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Seasonal dynamics of chemotypes in a freshwater phytoplankton community – A metabolomic approach

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ABSTRACT

Cyanobacteria are known to produce a huge variety of secondary metabolites. Many of these metabolites are toxic to zooplankton, fish, birds and mammals. Therefore, the toxicity of cyanobacterial blooms is strongly dependent on the cyanobacterial strain composition. These strains produce distinct bouquets of secondary metabolites, and thus constitute different chemotypes. Some of the cyanobacterial metabolites are potent inhibitors of gut proteases of the filter-feeder *Daphnia*. Here, we investigate the seasonal dynamics of secondary metabolites in a phytoplankton community from a hypertrophic pond, making use of a new metabolomic approach. Using liquid chromatography coupled with high-resolution mass spectrometry (LCMS), we obtained mass spectra of phytoplankton samples taken on different dates throughout the summer season. By applying multivariate statistics, we combined these data with the protease inhibition capacity of the same samples. This led to the identification of metabolites with cyanobacterial origin and as well of distinct cyanobacterial chemotypes being dominant on different dates. The protease inhibition capacity varied strongly with season, and only one out of 73 known cyanobacterial protease inhibitors could be confirmed in the natural samples. Instead, several so far unknown, putative protease inhibitors were detected. In conclusion, the creation of time series of mass spectral data of a natural phytoplankton community proved to be useful for elucidating seasonal chemotype succession in a cyanobacterial community. Additionally, correlating mass spectral data with a biological assay provides a promising tool for facilitating the search for new harmful metabolites prior to structure elucidation.

1. Introduction

Cyanobacteria play an important role in aquatic ecosystems worldwide (Dokulil and Teubner, 2000; O'Neil et al., 2012). Cyanobacterial blooms occur frequently, particularly in eutrophic ponds and lakes, and have detrimental effects on the water quality with respect to domestic, industrial and recreational usage of water bodies and also strongly influence zooplankton communities (Dokulil and Teubner, 2000; Ghadouani et al., 2003; O'Neil et al., 2012). Cyanobacteria are well known to negatively affect herbivorous zooplankton, e.g. the unselective filter-feeder *Daphnia* (Ghadouani et al., 2003; Wilson et al., 2006). Besides the fact that cyanobacteria provide low quality food for zooplankton (Porter and Mcdonough, 1984; Von Elert et al., 2003), the presence of cyanotoxins such as microcystins is a major issue (Rohrlack et al., 2001). A negative effect of microcystins on somatic growth and reproduction of *Daphnia* has been shown. Nevertheless, cyanobacterial strains lacking microcystins also lead to reduce fitness parameters of *Daphnia* (Lürling and van der Grinten, 2003; Tillmanns et al., 2008). Among other putative mechanisms, this reduction of fitness might be attributed to the presence of protease inhibitors, which are widespread in cyanobacteria, where they function as anti-herbivore defense (Agrawal et al., 2005; Gademann and Portmann, 2008; Von Elert et al., 2010; Schwarzenberger et al., 2010). Many of these protease inhibitors efficiently inhibit chymotrypsins or trypsins (Gademann and Portmann, 2008), which together are responsible for 80% of the proteolytic activity in the *Daphnia* gut (Von Elert et al., 2004). Natural lake phytoplankton frequently contains protease inhibitors (Czarnecki et al., 2006; Kuster et al., 2013); accordingly protease inhibitors could not only be obtained from pure cyanobacterial cultures (Shin et al., 1996; Bister et al., 2004; Von Elert et al., 2005), but also from cyanobacterial blooms (Agrawal et al., 2001; Grach-Pogrebinsky et al., 2003; Gesner-Apter and Carmeli, 2009; Lifshits et al., 2011).

The ability to produce high amounts of highly variable secondary metabolites is one of the major features of cyanobacteria

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(Carmichael, 1992; Dittmann and Wiegand, 2006; Ferrao and Kozlowsky-Suzuki, 2011). Therefore, cyanobacteria are a widely explored source for natural products (Gademann and Portmann, 2008) and new pharmaceutical compounds (Chlipala et al., 2011; Silva-Stenico et al., 2012). For this reason the search for bioactive compounds has been extended from cyanobacterial cultures to biomass obtained from natural cyanobacterial blooms (Jakobi et al., 1995; Banker and Carmeli, 1999; Agrawal et al., 2001; Reshef and Carmeli, 2001). In particular protease inhibitors are of interest with respect to their ecological relevance due to effects on zooplankton communities and their putative pharmaceutical relevance. In the present study, we address both aspects using a metabolomic approach.

The term "Metabolomics" in general refers to the scientific field concerned with the study of naturally occurring, low molecular weight organic metabolites within cells, tissues or biofluids (Bundy et al., 2009). Especially in chemical ecology this approach has increasingly attracted attention. Metabolomic data are useful for the elucidation of chemically mediated interactions between organisms, and many substances with pharmacological properties were discovered, while investigating such chemical interactions (Prince and Pohnert, 2010). Recent studies have shown, that the search for biologically active metabolites benefits from combining metabolomic profiles with data on biological activity, which led to the identification of single compounds (Gillard et al., 2013) or even the elucidation of pathways (Nylund et al., 2011). Thus, metabolomic profiling has proven to facilitate the traditional bioassay-guided isolation of bioactive compounds by identifying a limited number of target molecules via multivariate statistics prior to the isolation and structural elucidation of compounds (Prince and Pohnert, 2010).

The aim of this study was to analyze several consecutive natural freshwater phytoplankton samples, in order to test whether or not repeated metabolomic profiling is a promising approach for the identification of known and unknown protease inhibitors. For this purpose we applied extracts from natural phytoplankton samples from a hypertrophic pond to liquid chromatography coupled with high-resolution mass spectrometry (LCMS). We then used multivariate statistics to analyze the obtained metabolic profiles and combined these data with the previously determined potential of the phytoplankton to inhibit digestive chymotrypsins of *Daphnia* (Kuster et al., 2013). In addition to that, we measured protease inhibition potentials from fractions of LC-runs to attribute biological activity to selected metabolites.

A second goal of this study was the exploitation of the richness of cyanobacteria in secondary metabolites by using the same method to unravel seasonal succession patterns among cyanobacteria in terms of differing cyanobacterial secondary metabolite profiles, also referred to as chemotypes, present on different sampling dates. Cyanobacterial biomass often peaks in late summer and early fall (Sommer et al., 1986) due to high temperatures in the epilimnion (Jöhnk et al., 2008), and the cyanobacterial composition may vary strongly with season. Kuster et al. (2013) have recently shown that the inhibitory potential of natural phytoplankton varies seasonally in a similar pattern. However, so far no data with regard to the chemical identity of the secondary metabolites and the cyanobacterial chemotypes that might account for the differing inhibition potentials have been obtained. To our knowledge, the data presented here provide the first metabolomic analysis in terms of seasonal succession in freshwater phytoplankton samples.

2. Materials and methods

2.1. Phytoplankton samples

In order to screen for putative protease inhibitors in phytoplankton samples obtained from the Aachener Weiher (AaW; N50°56′2.40″, E6°55′40.81″), an urban pond that can be regarded as hypertrophic due to its Secchi depth of 1.6 meters (Carlson, 1977), eight samples were taken between the 26th of July 2007 and the 9th of October 2007 (26/06; 13/07; 26/07; 10/08; 21/08; 28/08; 12/09; 09/10). When investigating a hypertrophic pond with filamentous and colonial cyanobacteria, Müller-Navarra (2000) found that the ingestible fraction of phytoplankton displayed similar detrimental effects on *Daphnia* growth rates as the unfiltered phytoplankton samples, which indicated that even large-sized cyanobacteria are represented in the ingestible fraction. At each sampling date volumes of 10 litre of surface water were collected from different areas of the pond. These samples were subsequently mixed to account for any spatial heterogeneity in the pond, and 50–80 litre of this mixed surface water was screened with a 55 μm mesh to obtain the fraction, that is ingestible for daphnids.

The filtrate was then gently concentrated by hollow-fiber filtration and freeze-dried (Kuster et al., 2013). Lyophilized phytoplankton was homogenized using a mortar and pestle. We obtained the ash-free dry mass (AFDM) of the samples by heating a known amount of each sample in a muffle furnace at 550 °C for eight hours and measuring the weight before and afterwards. The mass loss was regarded as the AFDM and varied between 20% and 30%. For the LCMS measurements known amounts of dry mass were resuspended in 25 μl of 80% methanol (MeOH, HPLC-grade) per mg sample, sonicated (5 min) and then centrifuged (10 min, 4500 × *g*). For each sample 5 ml of the supernatant were transferred into new test tubes. Afterwards, the peptide Met-Arg-Phe-Ala (MRFA, 20 μl, 16.6 μM) was added as internal standard. Samples were then evaporated to dryness in a rotary evaporator (40 °C), re-dissolved in 1 ml 100% MeOH and subsequently evaporated in a vacuum centrifuge. Finally, the samples were redissolved in 200 μl 100% MeOH, centrifuged (2 min, 11,500 × *g*), and the supernatant transferred into glass vials.

2.2. Microcystis aeruginosa *PCC7806 and BM25*

Microcystis aeruginosa strains PCC7806 (Dittmann et al., 1997) and BM25 (Schwarzenberger et al., 2013) were cultured in cyanophycean medium (Von Elert and Jüttner, 1997) in a chemostat under constant light conditions (50 μE m^{-2} s^{-1}) at 20 °C, with a dilution rate of 0.23 per day. In the case of strain BM25 sample preparation was carried out as described for the phytoplankton samples using freeze-dried powder of a pure *M. aeruginosa* BM25 culture. Extracts of strain PCC7806 were obtained by re-suspending 150 mg of freeze-dried PCC7806 cells in 1.5 ml 60% MeOH. After vortexing and sonicating the sample for 10 min, the sample was centrifuged (2 min; 4500 × *g*). The supernatant was transferred into a new test tube, evaporated to dryness in a vacuum centrifuge and dissolved in 200 μl 100% MeOH.

2.3. Protease inhibition

The half maximal inhibitory concentration (IC_{50}) values for chymotrypsins and trypsins of methanolic extracts of freeze-dried phytoplankton samples have already been published (Kuster et al., 2013). Extracts were normalized to dry mass in that study, whereas here we determined the AFDM and normalized the IC_{50} values (Kuster et al., 2013) to the AFDM to rule out effects of inorganic material in the samples. For the sample from 12/09, no more material was available to determine the AFDM and thus we used the average of all other samples to calculate the IC_{50} value normalized to AFDM.

2.4. Liquid chromatography–mass spectrometry

Measurements of the relative amounts of protease inhibitors and other metabolites were carried out using an ultra-high