Natural organic matter (NOM) induces oxidative stress in freshwater amphipods *Gammarus lacustris* Sars and *Gammarus tigrinus* (Sexton)

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Abstract

Humic substances comprise the majority of natural organic matter (NOM) on Earth, including dissolved organic matter in freshwater systems. Recent studies show that these substances directly interact with aquatic organisms as chemical stressors. The aim of the present study was to investigate the mode of action of dissolved NOM on the freshwater amphipods *Gammarus lacustris* Sars and *Gammarus tigrinus* (Sexton), and in particular, to determine if NOM induces or promotes internal oxidative stress. NOM was isolated by reverse osmosis from a brown-water lake in Brandenburg State, Germany. Oxidative stress markers, such as lipid peroxidation, cell internal hydrogen peroxide concentration, as well as peroxidase, catalase and glutathione S-transferase activities, were quantified. Exposure of both amphipod species to NOM caused a significant increase in lipid peroxidation, hydrogen peroxide concentration, catalase, peroxidase and glutathione S-transferase activities. Both species showed a two-stage antioxidant response: the first stage allowed the organisms to effectively eliminate ROS and to protect cells from damage, whereas the second stage leads to $\text{H}_2\text{O}_2$ accumulation in combination with destruction of lipid structures in the cells and, finally, functional damage or even death of the organism.

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1. Introduction

Natural organic matter (NOM) is part of all aquatic ecosystems and mainly comprises humic substances (HS), which represent 60–80% of the total dissolved organic carbon (Steinberg and Münster, 1985; Thurman, 1985). Despite the fact that NOM is mainly formed as a
result of decomposition of terrestrial plants, NOM plays a crucial role in all aquatic ecosystems (Wetzel, 2001). The functions of NOM in aquatic systems include influence on energy regimes (Steinberg, 2003), modulation of concentration and toxicity of xenobiotics and metals, and decrease of pollution and aquatic organisms’ exposure to potentially toxic substances (Gjessing, 1981; Campbell et al., 1997; Haitzer et al., 1999).

Relatively little is known about direct adverse impact of NOM on freshwater organisms. Pioneering studies by Petersen and Persson (1987) showed direct adverse effects of dissolved NOM on Daphnia magna. As potential mode of action, they discussed an irritation of membranes by the lipophilic NOM fraction, which was already postulated by Münster (1985) based on electrophoretic studies of HS. It is also known that NOM can modulate the activity of biotransformation enzymes of organisms, and also directly inhibit photosynthetic oxygen production of plants (Pflugmacher et al., 1999, 2001). Further biomarkers indicating weak chemical stresses imposed by NOM have been identified: For instance, Wiegand et al. (2004) found elevated levels of the chaperon HSP70 in fishes and amphipods, and Timofeyev et al. (2004) reported modulation of peroxidase activity in amphipods after exposure to HS. A comprehensive perspective of the ecophysiological function of dissolved HS is given by Steinberg et al. (in press).

Recent studies have revealed a significant impact of NOM on the physiological condition and sex ratio in fishes and amphibians (Meinelt et al., 2004; Lutz et al., 2005). Applying oligonucleotide-based whole genome DNA microarray experiments to the nematode Caenorhabditis elegans, it has been shown that an artificial humic substances, as well as a NOM source, induce transcriptional changes, which were identified in chemosensors and olfactory receptors, and enzymes of the biotransformation system (CYP, UGT, GST). The results confirmed that HS are recognized as environmental signals and weak chemical stressors (Menzel et al., 2005). Very recently, also the induction of CYP1A in fishes has been identified (Matsuo et al., in press). Furthermore, the latter paper showed that also organic transporters were activated in C. elegans, indicating that HS are taken up, as reported previously based on 14C-uptake studies with 14C-labeled HS-like substances (Steinberg et al., 2003).

Several mechanisms of the direct impact of NOM on aquatic organisms are still unclear. One of the mechanisms under discussion is the potential of dissolved NOM to form various external reactive oxygen species (ROS), such as superoxide, hydroxyl radical and hydrogen peroxide (Paul et al., 2004) on photoexcitation. These ROS may subsequently be taken up by freshwater organisms. Another mechanism is internal ROS production. Due to the apparently low-molecular mass of the water-soluble and ionizable HS fraction (Hoque et al., 2003; Reemtsma and These, 2003; Cooper et al., 2004; Hatcher et al., 2004; Seitzinger et al., 2005), NOM (including HS) can easily be taken up by freshwater organisms (Steinberg et al., 2003). Metabolism of NOM was shown to generate ROS (Timofeyev et al., 2004). Among ROS, hydrogen peroxide and superoxide anions are main initiators of a number of cellular reactions, including the iron-catalyzed Fenton reaction (Droge, 2002). The main cellular components susceptible to damage by ROS are lipids with subsequent peroxidation of unsaturated fatty acids in membranes, proteins with subsequent denaturation, as well as carbohydrates and nucleic acids (Blokhina et al., 2003).

The aim of the present paper has been to study the mechanisms of NOM impact on freshwater organisms and, in particular, to assess the possible role of NOM in the promotion of oxidative stress in freshwater amphipods and to identify their response to the oxidative stress on the biochemical level. As test organisms, the amphipods Gammarus lacustris and G. tigrinus were used. The responses of the amphipods to NOM exposure include internal H2O2 level, modulation of enzyme activities, such as that of catalase, peroxidase, glutathione S-transferase and lipid peroxidation.

2. Materials and methods

2.1. Amphipods

Two amphipods (Crustacea, Amphipoda), considered to be typical inhabitants of diverse continental water bodies, were chosen for this study: G. lacustris Sars, which is a cold water inhabitant widely spread in Palaearctic lakes (Barnard and Barnard, 1983), and Gammarus tigrinus (Sexton), which has recently been introduced to Germany from North America (Szaniawska et al., 2003). Specimens of G. tigrinus were collected from Lake Müggelsee (Berlin, Germany) during October to November 2003 and 2004. Specimens of G. lacustris were collected from several shallow lakes in eastern Siberia (Russia) during August to September 2003 and 2004. The size of the collected individuals was 1–1.5 cm (G. lacustris) and 1.5–2 cm (G. tigrinus). Age and sex of amphipods were not determined (in order to minimize handling of the delicate organisms); however, we assumed that most of the amphipods were sub-adults because of the clearly sub-maximal body size.

All amphipods were reared for a few days prior to experiments in aerated 5-L tanks with distilled water.
with the addition of salts: NaHCO₃ 0.03 g L⁻¹, CaCl₂ 0.09 g L⁻¹ and commercial sea salts 0.03 g L⁻¹ (Sera GmbH, Germany). The amphipods were kept at a temperature of 6–8 °C. Commercial food was used (TetraMin, Tetra GmbH, Germany) ad libitum. The water was permanently aerated and the oxygen content was between 8 and 9 mg L⁻¹.

2.2. Isolation of natural organic matter

NOM was isolated by reverse osmosis from Lake Schwarzer See (a brown-water lake, Brandenburg State, northeastern Germany) according to Serkiz and Perdue (1990). The NOM comprised 24% organic carbon; the chemical characteristics of Lake Schwarzer See water and its NOM isolate are given in Table 1. The DOC concentrations were determined by high-temperature combustion (Shimadzu TC 5000) after acidification with phosphoric acid to remove inorganic carbonates (DIN EN 1484, 1998). The DOC was separated by a liquid chromatography fingerprint-technique with simultaneous UV- and DOC-detection according to Sachse et al. (2001, 2005) and classified as follows: HS 87.5%; low-molecular-weight acids 9.2%, polysaccharides 2.6%, low-molecular-weight substances 0% and specific UV absorption of 5.1 L (mg m)⁻¹. Approximately 90% of the DOC was comprised of HS, and an equalization of DOC and HS appears justified.

2.3. Exposure to NOM

Static exposure experiments were carried out in triplicate in well-aerated tanks with volumes of 500 mL⁻¹. Ten individual amphipods were placed in NOM solutions; G. lacustris was exposed to NOM concentrations of 0.6 and 1.2 mmol L⁻¹ DOC, while G. tigrinus was exposed to 0.6 mmol L⁻¹ DOC only. All other experimental conditions were identical including the acclimation period. Both exposure concentrations lie well below the ambient DOC concentration in Lake Schwarzer See; hence, they are environmentally realistic. Exposure times were 0.5 h, 2 h, 6 h, 24 h, 3 days and 6 days. Survival rates were evaluated in 10-day exposure experiments with the 0.6 mmol L⁻¹ DOC exposure. After exposure, amphipods were frozen in liquid nitrogen. The control animals were kept in the aqueous medium without NOM additions, and were checked at the start, after 3 days and at the end of the experiment for the same parameters as the exposed individuals. We took into account only experiments with little standard deviation of the controls, which was usually the case. Furthermore, in the control amphipods, the activity of all enzymes, and other studied biochemical parameters did not change significantly over several weeks. Because the exposed amphipods were kept in the laboratory not longer than 9 days, the control data apply to the entire exposure time.

2.4. Hydrogen peroxide measurements, enzyme extraction and measurements, and lipid peroxidation

Cell internal concentration of H₂O₂ was calorimetrically measured according to Jana and Choudhuri (1982). Three antioxidant enzymes, catalase (CAT), peroxidase (POD) and glutathione S-transferase (GST), were extracted from amphipod tissues as described by Wiegand et al. (1999). CAT activity was determined after Aebi (1984), POD after Bergmeyer (1983) and GST after Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene as the substrate. To quantify the impact of NOM exposure and subsequent oxidative stress on the lipid components, the primary products of lipid peroxidation (LP), the conjugated dienes, were measured as described by Kolesnichenko et al. (2001). All measurements were made using five replicates of the three independent samples. The Student’s t-test was performed to compare the results. All results were compared with values in control groups. The statistical significance was tested at the 95% level (p<0.05). All figures show means and standard deviation.

3. Results

Upon NOM exposure, cell internal H₂O₂ concentrations increase and the amphipods respond by modulation of the oxidative stress defense enzyme activity. If
the internal antioxidant capacity is exhausted, lipid peroxidation (LP) occurs.

3.1. Hydrogen peroxide accumulation

During the 0.5–24-h exposures, H$_2$O$_2$ levels did not exceed the control levels; at day 3 and day 6 exposures, significant increases of H$_2$O$_2$ concentrations were observed (Fig. 1).

3.2. Oxidative stress defense enzymes, CAT and POD

CAT activities in *G. lacustris* upon NOM exposure are shown in Fig. 2a. Only slight changes in CAT activity were detected with minima at 2 h, 3 and 6 days of exposure was observed (Fig. 2b).

Fig. 1. Hydrogen peroxide concentrations in the cytosol of *G. lacustris* after 0.5- to 6-day exposure to Lake Schwarzer See NOM in environmentally realistic DOC concentration (1.2 mmol L$^{-1}$ DOC). *Denotes significant difference as compared to the control ($p<0.05$).

Fig. 2. Catalase activity in cytosol of (a) *G. lacustris* and (b) *G. tigrinus* after 0.5- to 6-day exposure to Lake Schwarzer See NOM in environmentally realistic DOC concentrations (0.6 and 1.2 mmol L$^{-1}$ DOC). *Denotes significant difference as compared to the control ($p<0.05$).

Fig. 3. Peroxidase activity in the cytosol of (a) *G. lacustris* and (b) *G. tigrinus* after 0.5- to 6-day exposure to Lake Schwarzer See NOM in environmentally realistic DOC concentrations (0.6 and 1.2 mmol L$^{-1}$ DOC). *Denotes significant difference as compared to the control ($p<0.05$).

Fig. 4. Glutathione S-transferase activity in the cytosol of (a) *G. lacustris* and (b) *G. tigrinus* after 0.5- to 6-day exposure to Lake Schwarzer See NOM in environmentally realistic DOC concentrations (0.6 and 1.2 mmol L$^{-1}$ DOC). *Denotes significant difference as compared to the control ($p<0.05$).
Exposure to NOM resulted in a two-stage response of POD activity in the amphipods (Fig. 3). In *G. lacustris*, the POD elevation occurred after 0.5 h, with the enzyme activity being five times higher than in the control even with the 0.6 mmol L$^{-1}$ DOC exposure. After 6 h, the enzyme activity returned to control levels. A second POD activity increase occurred after 24 h and keeps at the high level till the end of the experiment (Fig. 3a). *G. tigrinus* responded similarly (Fig. 3b), except with a little time delay: the first peaks occurred at 2 h and the second at 3 days.

### 3.3. Biotransformation with emphasis on GST

GST activities in *G. lacustris* are presented in Fig. 4a. In the 0.6 mmol L$^{-1}$ DOC exposure, significant increase in enzyme activity was observed only on the third day of exposure (Fig. 5a), while at the 1.2 mmol L$^{-1}$ DOC exposure, the significant increase was seen only at 6 h after the onset, and then declined to control levels during the subsequent exposure (Fig. 4a). In *G. tigrinus*, no NOM-mediated modulation of the GST activity was observed (Fig. 4b).

### 3.4. Lipid peroxidation

During the first 2 h of exposure, LP levels were not significantly different from the control levels; thereafter, from 6 h to 6 days, there was a successive significant increase in LP (Fig. 5). In *G. tigrinus* (Fig. 5b), exposure to 0.6 mmol L$^{-1}$ DOC also led to an increase of LP with a small second maximum after 6 h.

### 4. Discussion

From their behavior in electric fields, Münster (1985) postulated that HS must be able to penetrate through and interact with biomembranes. Furthermore, after studying detergents, also Visser (1985) concluded by analogy that HS interact with membranes. In the very recent years, dissolved humic-like material has been shown to be taken up by freshwater organisms (Steinberg et al., 2003). This uptake is facilitated by the apparently low molecular mass of the water-soluble and ionizable fraction (Hoque et al., 2003; Reemtsma and These, 2003, 2005; Cooper et al., 2004; Hatcher et al., 2004). Non-specific organic anion transporters and/or organic anion transporting polypeptides may be the responsible biostructures, because several of these transporters exhibit a broad and overlapping substrate specificity and are expressed in a variety of different tissues (e.g. Pizzagalli et al., 2002) and, hence, may be able to even transport HS. When coming into contact with the membrane and during penetrating it, NOM may cause adverse biophysical and biochemical effects, such as depolarization of the resting potential (Steinberg et al., 2004) or membrane oxidation (Timofeyev et al., 2004).

The present study shows that NOM from Lake Schwarzer See promotes oxidative stress in the *G. lacustris* and *G. tigrinus*. The data correlate well with those previously obtained in other freshwater amphipods species *Eulimnogammarus cyaneus* (Dyb.) and *Chaetogammarus ischnus* (Stebbing) exposed to NOM isolated from Sanctuary Pond (Ontario, Canada) (Timofeyev et al., 2004; Wiegand et al., 2004). In particular, our data show that exposure of amphipods to NOM of the brown-water Lake Schwarzer See results in a significant increase of internal cell H$_{2}$O$_{2}$ and LP, as shown with *G. lacustris*; most likely, *G. tigrinus* should have responded very similar, because all oxidative stress defense parameters are similar to those of *G. lacustris*. This confirms the intrinsic property of NOM (including HS) to promote oxidative stress. As an antioxidant response, POD and GST activities are simultaneously significantly increased in *G. lacustris*, at least during periods of exposure. The CAT activity is differently modulated in the two *Gammarus* species studied: namely decrease in *G. lacustris* and increase in *G. tigrinus* during exposures. Nevertheless, any kind of modulation shows that NOM induces oxidative stress in
the amphipods and that they respond to it. Recent findings with the nematode *C. elegans* clarify that an increase in antioxidant enzyme activity is not only due to the activation of the existing enzyme pool, but also to transcriptional regulation (Menzel et al., 2005).

We are aware that abiotic factors, such as temperature (Abele et al., 1998) as well as biological factors, such as age (Viaenigo et al., 1991a; Arun and Subramanian, 1998; Correia et al., 2003) and reproductive cycle (Viaenigo et al., 1991b) may confound NOM exposure effects. However, we assume that in our experiments these factors were subordinate to the effects of NOM exposure, because of a constant exposure temperature for all experiments, only small variation in the body size, and a good and well comparable nutritional status (food ad libitum). Furthermore, with roughly 90% saturation, the oxygen concentration was rather stable and probably did not cause an oxidative stress.

There are two potential pathways of ROS formation, the internal and the external one. The internal ROS formation and LP may start with the entry of NOM itself, or its low-molecular mass components into tissues and cells of the organisms (Münster, 1985; Steinberg et al., 2003), followed by an increase in ROS production. The regular source of ROS is aerobic respiration, but also microosomal cytochrome P450 metabolism of xenobiotic compounds (Blokaina et al., 2003; Vasseur and Leguille, 2004) and transition metals (Manzl et al., 2004; Krumschnabel et al., 2005). Although we cannot exclude the contamination even of remote HS sources (see MacDonald et al., 2005), there are at least three indications that HS themselves have the potential of xenobiotic chemicals:

1. HS have a variety of functional groups, which resemble xenobiotic chemicals. For instance, roughly 20% of the building block of HS are comprised by alkylaromatic compounds. With a DOC concentration in brown-water lakes of 10 mg L\(^{-1}\) DOC, as much as approximately 2 mg L\(^{-1}\) may be alkylaromatics, with nonyl- and octylphenols being prominent anthropogenic representatives of this class of compounds. Roughly the same figure applies for the even more reactive phenols and lignin monomers (Schmitt-Kopplin et al., 1998). Admitting that not all of these chemical building blocks are bio-available, the figures still clarify the predominance of the natural chemical compounds.

2. To evaluate the effect of natural as well as artificial, non-contaminated, HS sources on the transcription of biotransformation and antioxidant enzymes, microarrays of *C. elegans* have been applied. Interestingly, both sources clearly induced a peroxidase gene (Menzel et al., 2005). 3. With the exception of Fe and Mn, the content of transition metals appear natural (cf. Table 1).

Another possible mode of action for the ROS production, however, is their external formation by HS or its colloids, precipitated to the amphipod surface, with subsequent penetration of ROS through epithelia or membranes. Recent studies on the net release of ROS from a variety of irradiated HS show that most of the ROS produced are immediately quenched by the HS molecules themselves (Paul et al., 2005). We assume that the external oxidative stress may be small compared to internal oxidative stress. Initial experiments with giant cells of the charophyte *Nitellopsis obtusa* confirm this assumption (Steinberg et al., 2004). However, also contrasting reports are available: Farjalla et al. (2001) showed that external H\(_2\)O\(_2\) production can reach amounts as high as approximately 160 nM h\(^{-1}\) with *Phragmites australis* leachates; yet, no internal H\(_2\)O\(_2\) production has been measured in this study.

Irrespective of the site of ROS production, ROS actively influence the metabolism within the cells, with strong effects on the lipid components, leading to an increase in LP. In order to defend itself, the cell activates the antioxidant reaction mechanism, making use of POD activity for direct elimination of ROS, and GST activity to eliminate LP products. In both amphipods species studied, one can differentiate two main stages of antioxidant system activity upon the oxidative stress exposure. The first stage includes instant peroxidase activation and lasts until the period of enzymatic activity decrease. The period of activity decrease, which was observed at 6 h, following the multiple increases in the activity, is likely to be connected with the exhaustion of cell resources necessary for supporting a high level of peroxidase activity. The second stage is characterized by increased peroxidase activity and persists at the high level up to 3 days (in *G. lacustris*) or 6 days of exposure (in *G. tigrinus*).

Comparing LP level and enzyme activities in *G. lacustris*, one can note the following: the LP level remains as low as the controls for 0.5 h and 2 h, and begins to increase after 6 h of exposure in both concentrations; the GST also remains as low as the control and a significant increase is observed after hour 6 at high exposure concentration and after day 3 at low exposure concentration. Thus, during the first stage after the initial increase, the cells seem to resist the oxidative stress and keeps H\(_2\)O\(_2\) concentrations and LP at low
levels. The beginning of the second stage gives an impulse to \( \text{H}_2\text{O}_2 \) accumulation, increasing LP and, consequently, increasing destruction of membranes and the activation of GST. Hence, LP increase is the ultimate indicator that the cell eliminates the ROS. The findings correlate with published data on xenobiotics-induced decrease of antioxidant enzyme activity followed by the accumulation of \( \text{H}_2\text{O}_2 \) (Schutzendubel et al., 2001; Boominathan and Doran, 2003). Although \( \text{H}_2\text{O}_2 \) is less reactive than other ROS in cells, it can be the source of hydroxyl radical formation through the Fenton reaction. Hydroxyl radicals, in turn, initiate lipid peroxidation (Carubelli et al., 1990; Blokhina et al., 2003).

It has previously been shown that GST isoforms could be involved in the internal cell-antioxidant defense by elimination of some LP products (Berhane et al., 1994; Fukuda et al., 1997; Bruns et al., 1999; Vontas et al., 2001; Raza et al., 2002). Here, we also propose that in amphipods, this mode of action applies during NOM exposure. The GST may act as an antioxidant agent, increasing its activity together with the LP level.

The possible reasons for peroxidase activation, de-activation and re-activation in \( \text{G. lacustris} \) and \( \text{G. tigrinus} \) are not yet well understood. However, the efficiency of the peroxidase activity during the second increase was lower than during the initial increase. The evidence is that, at similar levels of POD activity at 3 h and 3 days exposure, the degree of lipid structures damages (seen through the LP and \( \text{H}_2\text{O}_2 \) accumulation levels) notably differ. A similar picture as in \( \text{G. lacustris} \) was detected in \( \text{G. tigrinus} \). The increase of POD activity is divided in two stages, but no significant changes in GST activity could be detected.

### 5. Conclusion

The NOM from Lake Schwarzer See had an adverse impact on the amphipods \( \text{G. lacustris} \) and \( \text{G. tigrinus} \) by increasing the internal concentration of ROS that might destroy cell membranes and leading to lipid peroxidation. The exposed organisms respond by modulation of their oxidative stress defense. The mechanisms behind the increase of internal ROS concentrations induced by the presence of NOM are not yet known; but the response mechanisms in the studied amphipod species appears to be similar. Both species have an apparent two-stage antioxidant mechanism. The first stage allows the organism to effectively eliminate ROS and to protect cells from damage. The second stage does not effectively combat the oxidative stress, \( \text{H}_2\text{O}_2 \) accumulates, with destruction of lipid structures in the cells. Finally, the second stage may result in functional damage or even death of the organism.

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