Dissolved natural organic matter (NOM) impacts photosynthetic oxygen production and electron transport in coontail

*Ceratophyllum demersum*

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Abstract

Dissolved natural organic matter (NOM) is dead organic matter exceeding, in freshwater systems, the concentration of organic carbon in all living organisms by far. 80–90% (w/w) of the NOM is made up of humic substances (HS). Although NOM possesses several functional groups, a potential effect on aquatic organisms has not been studied. In this study, direct effects of NOM from various origins on physiological and biochemical functions in the aquatic plant *Ceratophyllum demersum* are presented. Environmentally relevant concentrations of NOM cause inhibitory effects on the photosynthetic oxygen production of *C. demersum*. Various NOM sources and the synthetic humic substance HS1500 inhibit the photosynthetic oxygen production of the plant as observed with 1-amino-anthraquinone, a known inhibitor of plant photosynthesis. 1-Amino-anthraquinone may serve as an analogue for the quinoid structures in NOM and HS. Most likely, the effects of NOM may be related to quinoid structures and work downstream of photosynthesis at photosystem (PS) II.

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Keywords: Humic substances; NOM; Photosynthetic oxygen production; Electron transport; Quinoid structures

1. Introduction

Natural organic matter (NOM) is dead organic matter. Its majority comprises humic substances (HS). Despite the microbial utilization of low-molecular weight NOM fractions (amino acids, saccharides, fatty acids), a variety of functional groups are able to react with chemicals in the environment. Dissolved NOM by far exceeds the concentration of organic carbon in all living organisms (Steinberg and Münster, 1985). In terrestrial systems, humic substances may be approximately three times more than the carbon bound in organisms. In freshwater, this factor may be one and in marine systems two orders of magnitude (Thurman, 1985; Killops and Killops, 1993; Schlesinger, 1997; Wetzel, 2001).
From a chemical point of view, HS are by no means dead in the sense of non-reactive. They are (weak) natural environmental chemicals (Steinberg et al., 2003) with a variety of functional groups, such as alcoholic, phenolic, methoxy, quinoid, keto, aldehyde, and carboxylic groups, and a range of molar masses from some 100 to several thousand daltons (Senesi and Loffredo, 2001). As chemicals, HS may have an impact on exposed organisms. However, our knowledge, understanding of how HS affect organisms is rather limited. In many instances, only modulations of bioavailability of nutrients or the suppression of pathogens have been the focus of studies (Chen et al., 1988, 1999; Chen and Aviad, 1990; Senesi and Loffredo, 2001). That means that the effects of HS upon organisms are thought to be more or less of indirect nature. One reason for this may be that HS, due to their large molecular mass, appear to be too big to penetrate bio-membranes.

Recent publications indicate that humic substances, due to their chemical properties, can display several more or less indirect effects on freshwater organisms, e.g. reduction of acid stress (Hargeby and Petersen, 1988; Petersen, 1990), reduction of harmful UV-irradiation (Schindler et al., 1996), indirect nutrition of microorganisms by increased bioavailability of inorganic micronutrients (Chen and Aviad, 1990), and fatty acids upon irradiation and photolytic cleavage (Wetzel et al., 1995; Tranvik and Bertilsson, 2001), or reduction in xenobiotic stress (Piccolo, 1994; Haitzer et al., 1999; Perminova et al., 1999; Steinberg et al., 2000). In general, a variety of ecosystem metabolism, particularly biodegradation pathways, is hampered in the presence of humic substance so that the term ‘entropy buffer’ has been established (Ziechmann, 1994).

From studies of the behaviour of HS in electric fields, however, Münster (1985) was the first to postulate direct interactions of HS with and penetration of biomembranes. Meanwhile, several xenobiotic effects of HS on aquatic organisms have been described (see review of Steinberg et al., 2003). In a previous paper, we reported on inhibitory effects on the photosynthetic oxygen production in the aquatic macrophyte Ceratophyllum demersum (Pflugmacher et al., 1999). The mechanisms behind this inhibition are still obscure and first thoughts were that contamination with xenobiotics, especially pesticides, could account for the measured effect. But taking the nature of HS into account there are a lot of chemical functional groups which are able to interfere with plant photosynthesis. Tollin and Steelink (1966) observed, for example, that electron spin resonance (ESR) parameters for HS were very close to those of hydroxyanthraquinones. The suggestion was that simple quinone–phenol structures could account for the ESR properties of HS. Also, in recent publications the possibility that HS could act as electron acceptors as well as electron donors in microbial respiration was shown, stating with ESR studies that quinone moieties are the primary electron-accepting groups in HS (Scott et al., 1998; Jezierski et al., 2000). In plant physiology, the inhibitory effect on the plant photosynthesis from quinones like 1-aminoanthraquinone is well known (Oettmeier et al., 1988).

In this study we focused on effects of NOM and one synthetic HS (HS1500) on photosynthetic oxygen production in the aquatic macrophyte C. demersum as well as on the influence of NOM on the electron transfer chain in the chloroplasts of this plant, to verify the hypothesis that quinoid moieties of HS can act as electron sinks and therefore inhibit the observed photosynthetic oxygen production of aquatic plants.

2. Material and methods

2.1. Plant material

C. demersum was collected during the summer season from different sites around Berlin. Identification of the plant species was performed according to Casper and Krausch (1988). Plants were cultivated non axenically prior to the experiment for 3 years in Provasoli’s medium (ESI\(_{15}\), 15 ml L\(^{-1}\)) in 100 L tanks. Supplementary light was provided by daylight lamps at a light/dark cycle of 14:10 h. Temperature was maintained at 22–24 °C.

2.2. NOM origin

Organic matter isolated by reverse osmosis is called natural organic matter (NOM). NOM samples
used in this study have been isolated from a range of different sources in Europe and the USA. Most of them were provided by the sources mentioned in Table 1 or purchased from the International Humic Substances Society (IHSS). Concentrations of NOM (mg L\(^{-1}\) dissolved organic carbon) in stock solutions were determined by high temperature combustion (Shimadzu TC 5000) after acidification with phosphoric acid to remove inorganic carbonates (DIN EN 1484, 1998). The synthetic HS1500 consists of condensed alkylated polyphenols with a mean molar mass of 1500 Da (Weyl, 1990).

2.3. Plant exposure to NOM, HS1500, and 1-aminoanthraquinone

Two grams fresh weight (fw) of each plant was exposed to NOM from different sources and also to 1-aminoanthraquinone in a volume of 200 mL each for 24 h under constant conditions concerning light and temperature. The pH was maintained at 7.5–8.0. The concentrations of NOM used in this study were 0.5 mg DOC L\(^{-1}\). The concentration of 1-aminoanthraquinone was 0.4 \(\mu\)g L\(^{-1}\) organic C. 1-Aminoanthraquinone was dissolved in ethanol. In the controls, NOM and quinone were left out of the medium, but they contained the same amount of ethanol as in the exposures (< 3% of the total volume). Each exposure was done in 12 replicates.

2.4. Preparation of C. demersum thylakoids

Thylakoids were prepared from C. demersum according to Böger (1993). In detail: whole plants of C. demersum were homogenized in buffer containing 0.4 M sucrose, 50 mM Tricine pH 8.0, 10 mM NaCl and 5 mM MgCl\(_2\). The homogenate was filtered through four layers of miracloth (Calbiochem, LaJolla, Cal., USA) and centrifuged for 1 min at 4000 \(\times\) g. The resulting pellet was re-suspended in the same buffer mentioned above for determination of whole chain electron transport, PSII and PSI electron transport. Total chlorophyll content of the thylakoid suspensions was estimated in 80% (v/v) acetone using the equations of MacKinney (1941).

2.5. Measurement of photosynthetic oxygen production

The measurement of photosynthetic oxygen production of the plant was performed using a Phosy Mess 4000 (InnoConcept, Strausberg), 100% light intensity (SI Unit: 2000 lx) and a dark/light/dark cycle of 10/12/10 min under at 20°C. Measurements were taken with a Clark electrode (WTW EO 196-1.5). The rates were calculated in \(\mu\)mol O\(_2\) h\(^{-1}\) gf w\(^{-1}\).

2.6. Determination of electron transport

Thylakoid measurements were done with the Phosy Mess 4000. Electron transport activities in

<table>
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<tr>
<th>Table 1</th>
<th>List of HS used in this study, their origin and source</th>
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<tr>
<td><strong>HS substance</strong></td>
<td><strong>NOM/HA/FA</strong></td>
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<tr>
<td>Suwannee River</td>
<td>NOM</td>
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<tr>
<td>Nordic Reference</td>
<td>NOM</td>
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<tr>
<td>Svatberget</td>
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<td>Lake Schwarzer See</td>
<td>NOM</td>
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<tr>
<td>HS1500</td>
<td>Synthetic, polymerization product of alkylated polyphenols</td>
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suspended thylakoid membranes (10 µg chlorophyll mL$^{-1}$) were measured under saturating light intensities. For measurement of whole-chain electron transport activity, 10 mM methylamine, 1 mM sodium azide, and 1 mM methyl viologen were added to the suspension. The assay medium for photosystem (PS) I electron transport activity contained 10 mM methylamine, 10 µM 3,4-dichlorophenyl-1,1-dimethylurea (DCMU), 1 mM sodium azide, 500 µM 2,6-dichlorophenolindophenol (DCIP), 2 mM sodium ascorbate, and 1 mM methyl viologen (paraquat). Activity of PS II electron transport was determined using 1 mM phenyl-β-benzoquinone as electron acceptor in the presence of 0.5 µM 2,5-dibromo-3-methyl-6-isopropyl-β-benzoquinone.

3. Statistics

Significance testing was performed using one way analysis of variances (ANOVA) and the Student’s t-test at α=0.05.

4. Results

Inhibitory effects on photosynthetic oxygen production in *C. demersum* were detected using different NOM in an environmental relevant concentration of 0.5 mg L$^{-1}$ DOC (Fig. 1). Highest reductions were caused by Valkeakotinen NOM (22.6 ± 2.9 µmol O$_2$ h$^{-1}$ fw$^{-1}$), Hietajärvi NOM (23.7 ± 2.4 µmol O$_2$ h$^{-1}$ g fw$^{-1}$) and Barschsee (22.1 ± 2.1 µmol O$_2$ h$^{-1}$ g fw$^{-1}$). The inhibition of photosynthetic oxygen production by various NOM isolates was comparable with the synthetic humic substance HS1500 (19.7 ± 7.3 µmol O$_2$ h$^{-1}$ g fw$^{-1}$). Exposure to 1-aminoanthraquinone showed the expected inhibitory effect on plant photosynthetic oxygen production, at a concentration three magnitudes lower than the NOM samples (Fig. 2).

Measurements of whole chain electron transport with isolated thylakoid membranes exposed to Svartberget NOM, HS1500, or 1-aminoanthraquinone showed reductions in the electron transport process (Fig. 3). The rate of electron transport mediated by PS
I only displayed a small decrease that was highest after exposure of the plant to Svartberget NOM, but no reduction was statistically significant. With the PS II electron transport, a similar pattern in transport reduction was measured as with the whole electron transport.

5. Discussion

There are numerous studies showing positive effects of humic substances on the growth of terrestrial plants (Chen and Aviad, 1990; Vaughan and Malcolm, 1985). Evidence exists that low-molar mass HS can be taken up by plants and directly interact with plant metabolic processes (Vaughan and Ord, 1981; Vaughan and Malcolm, 1985; Chen and Aviad, 1990; Steinberg, 2003). However, studies on direct, potentially adverse effects on terrestrial or aquatic plants are rare. For example, in a previous study Pflugmacher et al. (1999) presented evidence that HS and NOM may inhibit photosynthetic oxygen release in the aquatic macrophyte *C. demersum*. Hence, this study was set up to find out first details of the mode of action with environmentally realistic NOM exposures (0.5–4.0 mg L⁻¹ DOC; for environmental realism, refer to Thurman, 1985). The fact that the photosynthetic oxygen production after exposure to NOM still occurs, but at a lower rate, may be explained by the assumption that HS in the NOM are not able to catch some, but not all electrons between PS II and PS I. At the concentrations of NOM used, a few electrons still go the proper way via the electron transfer chain between PS II and PS I. Is there a structure relationship between HS and the measured inhibitory effect on plant photosynthetic oxygen production? HS are well known to contain stable free radicals of semi-quinone nature. These radicals may be formed by spontaneous charge-transfer
reactions through two single-electron steps between quinone and hydroquinone units (Senesi and Schnitzer, 1977). A further chemical reduction of quinoid structures leads to short-lived free radicals (Senesi and Schnitzer, 1977). For several decades, quinones have been known to have inhibitory effects on the electron transfer chain in PS II (Oettmeier et al., 1988) and to act as Hill oxidants by absorbing electrons in the chloroplast (Trebst, 1972; Büchel, 1972). Consequently, 1-aminoanthraquinone was used as an analogue for the quinoid structures in HS of the NOM with respect to the inhibition of photosynthetic oxygen production. Primary (QA) and secondary (QB) electron acceptors in the electron transport of PS II are plastoquinones. The one-electron carrier (QA) is connected via the two electron gate intermediate QB with the plastoquinone pool as two electron carrier (Oettmeier et al., 1988). Related to the carbon content, 1-aminoanthraquinone has the highest potential to reduce photosynthetic oxygen production. Hence, it is most likely that it is the quinoid structures of HS that can act as electron scavengers and therefore inhibit the photosynthetic oxygen production of aquatic plants. Comparing the specific impact on the photosynthetic oxygen production, the pure chemical (1-aminoanthraquinone) is more active than NOM by a factor of roughly $10^3$. This is due to the higher molar mass of NOM and HS and their potential to form aggregates. With an assumption that, at maximum, 5% of the NOM consists of quinoid structures (Schnitzer and Khan, 1972; Thurman, 1985) and that only 1–10% of these are available for interactions within the photosystems (the majority might be hidden inside the HS aggregate and, hence, not available for interactions with the photosystems), the specific activities of the NOM and the pure 1-aminoanthraquinone become well comparable.

Although the humic substances were isolated from some remote sites, we cannot exclude diffuse contamination by deposited atmospheric chemicals that are distributed worldwide. However, with the synthetic humic substance HS1500 exhibiting the same inhibitory effect, these effects can clearly be on quinoid moieties in HS samples and not to contamination e.g. with pesticides of the used HS. Experiments with isolated thylakoids from exposed plants showed that in fact only the whole electron transport chain and PS II electron transport is affected significantly by the different NOM with their HS or by 1-aminoanthraquinone. Hence, we may conclude that quinoid groups in NOM with their HS have a strong impact on electron transport and that the inhibition takes place mainly at the PS II site, which means downstream of the photosynthetic process.

In conclusion a new facet of the ecological role of HS is their possible herbicide-like mode of action on aquatic macrophytes. Various metabolic pathways seem to be involved and affected in the recognition by aquatic plants of HS as xenobiotics in general and as herbicides in particular (Steinberg et al., 2003). The ecological relevance may be found in the different susceptibility of aquatic plants to HS.

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