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Does quinone or phenol enrichment of humic substances alter the primary compound from a non-algicidal to an algicidal preparation?

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ABSTRACT

Dissolved organic matter (DOM) has been shown to affect phytoplankton species directly. These interactions largely depend on the origin and molecular size of DOM and are different in prokaryotes and eukaryotes. In a preceding study, however, two humic substance preparations did not adversely affect coccal green algae or cyanobacterial growth even at high concentrations of dissolved organic carbon (DOC). These results contradicted previous findings, showing a clear, negative response of different phototrophs to much lower DOC concentrations. To test whether or not at least defined building blocks of humic substances (HSs) are effective algicidal structures, we enriched two humic preparations with hydroquinone and *p*-benzoquinone, respectively, and exposed two different green algae, *Pseudokirchneriella subcapitata* and *Monoraphidium braunii*, and two cyanobacterial species, *Synechocystis* sp. and *Microcystis aeruginosa*, to the unmodified and enriched HSs. As response variables, growth rates in terms of biomass increase, chlorophyll-*a* content, and photosynthetic yield were measured. The highest concentration (4.17 mM DOC) of the modified HSs clearly inhibited growth; the cyanobacterial species were much more sensitive than the green algal species. However, realistic ecological concentrations did not adversely affect growth. Aerating the exposure solution for 24 h strongly reduced the inhibitory effect of the modified HSs. The algicidal effect was obviously caused by monomers and not by polymerised high molecular weight HSs themselves. Furthermore, the maximum quantum yield (Φ PSII max) was stimulated in the green algal species by low and medium DOC concentrations, but reduced in the cyanobacterial species upon exposure to higher HS concentrations. The quinone- and phenol-enriched HSs only showed algicidal activity at high concentrations of 4.17 mM DOC and lost their effects over time, presumably by oxidation and subsequent polymerisation. This study confirms that the applied humic substances themselves are not effective algicides even if enriched in effective structures.

1. Introduction

Humic substances comprise the majority of organic carbon in freshwater ecosystems and exceed the concentration in living organisms, by at least one order of magnitude (Wetzel, 2001; Steinberg et al., 2008). Dissolved organic carbon (DOC) originates from different sources, including peat, leaf litter, and plant debris in general. HSs vary not only by origin but also by molecular size and functional group compositions, such as carboxylic, phenolic, ketonic, saccharidic, peptidic, aromatic, and aliphatic molecules. These specific compositions are like fingerprints for each kind of dissolved organic carbon (Sachse et al., 2005). Depending on environmental conditions, drainage area, and dominating flora, the DOC can vary from less than 1 to 200 mg L^{-1} (Suhett et al., 2007).

With respect to direct effects, there have been several studies testing the sensitivity of eukaryotic and prokaryotic freshwater phototrophs against different kinds of DOM, such as natural organic matter (NOM) isolates (Pflugmacher et al., 2006; Prokhotskaya and Steinberg, 2007), fulvic acids (Steinberg and Bach, 1996; Ohkubo et al., 1998) and humic acids (Giesy, 1976; Hoeffner and Manahan, 1980; Vrana and Votruba, 1995; Imai et al., 1999; Pouneva, 2004; Sun et al., 2005, 2006). Furthermore, leachates of barley straw, rice straw, and leaf litter were exposed (Gibson et al., 1990; Welch et al., 1990; Pillinger et al., 1994; Everall and Lees, 1996; Ridge et al., 1999; Ball et al., 2001; Ferrier et al., 2005; Park et al., 2006; Murray et al., 2010). These studies showed diverse results, from promotion to total inhibition of growth with effective

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concentrations varying from <0.01 to 100 mg L^{-1} DOC. Overall, it appears likely that the growth response to different NOM is species specific.

Explanations for positive and negative effects on microalgal cultures and communities are varied. Paul et al. (2003) established a quantitative structure–effect-relationship relating photosynthetic oxygen release to spin densities of HSs, equivalent to quinoid structures. Pflugmacher et al. (2006) claimed the quinoid structures of different NOM to be effective in inhibiting the Photosystem II PSII of *Ceratophyllum demersum*. Prokhotskaya and Steinberg (2007) assumed a herbicide-like mode of action by blocking the electron transport chain. Internal oxidative stress and the development of intracellular H_2O_2 offer another explanation for the algicidal activity of HSs (Sun et al., 2005, 2006).

Natural chelates, like humic and fulvic acids, are able to positively and adversely affect algal growth by complexation reactions with essential metals, mainly iron (Giesy, 1976; Jackson and Hecky, 1980; Imai et al., 1999; Sun et al., 2005; Lee et al., 2009). Furthermore, due to reduced toxicity by complexation, algae can benefit from the chelating capacity of DOM if exposed to toxic heavy metals (Garvey et al., 1991; Ohkubo et al., 1998).

Several authors have presumed that chemical inhibitors are or derive from oxidised polyphenolic compounds (Pillinger et al., 1994; Ridge and Pillinger, 1996). These authors state that the potency of litter leachates to suppress algal growth depends on different aeration of the extract, the presence of decomposing bacteria, and the decomposition time of the organic matter itself. Pillinger et al. (1994) successfully suppressed the growth of *Chlorella vulgaris* and *Microcystis aeruginosa* by synthetic as well as natural tannic acids. In the same study, *M. aeruginosa* was also strongly inhibited by different quinones at concentrations below 100 μg L^{-1}. Everall and Lees (1996) analysed the major trace organic compounds in rotting barley straw, and significantly reduced general phytoplankton productivity and the cyanobacterial dominance by addition of decomposed barley straw. Continued oxidation can result in increasing polymerisation and in formation of HSs. Pillinger et al. (1994) observed first an increase in the toxicity of phenolic material during the quinone phase and then a decrease with ongoing polymerisation of the phenolic material. Ridge and Pillinger (1996) consistently showed that algae inhibitors are released from oxidised lignin by comparing the algicidal activity of lignin-enriched wood (brown-rotted) and lignin-depleted wood (white-rotted); high algicidal activity was seen with the former and no activity with the latter. This hypothesis has been supported by studies with high molecular HSs. Different freshwater phototrophs were not affected or even stimulated to grow when exposed to HSs (Hoeffner and Manahan, 1980; Vrana and Votruba, 1995; Steinberg and Bach, 1996; Pouneva, 2004; Lee et al., 2009; Bährs and Steinberg, 2012). In sum, it is difficult to predict if and where phenolics will be active and whether phenolic oxidation plays a key role in the ecological activity of DOM (Appel, 1993).

Microbial decomposition is not the only way to generate chemical inhibitors from DOM in freshwater ecosystems. Photolysis of humic substances plays a major role in the generation of organic radicals and reactive oxygen species (Baxter and Carey, 1983; Paul et al., 2003; 2006; Steinberg et al., 2008). Overall, it is obvious that low-molecular weight substances, such as oxidised phenols and quinones derived from plant debris, are potentially toxic to phototrophs and lose this potency with increasing molecular size due to condensation/aggregation. Furthermore, the specific algicidal activity of DOM depends to high degree on the state of decomposition and oxidation of phenolic compounds, whereby particularly photolysis of organic matter is central in the generation of bioactive compounds.

Based on the aforementioned state of knowledge, we enriched an already tested humic preparation HuminFeed® (HF) with hydroquinone and *p*-benzoquinone and tested whether or not the enrichment could change the antialgal activity of the humic preparation. To achieve this, we checked the growth rate and photosynthetic performance of the algal species on the one and the chemical composition of the different humic preparations on the other.

2. Materials and methods

2.1. Humic material

As humic material, we used commercially available HuminFeed®. It is composed of processed leonardite, with a high spin concentration (indicative of a high content of stable organic radicals, indicative of high reactivity), high C:CH_2 and C:H ratios, and high specific UV absorption. HuminFeed® consists of 82% humic substances, 18% low-molecular weight compounds and contains 43% organic carbon (Meinelt et al., 2007). It has demonstrated direct effects on aquatic and sediment organisms, for instance the water mould *Saprolegnia parasitica* Coker.

HuminFeed® was modified according to Perminova et al. (2005). The first modification was carried out with *p*-benzoquinone (Merck, Germany). 1000 mg of authentic HF were dissolved in 2.5 mL of H_2O and diluted to 50 mL with 0.1 M KH_2PO_4. Accordingly, 500 mg of *p*-benzoquinone were added and the mixture was stirred for 1 h at room temperature. Subsequently, the HSs were precipitated with 6 M HCl at a pH value of 1, filtered and freeze-dried. According to the modification, the product was called B500.

For the second modification, 1000 mg of authentic HF were dissolved in 2.5 mL of H_2O, diluted with distilled water to a volume of 50 mL and its pH value adjusted to 7. Addition of 500 mg of hydroquinone (Merck, Germany) was done in the presence of catalytic amounts of oxalic acid and 1 g of a 35% solution of formaldehyde. The mixture was stirred for 1 h at 100 °C. The dissolved HSs were precipitated by the addition of 6 M HCl at a pH value of 1, filtered and freeze-dried. The product was called Hy500. All three HSs were dissolved in 0.1 M KOH at a concentration of 5 g L^{-1} DOC and stored in the refrigerator.

2.2. Characterisation of HuminFeed® and modified humic substances

2.2.1. Absorption spectra

To exclude any light quenching effects when comparing the unmodified HF and the modified substances, the absorption spectra of the tested HSs was measured with a Shimadzu UV-2450 UV–Vis Spectrophotometer (Shimadzu, Kyoto, Japan).

2.2.2. Size exclusion chromatography

Fingerprints of the applied DOC samples were determined using size exclusion chromatography with UV_{254} detection and online carbon detection (Sachse et al., 2005). Liquid chromatography (LC) separation was carried out using an LC-DOC system (DOC Labor Dr. Huber, Karlsruhe, Germany). For separation, a Toyopearl HW-50S column was used (250 × 20 mm, particle size 20–40 μm, Tosoh Bioscience, Tokyo, Japan). The compounds were eluted with a phosphate buffer (20 mM) at a flow rate of 1.5 mL min^{-1}. After separation and detection of the UV absorption, the mobile phase passed through a Graetzel thin film reactor, where the organic compounds were oxidised after addition of concentrated phosphoric acid (0.4 mL min^{-1}) and irradiation with UV light at 184 nm. The produced carbon dioxide was transferred into an infrared detector with nitrogen as the carrier gas.