmus* species, which could result from the analysis approach (e.g. targeted or non-targeted). The number of identified metbolites was higher in the non-targeted (unbiased) metabolomics approaches, as shown in this study and by Bölling and Fiehn (2005), than in a targeted approach, described by Dunstan *et al*. (1992) and Brown & Jeffrey (1992).
Information about the other metabolites identified in *S. vacuolatus* extracts was not found in the literature and may be new information regarding the metabolic composition of 14 hours old *S. vacuolatus* cells under reference conditions.

4.3.2 Algal Growth during Reference Conditions

The metabolome of *S. vacuolatus* was studied during the 14 hour growth phase under light (Figure 1), in this study. Growth of the chlorophyte references was observable by increasing cell volumes (Figure 13). Additionally, individual metabolites as well as the detected metabolic composition of reference algae showed trends over time (Figure 10 & 12). In the following, dynamics in the metabolic composition of reference algae will be covered in relation to algal growth.

Time Trends in the Metabolome of *Scenedesmus vacuolatus*

In the metabolic composition of reference continuous changes with time showed up algae, due to visualisation of the metabolomics results with PCA (principal component analysis) (Figure 10). A tendency or trend in the algal metabolome with time was found over 14 hours of growth. Time trends and stage specificity of sample scores were comparable with the literature, e.g. by Viant *et al.* (2005) and Viant (2003). The authors illustrate a concept of metabolic "developmental trajectory", which summarises changes in the metabolome throughout embryogenesis of the fish medaka. In addition, the use of developmental trajectories to identify stage specificity of a toxicant mode of action was suggested.
During the development of an organism, cascades of metabolic changes occur. Thus, metabolites and pathways involved can be revealed due to the time resolution of metabolic actions in *S. vacuolatus* and changes in these metabolic pathways can support interpretation of effects of the chemical, e.g. throughout exposure to prometryn, if compared to

references. The time resolution can reveal the start of the molecular interaction and thus the start of the effect of a chemical, because the metabolites can react earlier than proteins or genes. The PCA score plot trends found in this study also support the discovery of stage specificities of the metabolome during the development or during exposure of *S. vacuolatus*. For example, Figure 12 demonstrates concentration dynamics of biochemically important compounds, such as sugars and amino acids, correlating to the increase of the cell volume during growth (described in the following section).

Using PCA loadings, biochemically important metabolites were selected for further investigation, as discussed above (see Section 4.1.5). Time specific dynamics of metabolites were found during growth of reference chlorophytes by studying individual metabolites from algae in detail (Figure 12). For example, in Figure 10 the scores of t_{12} samples departed from the right-tending time trend of hyrophilic and lipophilic metabolite compositions, which was found, for example in form of a concentration decrease of sugars, such as fructose and mannose (Figure 12 d & e). Stage specific dynamics of metabolites from *Scenedesmus vacuolatus* were shown at growth stage 12 hours. Such departures from the time trend of PCA samples scores were also noted by Mounet *et al.* (2007), who studied the metabolome of developing fruits of tomato. Stage specific dynamics in metabolite concentration levels may support the assumption that the time trend enables the finding of metabolic dynamics in form of cascades of biochemical pathways. The cell division of synchronised *S. vacuolatus* is reported to occur from growth stage 20 hours on (Altenburger *et al.*, 1990). Thus, metabolic composition of references at growth stage 12 hours can show cells in preparation for the subsequent cell division and prominent physiological stage specificity in the metabolite composition can be revealed, studying the metabolic composition from *S. vacuolatus* over the time and at the end of the growth.

x-fold Increase of the Metabolite Concentration and the Cell Volume during the Development of *S. vacuolatus*

The differences in the number of compounds found in the extracts from t_0 and t_{14} algal cells (see Section 3.1.1), indicate that the metabolite number increases over the first 14 hours of algal development. Dynamics in the metabolite composition during growth phase of references were also observed. While cells do not divide in the growth phase from 0 to 14 hours, they develop their photosynthesis apparatus and increase their cell diameter or cell volume.

Steuer *et al.* (2003), Camacho *et al.* (2005) and Morgenthal *et al.* (2006) reported the analysis of correlations between changes of metabolites and

changes of diverse physiological states with respect to environmental conditions. The authors conclude that this method can contribute to an understanding of complex biochemical regulations, reaching a comprehensive view of the metabolism as a sum of systematically changing activity of metabolic pathways. By applying this approach in this study, information on various methods (e.g. phenotypic and metabolic information) can be combined and enhanced information from the metabolomics approach can be revealed. Thus, this approach can also be suitable to find correlations in metabolic responses during algal growth.

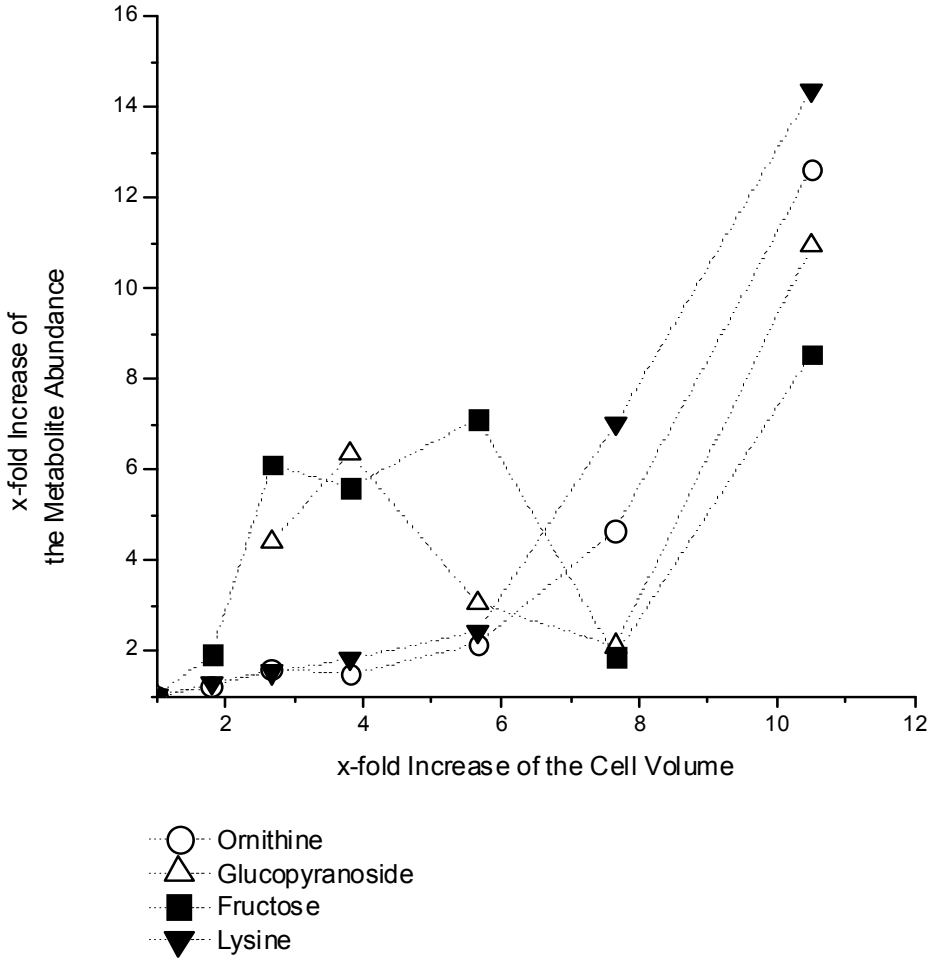


Figure 17: The cell volume (x-axis) and the abundance of 4 selected metabolites (fructose, glucopyranoside, lysine and ornithine) from *S. vacuolatus* are correlated. Samples were taken at growth stages 0, 4, 6, 8, 10, 12 and 14 hours of *S. vacuolatus* development. All values are given as the x-fold increase of the variable at a certain growth stage in relation to values at growth stage 0 hours.

In Figure 17, the x-fold increase of the mean cell volume (CV references) and of the abundances of four metabolites (fructose, glucopyranoside, lysine and ornithine) over seven developmental stages (0, 4, 6, 8, 10, 12 and 14 hours) of *S. vacuolatus* were correlated. All values demonstrate the x-fold increase of the metabolites and growth, in relation to values at

growth stage 0 hours, in relation to growth at a certain growth stage. Thus, the developmental stage 0 hours was set as value 1 of the x- and y-axis. While the increase of abundance of ornithine, lysine and the algal cell volume correlated exponentially, the trend of increase was dissimilar for fructose and glucopyranoside due to a decrease in concentration at growth stages from 8 to 12 hours. The amino acid metabolism in *S. vacuolatus* cells seems to correlate positively with the algal growth, whereas the sugar metabolism correlated positively with algal growth in the first 8 to 10 hours, followed by a change in trend. This change could have been caused by another metabolic mechanism, for example preparation for the end of the algal growth phase.

Metabolites, that show correlations between their abundance and the increase in cell volume, could be interesting as internal standards, as representatives for the biomass in a sample or for the growth stage of *S. vacuolatus*. Therefore, the analysis of correlations can increase information derived from the metabolomics approach, compared to the quantitative trend analysis of selected metabolites (Figure 12), to find and describe correlations between metabolite concentration and phenotypic dynamics.

Table 5: List of x-fold changes in the abundance of hydrophilic metabolites from *Scenedesmus vacuolatus* over 14 hours of algal development. X-fold changes in the chromatographic peak area abundance of the metabolites were divided into 5 groups. The number of the metabolites and names of metabolites are given as examples, which show these characteristics. Comments are given to describe specificities of the fold-change during time.

x-fold change	Number of hydrophilic *S. vacuolatus* metabolites	Examples	Comments
< 1	12	Methionine	Signal at t_0 but not at t_{14}
1-1.5	37	Phenylalanine, Oleic acid, Levoglucosan	-
1.5 – 10	43	Glycerol, Leucine, Proline, Threonine, Alanine, Glycine, Aspartic acid, Glutamine	-
10-20	6	Lysine, Fructose, Glucopyranoside, Phosphoric compound 1 and 3	-
>20	15	Valine, Furanose	No signal at t_0 but at late growth stages, such as $t_6 – t_{14}$

Besides the analysis of correlations between developmental trends from metabolites and growth, further dynamical trends of metabolic

concentrations have been found: (1) decrease, (2) no change - slight increase up to 1.5-fold, (3) large increase up to 10-fold or 20-fold. In Table 5, examples of hydrophilic metabolites of reference algae are listed to demonstrate alterations in the abundance over 14 hours of development. Within the total metabolic composition from *S. vacuolatus*, the metabolites show trends in their abundances with time and this complex systematic information may be suitable to understand metabolic activity during growth. The majority of metabolites, such as amino acids and sugars, increased during time and involvement in the development of *S. vacuolatus* can be revealed, for example in protein biosynthesis, protein turn-over and energy metabolism. In addition, the time related information from the analysis of correlations between metabolite concentrations and phenotypic information can reveal metabolic states, actions and networks in an organism, for example in response to a chemical, which can be suitable for the analysis of effects of toxicity using metabolomics.

4.4 Application of Metabolomics for the Analysis of *S. vacuolatus* Metabolites in Response to Time and Prometryn Toxicity

In the following chapters, the status of knowledge about the mechanism of action and the mode of action of prometryn will be discussed. In addition, observable effects on *Scenedesmus vacuolatus* after treatment with prometryn will be addressed regarding the results of this study, to demonstrate the benefits of the metabolomics approach for the analysis of metabolic effects of toxicity.

4.4.1 General Effects of *s*-Triazines observed on the Chlorophyte *Scenedesmus vacuolatus*

In this study, effects of prometryn on *Scenedesmus vacuolatus* growth and metabolites were examined by investigating the volume of algal cells and their metabolic composition, respectively. A literature search produced no evidence of previous applications of metabolomics to *S. vacuolatus* and its exposure to prometryn and this study can be the first one reporting effects of toxicity of prometryn on the metabolite level of the chlorophyte, using a metabolomics approach, and new information can support the understanding of the mechanism of action of prometryn.

However, in Table 6 parameters are listed, which were used, for example by Altenburger *et al.* (1990), Schmitt-Jansen & Altenburger (2007) and Neuwoehner *et al.* (2008), to study effects of toxicity from *s*-triazines. Published effect parameters are listed in the order of time of observation, such as algal reproduction, photosynthesis activity, ATP concentration,

algal photosynthetic fluorescence, oxygen production and photosynthetic electron transport.

For example, Altenburger *et al*. (2006) reported effects of the *s*-triazine atrazine after 45 seconds in the inhibition of the photosynthetic electron transport of *Chlorella fusca* (synonymous with *Scenedesmus vacuolatus*). This observed effect is known as the earliest effect of those reported in the literature. In the following first 4 minutes of exposure, *S. vacuolatus* photosynthesis fluorescence increased more than 80% compared with the references. On the basis of the study from Maretzek (1992), it is suggested that ATP also decreased in less than 5 minutes. The authors showed a decrease of ATP concentration in less than 5 minutes during exposure to dark after a light phase for two microalgae (*Chlorella fusca* and *Synechococcus leopoliensis*). The ATP concentration could be reduced in prometryn treated algae after 5 min as well, because prometryn inhibits photoautotrophic oxygen production in algae within the same time frame (Altenburger *et al*., 2006), which can be comparable to the effect observed if plants were exposed to the dark. Altenburger *et al*. (2006) show effects of atrazine on the photosynthetic fluorescence of *S. vacuolatus* in the first 10 minutes of exposure to atrazine (100 µg L^{-1}). After 15 min of exposure to atrazine, inhibition of algal photosynthesis has been shown (Altenburger *et al*., 1990), by measuring the oxygen production under light conditions. Moreover, Schmitt-Jansen and Altenburger (2007) demonstrated a decrease of the activity of the PS II by Water-PAM fluorometry in *S. vacuolatus*, after 1 hour of treatment with atrazine.

In addition, other parameters were observed to detect responses of *S. vacuolatus* to *s*-triazines (Table 6). For example, the cell volume after 14 hours of growth (t_{14}) was studied by Altenburger *et al*. (1990), the photosynthesis activity at t_{14} was studied by Neuwoehner *et al*. (2008) and algal reproduction at t_{24} was studied by Faust *et al*. (1992), Altenburger *et al*. (1990) and Neuwoehner *et al*. (2008). Hence, a gap of information has been found in the time range from 1 to 14 hours, but also between 14 and 24 hours of growth.

This gap of information between 1 and 14 hours has been filled with results of this study on the metabolic level of *S. vacuolatus*, with additional information about the inhibition of cell volume growth. Especially at early growth stages of the chlorophyte or at low exposure concentrations to prometryn the metabolite concentrations of references and treatments showed stronger changes than the parameter cell volume growth (see Section 3.2.4). Hence, metabolomics information of this approach can be useful for the extrapolation of effects of toxicity on the organism, a useful tool in the ecotoxicological risk assessment.

Table 6: Summary of observable effects in the *Scenedesmus vacuolatus* after exposure to prometryn or other *s*-triazines, published by the literature (see row references). The published observable effects of toxicity and the measured parameters on the chlorophyte are listed in order of time.

Time after application of prometryn to *S. vacuolatus* cultures	**45 s**	**5 min**	**< 10 min**	**15 min**	**1 h**	**14 h**	**24 h**	
Observable effects in treated *S. vacuolatus* samples compared to references	Inhibition of Hill-reaction	> 80 % inhibition of algal photosynthesis fluorescence	Decrease of ATP (GTP, NTP) Concentration	Inhibition of algal photosynthesis	Inhibition of algal photosynthesis	Inhibition of Cell Volume Growth	Inhibition of Cell Reproduction, Cell Volume Growth, Photosynthesis efficiency	Inhibition of Cell Reproduction
Measured parameter on/in *S. vacuolatus*	Photosynthetic electron transport (Dichlorphenolindophenol-Test)	Increase of Fluorescence (photoautotrophic oxygen production (Clark-type electrode)	ATP concentration (NMR)	O_2-Production (hydrophilicographical measurement under light cond. / Oxygen-Monitor measurement of respirational O2 consumption in darkness)	Activity /Efficiency of PSII (Fluorescence based quantum yield measurement; Water-PAM fluorometer)	Cell volume (Cell Coulter Counter)	Cell volume (Cell Coulter Counter)	Cell number (Cell Coulter Counter)
Exposure conditions	Atrazine ~0.6 µmol L^{-1}	Atrazine ~100 µg L^{-1}	Exposure to the dark	Atrazine ~0.7 µmol L^{-1}	Atrazine ~0.15 µmol L^{-1}	Atrazine ~0.2 µmol L^{-1}	Diuron ~0.1 µmol L^{-1}	Atrazine ~0.1 µmol L^{-1}
References	Altenburger *et al.* (1990)	Altenburger *et al.* (2006)	Maretzek (1992)	Altenburger *et al.* (1990)	Schmitt-Jansen & Altenburger (2007)	Altenburger *et al.* (1990)	Neuwoehner *et al.* (2008)	Altenburger *et al.* (1990) Faust *et al.* (1992)

4.4.2 Observed Effects of Prometryn on the Metabolome and the Cell Volume of *Scenedesmus vacuolatus*

S. vacuolatus was exposed to prometryn over a time period of 14 hours. Both metabolic and phenotypic parameters were measured in this study and the suitability of the developed metabolomics approach for the effect translation of prometryn in the chlorophyte was investigated. Effects of 0.1 µmol L^{-1} prometryn treatment observed within a time range from 4 to 14 hours are listed in Table 7. In the same table, the self-obtained data for the metabolome and the cell volume of *S. vacuolatus* were listed in the rows measured parameters and x-fold differences between references and treatments. The proposal of the effects of prometryn on *S. vacuolatus* was made on the results of this study or on the basis of information about the mechanism of action of prometryn, for example published by Taiz and Zeiger (2006) or Wakabayashi and Böger (2004) (see Table 7).

In this study, effects on the phenotype of *S. vacuolatus* were observed by measuring the cell volume or cell size. As shown in Table 7, after 4 hours of treatment with prometryn (0.1 µmol L^{-1}). and in comparison to references, the cell volume was less clear affected (~1.5-fold decreased) than the metabolome (> 4-fold decrease) of the chlorophyte. After a 6 hour treatment of *S. vacuolatus* with 0.1 µmol L^{-1} prometryn comparable results were found. In addition, effects were often found in many metabolites of the metabolic composition. Hence, the major advantage of metabolomics in ecotoxicology is demonstrated in the multivariate results, where the effect of a chemical can be demonstrated by many metabolites and also unknown components. Furthermore, the identified metabolites can be used for ecotoxicological interpretation of effects and many metabolites responded with prominent changes after 4 hours of growth, larger than 3-fold. This change is equivalent to twice the change of the cell volume growth in response to prometryn. Additional information about the interaction of prometryn with *S. vacuolatus* was obtained, as metabolomics results can be more clear than phenotypic effects at early developmental stages of the chlorophyte. Therefore, the developed metabolomics approach with *S. vacuolatus* could be useful to identify possible biomarkers indicating effects of toxicity prior to their expression in the phenotype.

Table 7: List of proposed effects in *S. vacuolatus* exposed to prometryn (0.1 µmol L^{-1}). Measured parameters and x-fold changes from 4 to 14 hours of the growth phase of the chlorophyte are summarised. Information was taken from results in this study and proposals were made in comparison with the literature (see references in this Table).

	Time after application of prometryn to *S. vacuolatus* or other plant systems												
	4 h					**6 h**			**6 -10 h**	**12 h**	**14 h**		
Measured parameters on *S. vacuolatus* in this study	Cell Volume (Cell Coulter Counter)	Whole metabolic composition and individual 3 Amino acids	sugar (e.g. mannose-6-p, fructose, galactose) concentration	aspartic acid concentration	glutamine	Cell Volume (Cell Coulter	aspartic acid concentration	Linolenic acid	sugar (e.g. mannose-6-p, fructose, galactose) concentration	Lysine, mannose-6-phosphate, aspartic acid concentration	Whole metabolic composition	Methionine, aspartic acid, propanoic acid concentration	Lysine, glycine, levoglucosan, glucopyranoside concentration
x-fold difference between treatments and references	~ 1.5-fold decreased	Clearly different	> 4-fold decreased	3.3-fold increased	~ 3-fold decreased	~ 2 fold decreased	~ 8-fold increased	~ 2-fold decreased	~ 10-fold decreased	Change to control levels at earlier growth stages (2.5-fold decrease in cell volume)	Clearly different at various concentrations of prometryn	~3-fold increased	≥ 2-fold decrease
Proposed effects of 0.1 µmol L^{-1} prometryn on *S. vacuolatus* in comparison to the literature	Inhibition of Cell Volume Growth	Change of hydrophilic metabolic composition of *S. vacuolatus*	Inhibition of energy metabolism	Inhibition of asparagine synthetase due to the lack of ATP	Dissection of glutamine synthetase (GS) or glutamate synthase (GOGAT) within the GS/GOGAT cycle	Inhibition of Cell Volume Growth	Inhibition of asparagine synthetase due to the lack of ATP	Lipid peroxidation	Inhibition of energy metabolism	An alternative pathway may have been established for controlled regulation of amino acid and sugar synthesis in the chlorophyte?	Changed metabolic composition of *S. vacuolatus*	Changed individual metabolites	Changed individual metabolites
References	Phenotype of *S. vacuolatus* in this study	Metabolomics results in this study	Wakabaya-shi & Böger, 2004	Taiz and Zeiger (2006)	Zulak *et al.* (2008)	Phenotype of *S. vacuolatus* in this study	Metabolomics results in this study	Fuerst and Norman, 1991	Wakabayashi & Böger, 2004	Metabolomics (+Phenotypic) results in this study	Metabolomics results in this study	Metabolomics results in this study	Metabolomics results in this study

The information derived from the study of time- and concentration series from 4 to 14 hours can enhance information about the translation of effects caused by the mechanism of toxicity of prometryn. Hence, these metabolic effects underlying a visible effect in algae can therefore support the understanding of the effect development. Examples of the enhancement of understanding are given in the following sections.

Energy Metabolism

Due to the treatment of *S. vacuolatus* cells, a decrease of the concentration of sugars, such as fructose, galactose and glucopyranoside, of mannose-6-phosphate and of amino acids, like glutamine was detected compared to references. In parallel, the growth of algal cells was inhibited. Prometryn is reported to inhibit the photosynthetic electron transport (PET) by binding to the plastochinone QB niche of the D1 protein of the photosystem II reaction centre, thus displacing the electron acceptor (Wakabayashi & Böger, 2004; Fuerst & Norman, 1991). The function of the PET is to convert light energy into ATP and NADPH. The chemical energy, in form of ATP and NADPH, is subsequently used to reduce CO_2 and produce sugars, which are the basic components of plant primary biomass production (Wakabayashi & Böger, 2004). The interaction of prometryn with the PET should disturb the plant energy metabolism, resulting in a deficit of ATP and NADPH availability and therefore in a decrease in biomass.

Some compounds increased during treatment with prometryn, compared with the references. As shown in Table 3, the concentrations of the amino acids aspartic acid and glycine increased after 4 hours of treatment 3.3 and 1.6-fold, respectively, compared with references. During further treatment, the concentration of glycine decreased below the level of references. In contrast the concentration of aspartic acid was always higher in treatments compared to those of references, but decreased continuously from growth stage 8 to 14 hours.

One possible cause of aspartate accumulation under the influence of prometryn could have been the inhibition of asparagine synthetase (AS). The enzyme requires ATP for the synthesis of asparagine and glutamate from aspartate and glutamine (Taiz and Zeiger, 2006). Hence, where insufficient ATP is available, synthesis of asparagine could have been inhibited, resulting in an accumulation of aspartate and glutamine. As accumulation of aspartate concentration was found during the time course of 14 hours in this study, the plant energy metabolism may have been disturbed already after 4 hours of treatment.

Because glutamine concentration was greatly decreased after 4 hours of treatment compared with references (see Section 3.2.2) glutamine

resources were possibly metabolised but not replenished during exposure to prometryn. Glutamine serves mainly as nitrogen donor for the biosynthesis of compounds, such as amino acids, nucleotides, chlorophylls, polyamines and alkaloids (Zulak *et al.*, 2008). Glutamine could have been decomposed to synthesise those compounds during algal treatment. The decreased concentration of glutamine in *S. vacuolatus*, after 4 hours of treatment, could indicate a disturbed synthesis of amino acids. A possible effect of prometryn could be an uncoupling of the glutamine synthetase (GS) or glutamate synthase (GOGAT) within the GS/GOGAT cycle. Because GS requires ATP for the synthesis of glutamine, a lack of ATP and thus a possible reduction of activity of GS could explain a decrease of glutamine during prometryn exposure.

Galactose, Galactonic acid and Ascorbate

The response of galactose, galactonic acid and ascorbic acid under treated conditions seem to be related in kind of a cascade, as seen for growth stages 4 and 14 hours (see section 3.2.2). Galactonic acid and galactose in treatments at growth stage 4 hours show a decreased abundance compared with references, while the concentration of ascorbic acid increased above the references. The references show a quite stable level of concentration of galactose at growth stage 4 and 14 hours, while that of galactonic acid increased strongly within 14 hours of growth. Galactose and mannose are efficient precursors for ascorbate synthesis (Wheeler *et al.*, 1998). Moreover, ascorbate production is enhanced by adding l-galactonic acid γ-lactone in yeast (Onofri *et al*. 1997). Thus, regulation of galactose, galactonic acid and ascorbate can be related in a cascade of metabolic processes.

Furthermore, ascorbic acid is reported as a compound involved in the defence mechanisms of plants (Fuerst and Norman, 1991). Smirnoff (2000) described ascorbate peroxidase as a key enzyme in the regulation of H_2O_2 concentration and Fuerst & Norman (1991) report ascorbate as a protection metabolite. As prometryn is a xenobiotic chemical causing effects of toxicity in *S. vacuolatus*, the defence metabolism of the algae could have been activated. Thus, compared with references the higher concentration of ascorbic acid in the treated t_4 cells can indicate an increased synthesis of this organic acid for defence.

Phenylalanine

After 14 hours of treatment, a higher abundance of phenylalanine was found in algal extracts from treatments compared with the references and the responses were in the opposite direction (Table 3). Phenylalanine is an intermediate in the biosynthesis of most plant phenolics, which are

secondary metabolites and have been found to serve as defence compounds, absorbing ultraviolet radiation in plants (Taiz and Zeiger, 2006). Hence, increased concentration of phenylalanine at growth stage 14 hours in *S. vacuolatus* cells can be a result of disruption of secondary cellular processes by prometryn.

Summary for the Hydrophilic Metabolite Composition

In summary, differences between references and treatments were found for various metabolites, such as amino acids, sugars and organic acids (Table 3), after 4 hours of treatment with prometryn. Algal metabolites concentrations, for example of sugars, were often higher expressed after 14 hours than after 4 of exposure to prometryn, but mostly less apparent in treatments compared with references. Furthermore, most of the identified organic acids increased in their concentration during the growth phase during treatment and were more abundant at growth stage 14 hours compared to autospores and 4 hours old algae. In addition, the most influential metabolites, for the PCA separation of data in the time course study, were less abundant in treatments compared to references.

Lipophilic Metabolite Composition

PCA score plots from the time course and the concentration series (Figure 7 b & 10 b) show the responses of the lipophilic metabolite compositions from *Scenedesmus vacuolatus* to prometryn. The zigzag pattern of sample scores may show protection mechanisms from prometryn.

Linolenic acid has been assigned in *S. vacuolatus* extracts. For algae at stage 4 and 14 hours, linolenic acid abundance was lower in treated algae compared with the references. Linolenic acid is one of the most abundant fatty acids of thylakoid membranes, which is prone to lipid peroxidation when abnormally large quantities of triplet-chlorophyll (^{3}Chl) and radicals of oxygen ($^1O_2$, H_2O_2 and O_2^-) are present in the plant cell exposed to a PSII electron transport inhibitor (Fuerst & Norman, 1991). Moreover, the authors described the herbicide-induced inhibition of PET resulting in production of triplet chlorophyll and singlet oxygen which can result in photooxidation of photosynthetic membranes followed by lipid peroxidation. Thus, the results for linolenic acid could support the study of the authors, who described that linolenic acid was degraded by lipid oxidation process, thus potentially destroying the integrity of membranes in response to phytotoxicity. Fuerst & Norman (1991) describe the aptitude of plants using mechanisms protecting from lipid peroxidation or lipid peroxide radicals. The main products of lipid peroxidation are malondialdehyde and ethane. Lipid peroxidation is supposed to lead to cellular disorganisation, destruction of the integrity of photosynthetic

membranes, loss of cellular compartments and phytotoxicity. Hence, lower concentration of linolenic acid in treatments than in references can show increased degradation processes as a consequence of protection mechanisms from prometryn, probably representative for the long treatment with a high concentration (EC 86) of this compound. Therefore, lipid peroxidation can be an effect, unrelated to the specific mode of action of a PSII inhibitor.

4.4.3 Primary and Secondary Responses to Toxicity over Time

Molecular effects in a biological system can occur within the first interactions of a chemical with its site of action. During the following mechanism of action secondary effects occur. In this study, the first changes to the metabolite concentration found in treated samples compared with references dynamics over time were termed primary molecular effects. All following alterations of metabolites under exposure conditions were defined as secondary molecular effects. Please note that these primary and secondary metabolites may not necessarily be involved into the primary and secondary metabolism of plants.

During the time series, stage specificities can be shown in PCA score plots (Figure 10). These stage specificities were also found for individual metabolites (Figure 12). The division of the stage specificities into primary or secondary metabolic responses to prometryn exposure is discussed in the following.

Primary and Secondary Effects

The metabolic composition of 14 hours old algae under exposure to various concentrations of prometryn showed concentration-response profiles (Figure 7 a and b). The concentration-response relationship was more clearly demonstrated by the hydrophilic metabolites than by the lipophilic ones. As shown in Figure 7 a for the hydrophilic fraction of *S. vacuolatus*, all of the concentrations of prometryn studied caused a strong discrepancy between the reference group and treatments, where the discrepancy increased with higher concentrations. In the PCA plots (Figure 7 a & b), a trend of treatment scores away from the reference group was found. This trend was comparable to the literature, e.g. by Hole *et al*. (2000), who studied metabolic effects of five different concentrations of sulfometuron on maize (*Zea mays*) and soy bean (*Glycine max*) plants, and Viant *et al*. (2006), who studied metabolic responses from fish embryos of medaka (Oryzias latipes) exposed to two sublethal dinoseb concentrations. The authors of these studies also outlined trends of PCA sample scores of treatments away from the

reference group with increasing exposure concentration. Viant *et al*. (2006) linked the metabolomics data with that of fish eye growth. As a result, the authors suggest that the concentration-related trend in the fish embryo metabolome shows an increase of perturbation of the same biochemical pathways. In addition, the authors suggest an induction of an entirely different metabolic condition from significant distances of treatment scores from that of references. As the hydrophilic fraction was clearly different in references and treatments in this study, an entirely different metabolic condition can exist in the stronger separated samples to groups in a PCA score plot. The further concentration-dependent increase of distance to the reference group may be the result of an increased disruption of the same or of several biochemical pathways.
In the PCA score plot of lipophilic fractions from *S. vacuolatus* (Figure 7 b), a concentration dependent trend was also found, but less clearly compared with the score plot for the hydrophilic fraction (Figure 7 a). In addition, the highest concentrations of prometryn showed a trend back into the direction of the reference group. Because prometryn is a PSII inhibitor, the metabolic condition of treated *S. vacuolatus* can represent indirect effects of prometryn on the lipophilic metabolic composition.

Investigation of Time Dependencies of Prometryn Exposure

In Figure 12, the concentrations of 6 individual metabolites are shown for references and treatments. As amino acids and sugars belong to the primary metabolites, involved for example in the organisms maintenance and survival, the time window studied here can show effects of prometryn in the primary metabolism of *S. vacuolatus*. After treatment metabolite concentrations of aspartic acid and mannose-6-phosphate (Figure 12 a & f) showed a strong difference to the references after 4 hours of treatment possibly demonstrating primary plant responses to prometryn exposure. At late growth stages the concentrations of aspartic acid and mannose-6-phosphate changed in treatments to the level of references. Alterations in the metabolite concentration, e.g. of aspartic acid, lysine, glycine, mannose and mannose-6-phosphate (Figure 12), which occurred at late growth stages, can show downstream dynamics of metabolites, such as secondary responses.

4.4.4 Combining the Information of the Metabolome with that of the Phenotype from *S. vacuolatus*

The application of the concept of phenotypic anchoring is demonstrated in the following two examples.

Example 1

Alterations in *S. vacuolatus* cell size were found in both time and concentration dependent studies. Between 4 and 14 hours of exposure with prometryn, algal cell size rarely increased, but metabolite composition altered very clearly. Hence, linking of information from algal cell size and algal metabolites, information could be used to explain mechanism of toxicity during exposure. For example, the difference between references and treatments after 4 hours of exposure was more clearly shown with the metabolite expression than with the algal cell size. Thus, it may be concluded that stage specific metabolic effects occurred prior to manifestation in the phenotype, e.g. the cell volume.

Similar conclusions can be made for the concentration-dependent study of hydrophilic and lipophilic metabolites, but responses from hydrophilic metabolites were clearer for prometryn concentrations in the range from 0.004 up to 0.02 µmol L^{-1}. The hydrophilic and lipophilic metabolite extracts from *S. vacuolatus* show responses to these concentrations, while no responses were observed in the cell size of the chlorophyte. Hence, the algal metabolome responded at lower concentrations than the cell volume growth of *S. vacuolatus*. At low concentrations of prometryn, hydrophilic metabolic effects provide more indications for the reported mode of action of prometryn (Wakabayashi & Böger, 2004) than the classical parameter algal growth inhibition.

Example 2

In another example, growth inhibition of 65% was found for *S. vacuolatus* exposed to 0.1 µmol L^{-1} prometryn for 14 hours. After 14 hours of treatment, the cell size was similar the reference cell size after 4 to 6 hours (Figure 13). During the growth phase, cell size of treatments did not increase very much, but remained almost stable. In contrast, the metabolite composition of *S. vacuolatus* changed over time. The metabolite composition of treatments at growth stage 14 hours was found to be similar to references after 4 and 6 hours of growth. The metabolome from *S. vacuolatus* showed stronger changes during growth than the cell size and therefore enhances the information available to understand the effects of prometryn. As mentioned above, atrazine can cause toxicity effects in plants which are similar to prometryn. Faust *et al*. (1992) studied *S. vacuolatus* under the influence of various s-triazine herbicides.

The authors showed the influence of atrazine on growth and reproduction regarding algal toxicity. The influence of prometryn on the growth of *S. vacuolatus*, in this study, was rather similar to that of atrazine (described by Faust *et al.*, 1992). Hence, in the present study the observed cell size of *S. vacuolatus* at growth stage 14 hours revealed insufficiency for a subsequent cell division at the end of the cell cycle and therefore an inhibition in reproduction or population increase is expected in proportion to the growth inhibition.

In summary, combining the phenotypic with the metabolic information can support the understanding of alterations in the metabolome. Moreover, metabolomics in combination with the concept of phenotypic linking can be useful for the extrapolation of metabolic information to the potential of toxicity of a chemical. If this metabolic response of *S. vacuolatus* could be generalised for classes of chemical compounds, the developed metabolomics approach could also be useful to characterise the mechanisms of action of unknown chemicals or chemical mixtures.

Conclusions and Perspectives

5 Conclusions & Perspectives

In this study, a metabolomics approach was developed with a gas chromatography-mass spectrometry (GC-MS) system to analyse hydrophilic and lipophilic metabolite extracts from the chlorophyte *Scenedesmus vacuolatus*. Within the chromatographic results, lower intra- and inter-experimental variability was shown to be half of that in published plant metabolomics studies. Thus, the advantage of synchronous culturing of the organism in a clonal population for metabolomics approaches was demonstrated. The developed Metabolomics approach enabled the demarcation of time- and concentration-related metabolic changes in the algal metabolome in response to the exposure to the chemical prometryn. As shown with the metabolomics results over the 14 hours growth phase of the chlorophyte, the metabolome was found to be very specific regarding time. At different developmental stages of the alga specific changes between treatment- and reference-metabolites were found. Therefore, the developed metabolomics approach can be suitable to classify states of the metabolome in response to environmental stimuli, such as chemicals with different mechanisms of action, and support the classification of chemicals regarding their mechanism of action.

Over the developmental phase from 0 to 14 hours different dynamics of the abundances of hydrophilic and lipophilic metabolites were found in references and prometryn treatments. Measuring seven algal developmental stages (0, 4, 6, 8, 10, 12 and 14 hours), the hydrophilic metabolite composition was responding after 4 hours of prometryn exposure more clearly than the lipophilic metabolite fraction. Also after 14 hours of *S. vacuolatus* exposure to the lowest used prometryn concentrations (0.002 µmol L^{-1}), the effects were more clearly in hydrophilic than in lipophilic extracts. Thus, studying the metabolic composition in response to other time frames, e.g. 0 to 4 hours or 14 to 24 hours, and to lower prometryn concentrations, should increase the time resolution of information about metabolic processes and therefore support the discovery of the begin and the mechanisms of metabolic interactions between the chemical and the chlorophyte. The understanding of metabolic dynamics, such as cascades within metabolic pathways and metabolic functions until the phenotypic expression of the effect of the chemical should be supported as well. For example, within the time range from 0 to 4 hours, primary interactions between *S. vacuolatus* cells and

the chemical prometryn are expected, which should be mainly observable within the hydrophilic composition of metabolites from the alga. In addition, the detailed knowledge about the metabolic status over the time range between 10 and 14 hours in reference algae could support the clarifying and understanding of the metabolic actions at growth stage 12 hours, where the hydrophilic and lipophilic metabolite composition was found to be similar to that at earlier growth stages. Moreover, increased information about the time range between the developmental stages 14 and 24 hours of *S. vacuolatus* may be important to improve the knowledge about the metabolic status in reference algae and to support the understanding of metabolic actions in the alga, e.g. under light and reference conditions, with or without exposure to toxicity and over time.

Multivariate changes in the metabolome of *S. vacuolatus* were found at earlier growth stages and lower prometryn concentrations than the univariate change in the phenotype, which was quantified by changes of the cell volume or size of the chlorophyte. Therefore, metabolomics may be applicable for the assumption of single and/or multiple metabolic biomarkers of toxic effects of prometryn, or other chemicals, earlier than the classical ecotoxicological parameter algal growth.

The clear effects in the metabolite composition from *S. vacuolatus*, shown in this work, may represent a specific effect pattern for prometryn, acting as a PSII inhibitor (*s*-triazine). All metabolites of a biological system could theoretically demonstrate the organism's response to the exposure to a chemical and many of them are unknown. Due to the wide biochemical range, metabolomics has the potential to identify these metabolites and to classify chemicals regarding their mechanism of action which can cause specific metabolite patterns. For example, the chemical prometryn was found to cause more clear effects in the hydrophilic metabolite composition from *S. vacuolatus* than in the lipophilic metabolite composition. The data evaluation led to the conclusion that the energy metabolism of the microalga was affected, as shown with altering abundances of sugars in the metabolite extracts. Toxicants of other chemical classes with other mechanism of actions are expected to cause other characteristic metabolic effect patterns. For example, the chloroacetamide metazachlor was described by Böger (2003) to affect the synthesis of very long chain fatty acids and to increase the concentration of other fatty acids, such as hexadecanoic, oleic and linolenic acid and thus, clear effects of toxicity are expected in the lipophilic metabolic composition of *S. vacuolatus*. On the basis of the metabolite pattern as well as on identified metabolites, the metabolic composition of *S. vacuolatus* could be specific for different states of the organism in

response to chemicals. If it would be possible to classify chemicals on the basis of effects on the metabolic composition regarding different mechanisms of action, such as inhibitors of the PSII or of the fatty acid- and the amino acid synthesis, it might also be possible to support the understanding of the mechanisms of action of chemicals with unknown or multiple mechanisms.

In this work, metabolic effects of prometryn were detected prior to its phenotypic effect on *S. vacuolatus*, after 4 hours of treatment with 0.1 µmol L^{-1} prometryn and after 14 hours of treatment with 0.002 µmol L^{-1} prometryn. Thus, the approach showed higher sensitivity, e.g. with shorter exposure-times and lower concentrations of prometryn and metabolomics may be applicable to gain specific information on the metabolome suitable to notify the presence of low dose chemicals after short time exposure in the environment of *S. vacuolatus*, and their toxic potential with respect to the organisms health status.

The combination of the information from metabolic composition with physiological and phenotypic information, with respect to both time and various concentrations of prometryn provided additional information, such as a change in the abundance of sugars at a specific growth stage, while the phenotypic information showed a consistent trend. Thus, the plant metabolites, responding within seconds to environmental stimuli, might be suitable to discover new physiological knowledge of the developmental phase of *S. vacuolatus*, but also to discover unknown short time effects of toxicity or defence mechanisms of the organism. The multivariate metabolic information could also provide a basis for the decision making of further more detailed targeted studies of metabolites or metabolic pathways.

In further studies, the potential of metabolomics to gain additional information about the metabolites from *Scenedesmus vacuolatus* responding to a chemical, should be estimated. For example, the identification of metabolites or the application of further data evaluation methods should be improved. The data evaluation methods PLS-DA (partial least squares - discriminant analysis) and the application of significance tests ((M)ANOVA and Post hoc test) are promising methods to improve the biological and physiological understanding of the results. The additional information about significancies of differences between metabolic patterns as well as about classification or grouping of these patterns could be suitable for the ecotoxicological classification of metabolic patterns regarding their status. This can support the classification of chemicals regarding their mechanism of action as well as the assessment of the toxic potential of a chemical. The developed

metabolomics protocol should further be optimised (1) regarding the harvest method of *S. vacuolatus* cells, to ensure similar harvest of samples and therefore to improve the metabolomics results for specific algal states, (2) regarding the range of detected and identified metabolites, such as basic primary metabolites, and (3) regarding the automatism of data evaluation, to decrease the effort of this protocol step.

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Appendix

Appendix

List of the sum of identified metabolites in three chlorophytes: (1) *Scenedesmus vacuolatus* from this study (2) *Chlamydomonas reinhardtii* from Bölling and Fiehn (2005) and (3) three *Chlorella* species from Dunstan *et al.* (1992) and Brown and Jeffrey (1992). Positive results in assignment are marked with 'xxx'. Metabolites are divided into 7 classification groups. Assigned metabolites are listed independently from their mass spectral match quality factor. The 77 metabolites from *S. vacuolatus* and *C. reinhardtii* were analysed with a metabolomics approach for hydrophilic metabolites, while the targeted analysis (*) of metabolites from *Chlorolla* species resulted in the sum in 21 metabolites.

Counts	Metabolite assignments	*Scenedesmus vacuolatus* (this study)	*Chlamydomonas reinhardtii* Bölling and Fiehn (2005)	3 *Chlorella* species (Dunstan *et al.* (1992); Brown and Jeffrey (1992)
	Sum of assigned metabolites:	**77**	**77**	**21 ***
Amino Acids (∑ 19)				
1	ß-Alanine (Ala)	xxx	xxx	xxx
2	Asparagine (Asn)		xxx	xxx
3	Aspartic acid (Asp)	xxx	xxx	xxx
4	Cysteine (Cys)		xxx	
5	Glutamate (Glu)		xxx	xxx
6	Glutamine (Gln)	xxx	xxx	
7	Glycine (Gly)	xxx	xxx	xxx
8	Histidine (His)		xxx	
9	Isoleucine (Ile)		xxx	
10	Leucine (Leu)	xxx	xxx	xxx
11	Lysine (Lys)	xxx	xxx	xxx
12	Methionine (Met)	xxx	xxx	xxx
13	Phenylalanine (Phe)	xxx	xxx	xxx
14	Proline (Pro)	xxx	xxx	xxx
15	Serine (Ser)	xxx	xxx	
16	Threonine (Thr)	xxx		xxx
17	Tryptophan (Trp)		xxx	
18	Tyrosine (Tyr)	xxx	xxx	xxx
19	Valine (Val)	xxx	xxx	xxx
Amino Acid Derivatives (∑ 8)				
1	*N*-Acetyl-Glutamate		xxx	
2	Cystine (Amino acid dimer)		xxx	
3	Norleucine	xxx		
4	Ornithine	xxx	xxx	xxx
5	*N*-Acetyl-Ornithine		xxx	
6	4-Hypdroxyproline (4-Hyp)		xxx	
7	Oxoproline		xxx	
8	Ketovaline		xxx	

Counts	Metabolite assignments	*Scenedesmus vacuolatus* (this study)	*Chlamydomonas reinhardtii* Bölling and Fiehn (2005)	3 *Chlorella* species (Dunstan et al. (1992); Brown and Jeffrey (1992)
Sugars (∑ 14)				
1	Arabinofuranose	xxx		
2	Arabinose	xxx		
3	Erythrofuranose	xxx		
4	Erythrose	xxx		
5	Fructose	xxx	xxx	
6	Furanose	xxx		
7	Galactose	xxx		xxx
8	Glucose	xxx	xxx	xxx
9	Levoglucosan	xxx		
10	Maltose		xxx	
11	Mannose	xxx		xxx
12	Ribose	xxx	xxx	
13	Sucrose	xxx		
14	Trehalose	xxx	xxx	
Sugar Derviatives (∑ 14)				
1	Arabino-Hexo-2-ulose	xxx		
2	D-Fructose-1,6-bisphosphate		xxx	
3	Fructose-6-phosphate		xxx	
4	Galactinol		xxx	
5	Galactose-6-phosphate	xxx		
6	Glucopyranosid	xxx		
7	Glucose-1-phosphate		xxx	
8	Glucose-6-phosphate		xxx	
9	Glycerol	xxx	xxx	
10	(sn)-Glycerol-3-phosphate		xxx	
11	Mannose-6-phosphate	xxx		
12	Ribose-5-phosphate		xxx	
13	Sedoheptulose-1,7-bisphosphate		xxx	
14	Tramadol	xxx		
Fatty Acids (∑ 5)				
1	Octadecanoic acid	xxx		xxx
2	Hexadecanoic acid	xxx		xxx
3	Linolenic acid	xxx		
4	Oleic acid	xxx		xxx
5	Tetradecanoic acid	xxx		
Organic Acids (1 – 17 of ∑ 39)				
1	PyroGlutamic acid	xxx		
2	Threonic acid		xxx	
3	Citramalic acid	xxx	xxx	
4	Erythronic acid	xxx		
5	Fumaric acid	xxx	xxx	
6	Fumaric acid	xxx	xxx	
7	Galactonic acid			xxx
8	Gamma-lactone Xylonic acid	xxx		
9	Malic acid	xxx	xxx	
10	Succinic acid	xxx	xxx	
11	Glyceric acid		xxx	
12	Benzenedicarboxylic acid	xxx		
13	Benzoic acid		xxx	
14	Butanedioic acid	xxx		
15	Butanoic acid	xxx		
16	Carotenic acid	xxx		
17	Cinnamic acid	xxx		

Counts	Metabolite assignments	*Scenedesmus vacuolatus* (this study)	*Chlamydomonas reinhardtii* Bölling and Fiehn (2005)	3 *Chlorella* species (Dunstan et al. (1992); Brown and Jeffrey (1992)
Organic Acids (18 – 39 of ∑ 39)				
18	Citric acid	xxx	xxx	
19	Dihydroxybutanoic acid	xxx		
20	Glycolic acid		xxx	
21	Glyceric acid-3-phosphate	xxx		
22	Hydroxybenzoic acid		xxx	
23	3-Hydroxybutyric acid		xxx	
24	Isopropylmalic acid		xxx	
25	2-Ketoglutaric acid	xxx	xxx	
26	Myristic acid		xxx	
27	Oxalic acid	xxx	xxx	
28	Pentanedioic acid	xxx		
29	Pentenoic acid	xxx		
30	Phosphoenolpyruvic acid		xxx	
31	6-Phosphogluconic acid		xxx	
32	2-Phosphoglycolic acid		xxx	
33	Phosphoric acid	xxx		
34	Prephenic acid		xxx	
35	Propanoic acid	xxx		
36	Propantricarboxylic acid	xxx		
37	Pyrrolidone carboxylic acid	xxx		
38	Pyruvic acid		xxx	
39	Shikimic acid		xxx	
Other Compounds (∑ 28)				
1	1,3-Dihydroxyacetone		xxx	
2	1H Indole	xxx		
3	2-Aminoadipate		xxx	
4	4-Hydroxypyridine		xxx	
5	Adenine		xxx	
6	Adenosine	xxx		
7	Butanediamine	xxx		
8	Butylated hydroxytoluene	xxx		
9	Cadaverine	xxx		
10	Citrulline		xxx	
11	Cyclolanostan	xxx		
12	Decane	xxx		
13	Dihydroxyacetonephosphate		xxx	
14	Disilatetradecane	xxx		
15	Dithia-oxaspirol	xxx		
16	*epi*-Inositol	xxx		
17	Ethanedione	xxx		
18	Glyceraldehyd-3-phosphate		xxx	
19	Glycerate-3-phosphate		xxx	
20	Hydroxylamine		xxx	
21	Inositol-ß-galactoside		xxx	
22	*myo*-Inositol	xxx	xxx	
23	*N*-acetyl-galactosamine		xxx	
24	Nicotinamide		xxx	
25	Octadecane	xxx		
26	Phosphine	xxx		
27	Putrescine	xxx	xxx	
28	Uracil		xxx	

Liste der Publikationen

Zeitschriftenartikel

Kluender, C., Altenburger, R., Sans-Piché, F., Riedl, J., Härtig, C., Laue, G. and Schmitt-Jansen, M. (2008) A metabolomics approach to assessing phytotoxic effects on the green alga *Scenedesmus vacuolatus*. Metabolomics (DOI: 10.1007/s11306-008-0139-x)

Sabater, S., Guasch, H., Ricart, M., Romani, A. Vidal, G. Klünder, C. and Schmitt-Jansen, M. (2007) Monitoring the effect of chemicals on biological communities. The biofilm as an interface. Analytical and Bioanalytical Chemistry, 387, 1425-1434

Vorträge

Kluender, C., Altenburger, R., Laue, G., Härtig, C., Sans-Piché, F. and Schmitt-Jansen, M. (2007) Downstream of Genomics – Metabolomics an der Grünalge *Scenedesmus vacuolatus*. SETAC GLB (Leipzig, Deutschland)

Poster

Kluender, C., Altenburger, R., Härtig, C., Laue, G. and Schmitt-Jansen, M. (2007) A Metabolomics Approach to Detect Toxic Effects in the Green Alga *Scenedesmus vacuolaus*. SETAC Europe (Porto, Portugal)

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