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Flow cytometry as a diagnostic tool for the effects of polyphenolic allelochemicals on phytoplankton

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ABSTRACT

We investigated the impact of the polyphenol tannic acid (TA) on the green algae *Desmodesmus armatus* and *Scenedesmus vacuolatus* and the diatom *Stephanodiscus minutulus* in order to find new diagnostic tools for allelopathic effects on phytoplankton. Esterase activity, membrane integrity and production of reactive oxygen species (ROS) were tested using flow cytometry with specific fluorescent markers. For comparison, growth rate and photosynthesis, two variables known to be affected by TA, were evaluated. Algae were exposed to TA concentrations between 0.6 and 30 μmol L^{-1} for 3, 14 and 24 h. A significant inhibition of esterase activity was detected at every time point in all three tested algal species at 30 μmol L^{-1} TA and in most other treatments when TA concentrations exceeded 3 μmol L^{-1}. A significant production of ROS could also be detected in all three algal species, but only after a longer exposure period. Changes in membrane rigidity revealed no consistent patterns of enhancement or inhibition when tested with different TA concentrations, algal species and exposure time. Growth rates of all algae were significantly inhibited after 24 h, whereas *D. armatus* was the only species for which the photosynthetic yield did not decline.

The effects on esterase activity and ROS production indicate a general influence of polyphenolic allelochemicals on phytoplankton, but also reveal patterns which vary between species, concentrations and exposure times. Changes in esterase activity were the most sensitive variable, and could be detected after short exposure periods and at naturally occurring concentrations. Thus, esterase activity may be a suitable variable for future investigations into the allelopathic effects of submerged macrophytes on phytoplankton.

1. Introduction

Allelochemicals exuded from submerged macrophytes may inhibit other aquatic primary producers such as phytoplankton or epiphyton, providing macrophytes with a competitive advantage for light and nutrients. Numerous aquatic macrophytes contain and release allelochemicals into the ambient water body (Gross et al., 1996). Most studies into the effects of allelochemicals on phytoplankton have used plant extracts or purified plant compounds, even though such procedures fail to reflect natural lake conditions (Hilt and Gross, 2008). Coexistence experiments placing phytoplankton (typically in dialysis bags) among macrophytes are closer to *in situ* conditions, but often suffer from interfering processes that complicate the isolation of allelopathy as a primary mechanism. One of the major confounding factors is the potential simultaneous competition for nutrients between macrophytes and phytoplankton (Inderjit and Del Moral, 1997; Hilt et al., 2006; Gross et al., 2007). This could be prevented by short-term experiments. Such experiments, however, would require a sensitive observation variable that detects a phytoplankton response to allelochemicals within a short exposure period.

A few specific modes of action on phytoplankton have been identified for some of the known aquatic allelochemicals (*e.g.*, tellimagrandin II, Gross et al., 1996; Leu et al., 2002 and ethyl 2-methyl acetoacetate, Hong et al., 2008). Polyphenols, a common and well investigated class of allelopathically active compounds in submerged macrophytes of the genus *Myriophyllum* (Gross et al., 1996; Gross, 2003; Bauer et al., 2009), have been shown to inhibit two processes in algae: (1) alkaline phosphatase (APA) activity (Gross et al., 1996) and (2) photosystem II (PS II) activity (Körner and Nicklisch, 2002; Leu et al., 2002). Both processes, however, have some drawbacks when used in coexistence experiments. The exoenzyme APA is only produced during periods of inorganic phosphorus limitation, and effects on PS II activity are also influenced by nutrient limitation (Lippemeier et al., 2003). In addition, significant inhibitions of

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APA and PS II were only found after at least 3 d of exposure. Studies on other modes of action that respond more rapidly are lacking.

A second important aspect that requires the detection of various observation variables is the different sensitivity of phytoplankton groups and species towards allelochemicals. Chlorophytes generally seem to be less sensitive than diatoms and cyanobacteria (Hilt and Gross, 2008) in terms of growth and photosynthetic inhibition (Körner and Nicklisch, 2002), and epiphytes appear to be less sensitive than planktonic species (Hilt, 2006). Whether this holds true for other variables is not yet known. A first comparison of different methods (co-incubation with and without macrophytes *in situ* and in aquaria, with or without tannic acid addition) and variables (fluorescence-based chlorophyll a concentrations, PS II activity, cell counts, or biovolume) used to detect the allelopathic effects of macrophytes on two green algal species revealed significant differences between variables (Hilt et al., 2012).

Despite recent advances in this field of study, the molecular interaction between allelochemicals and possible cellular targets remains unclear. Every organic chemical exhibits a non-specific or baseline toxicity to an organism, due to the fact that chemicals penetrate biological membranes according to their lipophilicity. As this process is driven by partitioning between phases, baseline toxicity correlates with the K_{ow} (octanol–water partition coefficient) of a substance and thus represents the minimum toxicity of a given substance towards an organism. These empirical relationships can be modelled (*e.g.*, Altenburger et al., 2004) and used to predict the minimum toxicity of untested substances. More specific interactions, such as binding to enzymes or receptors, typically result in a higher toxicity than the baseline toxicity. A comparison of the effect levels between different observation variables and baseline toxicity may indicate the specificity of a given variable. There are, however, prerequisites for the applicability of these models. For instance, the molecular structure of an allelopathically active substance must be known, thus confining most studies to natural products. Another problem arises due to the complexity of the known polyphenolic allelochemicals (Gross et al., 1996). All models that have estimated the effects of chemicals on algae (*e.g.*, Altenburger et al., 2004) have used well-defined molecules with low molecular weights. For natural products with high molecular weights, it is unlikely that the existing models are suitable. Natural products are difficult to characterize and often highly degradable (Müller et al., 2007; Bauer et al., 2012), thus complicating analyses. One further aim of this study was therefore to consider the applicability of available baseline toxicity models to polyphenols.

The diagnostic technique of flow cytometry can be used to evaluate the metabolic status of cells, and was initially shown to be a suitable tool for detecting the disturbance of specific cellular algal characteristics by metals (Franklin et al., 2001; Stauber et al., 2002) and paraquat (Franqueria et al., 2000). Fluorescence signals can be derived *via* direct auto-fluorescence measurements of the cells, or can be mediated after staining with suitable fluorescence markers. In the green alga *Scenedesmus vacuolatus*, fluorescence markers for membrane permeability and potential, as well as mitochondrial respiration and esterase activity were used to investigate the effects of various xenobiotics (Adler et al., 2007). In addition, fluorescence markers for the production of reactive oxygen species (ROS) were established (Le Bel et al., 1992) and used in flow cytometric approaches with algae (Szivak et al., 2009). Hong et al. (2008) were the first to test whether an allelochemical, ethyl 2-methyl acetoacetate (EMA) produced by reeds, inhibits processes in algae; namely esterase activity and ROS-production with established fluorescence markers. *Microcystis aeruginosa* cultures exposed to the EMA, however, revealed either enhanced or decreased enzyme activity depending primarily on exposure time (Hong et al., 2008). The same was found to hold true for esterase activity in *M. aeruginosa* and *Selenastrum capricornutum* cultures exposed to acid mine drainage water (Regel et al., 2002). Furthermore, an enhanced ROS production was detected in phytoplankton and cyanobacteria cultures exposed to metals and EMA, and has been interpreted as a consequence of the inhibition of detoxification enzymes such as esterases (Szivak et al., 2009) or as acute cell damage (Hong et al., 2008). In the latter case, even a subsequent increase in detoxification enzymes such as esterases was proposed (Hong et al., 2008). Still, the enhanced production of ROS may be accepted as a general early response of algae to a stressor (Szivak et al., 2009).

Polyphenols are able to penetrate cell membranes due to their amphiphilic or lipophilic structure (Leu et al., 2002). Possible impacts on cell membrane integrity in algal cells due to polyphenols might therefore be detectable with fluorescence dyes established for other phytotoxicants (Franklin et al., 2001; Adler et al., 2007). Gram-negative bacteria exposed to polyphenols increased their membrane permeability (Yi et al., 2010), whereas *in vitro* studies revealed that the polyphenol tannic acid aggregated phospholipid bilayers, thus reducing the fluid spacing between them (Simon et al., 1994). At higher concentrations of tannic acid, however, phospholipid bilayers became unstable (Simon et al., 1994). Polyphenols can, depending on redox conditions (primarily oxygen availability and pH), be oxidized and bind to other metabolites by hydrogen bonding and hydrophobic interactions, thus acting as potential enzyme inhibitors (Gross et al., 1996; He et al., 2006). However, studies on these possible effects of polyphenols on phytoplankton are lacking.

The aims of the present study were to investigate (I) whether new effect variables of polyphenols on phytoplankton can be detected by the use of flow cytometry, and (II) whether these effects can be detected after short-term exposure and at naturally occurring allelochemical concentrations. We therefore modelled EC_{50} values for TA on three algal species after measuring changes in membrane integrity, production of ROS and esterase activity with specific fluorescence markers. Results were compared to the inhibition of growth rates and photosynthetic activity.

2. Methods

2.1. Test organisms and culture conditions

A synchronized, unicellular non-axenic culture of the green alga *S. vacuolatus* Shihira et Krauss (strain 211-15; SAG University of Göttingen, Germany) was photoautotrophically grown in 2-fold Gimme–Bordman medium (pH 7.2) at $28 \pm 0.5\,^{\circ}C$ under 14:10 h light:dark conditions at 370 μmol photons $m^{-2}\,s^{-1}$ (Altenburger et al., 2004). The non-axenic green alga *Desmodesmus armatus* Chodat (SAG University of Göttingen, Germany) and the diatom *Stephanodiscus minutulus* Kütz (Kleve et Möller) (SAG University of Göttingen, Germany) were grown in modified MIII medium (Nicklisch, 1992) at pH 7.5–7.9 at $20 \pm 0.5\,^{\circ}C$ at 80 μmol photons $m^{-2}\,s^{-1}$ under 12:12 h light:dark conditions in a conditioning cabinet. The MIII medium contained $CaSO_4$ 0.5 mM, $CaCl_2$ 0.5 mM, $MgSO_4$ 0.25 mM, $NaNO_3$ 0.5 mM, KH_2PO_4 0.05 mM, KCl 0.1 mM, Na_2SIO_3 0.4 mM, HCl 0.75 mM, $NaHCO_3$ 2 mM, $FeCl_3$ 0.01 mM, Na_2 EDTA 0.02 mM, trace elements H_3BO_3 4 μM, $MnSO_4$ 0.8 μM, $ZnSO_4$ 0.08 μM, Na_2MoO_4 0.04 μM, $CuSO_4$ 0.04 μM, $AlK(SO_4)_2$ 0.08 μM, $CoCl_2$ 0.04 μM, $NiSO_4$ 0.04 μM, KBr 0.08 μM, KJ 0.04 μM and H_2SO_3 0.06 μM. Both cultures were shaken gently at 60 rpm. All algal cultures grew exponentially when applied in the experiments.

2.2. Inhibition tests with tannic acid (TA)

Solutions of the hydrolysable polyphenol TA (Fluka, filling code: 403955/1 64400) were freshly prepared for each