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Dissolved humic substances – ecological driving forces from the individual to the ecosystem level?^{*}

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^{*}We dedicate this paper to the memory of Profs. Robert G. Wetzel and Richard C. Playle, great pioneers in the field of humic substances ecology and physiology, and personal friends who died much too young.

Summary

1. This review focusses on direct and indirect interactions between dissolved humic substances (HS) and freshwater organisms and presents novel opinions and hypotheses on their ecological significance. Despite their abundance in freshwaters, the role of HS is still inadequately understood. These substances have been considered too large to be taken up by freshwater organisms. On the contrary, here we present evidence that dissolved HS are indeed taken up and interact directly and/or indirectly with freshwater organisms.
2. We show that dissolved HS exert a mild chemical stress upon aquatic organisms in many ways; they induce molecular chaperones (stress shock proteins), induce and modulate biotransformation enzymes, and modulate (mainly inhibiting) the photosynthetic release of oxygen by freshwater plants. Furthermore, they produce an oxidative stress, which may lead to membrane oxidation. Humic substances modulate the multixenobiotic resistance activity and, probably, other membrane-bound pumps. This property may lead to the increased bioaccumulation of xenobiotic chemicals. Furthermore, they can modulate the numbers of offspring in a nematode and feminise fish and amphibians. The ecological consequences of this potential remain obscure at present. Humic substances also have the potential to act as chemical attractants (as shown with a nematode).
3. In some macrophytes and algae we show that HS interfere with photosynthesis and growth. For instance, the presence of HS suppresses cyanobacteria more than eukaryotic algae. By applying a quantitative structure activity relationship approach, we

show that quinones in the HS interfere with photosynthetic electron transport. We show that even *Phragmites* leachate can act as a kind of phytotoxin. Humic substances also have the potential to suppress fungal growth, as shown with the water mould *Saprolegnia parasitica*, and force the fungus to respond by spore production.

4. In very soft, humic freshwaters, such as the Rio Negro, Brazil, HS stimulate the uptake of essential ions, such as Na and Ca, at extremely low pH (3.5–4.0) and prevent the ionoregulatory disturbance induced by acid waters, thereby enabling fish to survive in these environments.
5. We discuss whether or not HS are directly utilised by aquatic microorganisms or via exoenzymes which may be washed in from the terrestrial catchment. There is accumulating evidence that the quality of the HS controls microbial growth. In total, net-heterotrophy may result from HS-mediated suppression of primary production by the quinone structures and/or from HS-mediated support of microbial growth. Since there is also evidence that HS have the potential to support photoautotrophic growth and suppress microbial growth, the opposite community effect could result. Consequently, DOC has to be chemically characterised, rather than simply measuring bulk DOC concentration.
6. In sum, dissolved HS interact with freshwater organisms in a variety of ways in unenriched humic lakes. In addition to the well known effects of HS on light regime, for example, and the direct and indirect supply with carbon (energy), other interactions may be much more subtle. For instance, HS may induce internal biochemical stress defence systems and have the potential to cause acclimatisation and even adaptation. We are just at the beginning of understanding these interactions between dissolved HS and freshwater organisms.

Keywords: chemical stress defence, feminisation, natural herbicides, membrane irritation, dissolved humic substances

Introduction

Humic substances (HS) are complex organic molecules that make up most (50–80%) of the dissolved organic matter in freshwater ecosystems (Thurman, 1985; Wetzel, 2001). In oligotrophic freshwater ecosystems, with a DOC concentration between 1 and 100 mg L⁻¹, HS exceed the organic carbon in all living organisms by roughly one order of magnitude (Thurman, 1985; Wetzel, 2001; Steinberg & Münster, 1985; Steinberg, 2003). The concentration of DOC may occasionally be even greater, as in Brazilian coastal lagoons (~160 mg L⁻¹ DOC [Suhett *et al.*, 2004]) or Canadian wetlands (up to 300 mg L⁻¹DOC [Blodau *et al.*, 2004]), for instance. Jones (1998, 2005) emphasises that **all** freshwater systems contain some HS of allochthonous and at least autochthonous origin.

In clear contrast to the quantitative significance of dead organic carbon in oligotrophic freshwater ecosystems, our knowledge of its qualitative significance (ecological function) is still small. Only the role of HS as a direct or indirect external energy source has been studied intensely in the last two decades, after the energy budget calculation of a humic lake by Sarvala *et al.* (1981) and the pioneering mechanistic laboratory studies of Geller (1985, 1986) and Tranvik & Höfle (1987). However, the most important objects of limnological study are still the living organisms and their interactions with the ambient biotic and abiotic world. Evidently, there has been relatively little interest in the role of dissolved organic carbon (including HS) in the ecology of freshwater organisms.

Based mainly on experience from Scandinavian lakes with high humic content, Jones (1992) summarised the influence of HS on lacustrine planktonic food web, suggesting that HS might influence planktonic food chains in lakes in two ways: (1) by altering the physical and chemical environment and thus modifying autotrophic primary production and the dependent food web, and (2) by acting as a direct carbon and/or energy source to the food web.

Approximately ten years ago, Wetzel (1995) also commented: *‘Population fluxes are not representative of the material and energy fluxes of either the composite pelagic region or the lake ecosystem. Metabolism of particulate and especially dissolved organic detritus from*

many pelagic and non-pelagic autochthonous and from allochthonous sources dominates both material and energy fluxes. Because of the very large magnitudes and relative chemical recalcitrance of these detrital sources, the large but slow metabolism of detritus provides an inherent ecosystem stability that energetically dampens the ephemeral, volatile fluctuations of higher trophic levels. This statement is equivalent to the general designation of HS as 'entropy buffers' by Ziechmann (1994). Wetzel (1995) further explained that 'continued application of animal-orientated relationships to the integrated, process-driven couplings of the aquatic ecosystems impedes understanding of quantitative ecosystem pathways and control mechanisms.' Thomas (1997) expanded Wetzel's concept and wrote: 'The central dogma of the food web, and its implicit assumption that the energy flow in aquatic ecosystems can be quantified solely by measuring rates of photosynthesis, ingestion of solid food and its digestion by higher organisms, is invalid.'

Recently, Jones (2005) summarised the current state of ecological knowledge. Studies of humic waters provided the initial impetus for the realisation that many lakes are net heterotrophic; that is, the total respiratory production of CO₂ exceeds the fixation of CO₂ by primary production. The first evidence came from Finnish lakes (Sarvala *et al.*, 1981; Salonen *et al.*, 1992), but this phenomenon has been reported from many other humic lakes all over the world (e.g. del Giorgio & Peters, 1994; Cole *et al.*, 1994; Jansson *et al.*, 2000). These papers laid the foundations of an ecological understanding of dead organic matter in freshwater, with emphasis on energy flow and the utilisation by microbes of dissolved organic carbon compounds after the latter's photodegradation. However, these previous views do not consider HS as natural chemicals. In freshwater systems, the majority of HS derive from terrestrial plant debris, lignins (cumaryl alcohol, coniferyl alcohol and sinapyl alcohol) and terpenes being the main source material. All of these alcohols are basically polyvalent alkylphenols, i.e. they are natural environmental chemicals in the aquatic environment (Steinberg *et al.*, 2003). With their functional groups, they are able to react with many chemicals, be it a compound in a water column or in an organism. From studies of the electromobility of HS, Münster (1985) was the first to postulate the ability of HS to interact with biomembranes. Subsequently, when studying the potential toxicity of acidified waters, Petersen & Persson (1987) attributed to protonated, lipophilic HS the ability to be the most toxic fraction of humic material. Furthermore, Visser (1985) speculated on the interaction of HS with biomembranes. He described how low humic concentration could strongly stimulate, and high concentration inhibit, microorganisms – a "hormesis" effect (see Glossary). He explained this in terms of interactions with membranes and subsequent increased permeability to essential materials. Visser (1985) concluded that the effect of fulvic acids (FA) was analogous to that of laboratory surfactants (such as Tween 80). Empirical evidence, however, was only presented much later (Wang *et al.*, 1999; Steinberg *et al.*, 2003).

Organisms exposed to chemicals have several stress response reactions in common, which are almost identical from bacteria to vertebrates. This indicates that these reactions must have developed very early during the evolution of life on Earth. One chemical trigger of this development may have been primordial HS-like substances, which are likely to have developed very early during biological and chemical evolution (Miller 1955; Bada & Lazcano, 2000) or even completely abiotically (Ziechmann 1994, 1996; Kissel *et al.* 2004).

This review of the ecology of humic substances will focus mainly on various interactions of HS with freshwater organisms and whether these interactions could have an influence at the ecosystem scale. In addition to the view of HS as an energy/carbon source, we want to call attention to an emerging view that HS may be mild chemical stressors. We will emphasise the individual level of organisation, since

a) Any adaptation of a species to its environment starts at this level,

b) This kind of interaction between individuals and their biogeochemical environment has been almost totally ignored in textbooks of freshwater ecology. We also formulate hypotheses on the long-term role of HS-organism interactions with the aim of stimulating further, novel empirical research.

Uptake of Humic Substances

The question as to whether or not HS are taken up by organisms has been argued intensely in the literature. Currently, empirical evidence is accumulating that HS are indeed taken up. In a cell culture study, Wang *et al.* (1999) showed that HS, or at least fractions thereof, were found inside the cells and even in the DNA. More recently, Nardi *et al.* (2002) showed that the physiological effects of HS on terrestrial plants depend on the source, concentration and molecular mass of the HS. The authors presented evidence that HS <3.5 kDa easily pass the cell membrane of higher plant cells and were taken up. Steinberg *et al.* (2003) presented evidence that ¹⁴C-labelled HS-like substances (caffeic acid oxidation products) were taken up and bioconcentrated by freshwater organisms. The macrophyte *Ceratophyllum demersum* L., the invertebrate *Gammarus pulex* (L.), and a vertebrate, tadpoles of the moor frog *Rana arvalis* Nilsson, were able to bioconcentrate considerable amounts of ¹⁴C in their bodies during short-term exposures. Recently, similar results were obtained with ¹⁴C-labelled natural organic matter (NOM) from compost and the waterflea, *Daphnia magna* Straus (Wiegand, Humboldt-University, pers. comm).

Arguably, it is not the intact caffeic acid oxidation product or NOM, but smaller (photo)-degradation products, which account for the bioconcentration of ¹⁴C. Low-molecular mass products of the caffeic acid oxidation products may be in the molecular mass range of <1.0 kDa and include the molecular masses of fulvic acids (FA) in freshwater ecosystems: Very recent environmental-chemical studies consistently show that water-soluble and ionisable HS are relatively small by molecular weight (~0.5 kDa) and very regularly structured (Hoque *et al.*, 2003; Reemtsma & These, 2003, 2005; Cooper *et al.*, 2004; Hatcher *et al.*, 2004; Seitzinger *et al.*, 2005). This water-soluble and ionisable fraction of the HS may serve as building blocks of the larger associations that are often reported from freshwater studies. They seem to be built up by polyvalent cation bridges (Cooper *et al.*, 2004; Hatcher *et al.*, 2004), ester bonding or hydrogen bridges (Anja These, Technical University Berlin, pers. comm.). In general, it is not surprising that some freshwater organisms take up HS. Recent results show that HS may be actively taken up via transporter protein, because in the threadworm *Caenorhabditis elegans* Maupas the exposure to HS caused an up-regulation of a gene with a product similar to an aromatic amino acid transporter (Menzel *et al.*, 2005). Even if the water-soluble and ionisable proportion is small, it appears to be quantitatively sufficient to explain the effects described below.

Interaction of humic substances with organisms

Once taken up, HS are able to migrate to organs or organelles and may provoke stress response reactions (Steinberg *et al.*, 2003). They have non-specific as well as specific effects. Non-specific effects are physical and chemical membrane irritation, induction and modulation of biotransformation activity, induction of chemical defence proteins (stress proteins, such as HSP70), and the development of internal oxidative stress by creating free radicals and reactive oxygen species (ROS) with subsequent lipid oxidation and induction of ROS defence enzymes. So far, specific effects comprise reduction of the photosynthetic oxygen production, oestrogenicity or chemical attraction. Very preliminary results with *Xenopus laevis* (Daudin) hepatocytes even show that apoptotic (programmed cell death) reactions may be induced upon exposures to HS (Zhang & Steinberg unpublished). Apoptosis may be considered as a cancer defense mechanism and has very recently also been observed with human cells upon exposure to HS (Cheng *et al.*, 2003; Hseu *et al.*, 2002; Yang *et al.*, 2004). These findings even attribute

anti-carcinogenic properties to HS. In this contribution, we follow very briefly the pathway from extracellular sources to signal transduction and transcription, present some representative results and try to put them into a conceptual framework of the ecological importance of dissolved HS.

Interactions with membranes

The giant cell of *Nitellopsis obtusa* (Desvaux) Groves and other charophytes has been shown to be a highly suitable model for studying the electrical characteristics and ion-transport phenomena of plant membranes under normal as well as under stress conditions (Manusadžianas *et al.*, 1999, 2002). For the interaction of chemical stressors with living systems, the cell membrane is the very first sensing site, from which chemical or physical signals are redirected toward a target site through a multilevel network of cellular reactions. Changes in membrane bioelectrical parameters may serve as a key indicator of any adverse impact on the cell. Manusadžianas *et al.* (2002) showed that the depolarisation of the resting potential of *N. obtusa* cells could be used as a reliable parameter for assessing a variety of chemical stresses. Steinberg *et al.* (2004) employed this method and found a clear NOM-concentration-dependent depolarisation of the membrane.

Exposed to HS, biomembranes are not only physically irritated, but also chemically affected. If the internal pool of reducing agents (mainly glutathione, ascorbic acid, α -tocopherol) is exhausted, membrane peroxidation will occur. One example is given in Fig. 1 with amphipods (Timofeyev *et al.*, 2006). The underlying mechanism may be the external release of reactive oxygen species (ROS) from illuminated dissolved HS and their subsequent uptake, as well as the internal production of ROS. Internal NOM is processed like a xenobiotic chemical (*i.e.* non-native compound) with ROS as by-products¹. The mechanisms are by no means mutually exclusive; rather they may reinforce each other. Recent results show that there appear to be clear differences in susceptibility towards lipid peroxidation between amphipods from Lake Baikal and those from the Palaeartic, whereby the Baikalian species seem to resist exposure to NOM better than the Palaeartic ones (Timofeyev *et al.*, 2006), although the underlying mode of action and any evolutionary benefits remain obscure.

Membrane protective effects in extremely soft waters

In the extreme environment of Amazonian blackwaters, HS even may have beneficial effects. Humic substances have been reported to protect fish against ionoregulatory effects induced by low pH. Numerous streams along the Rio Negro have pH as low as 3.0, which should render them fishless. However, the great diversity of fish in the Rio Negro (>1,000 species; Chao, 2001) indicates that the fish of these blackwaters are highly adapted to extreme conditions and, in particular, to low pH and low Ca^{2+} concentration (for reviews, see Matsuo & Val, 2003; Gonzalez *et al.*, 2005). Wood *et al.* (2003) reported that HS from the Rio Negro prevented ionoregulatory disturbance in freshwater stingrays (*Potamotrygon* spp. Garmann) exposed to pH 4.0. Peat bog HS (Aldrich humic acid) did not display similar protective effects for stingrays, probably because of the differences in the geochemical nature of the commercial HS, and because the fish were not fully acclimated to Aldrich humic acid. There seem to be major differences in the results of studies on HS in freshwater organisms, particularly when the exposure regime to HS is acute or chronic. For example, Matsuo *et al.* (2004) noticed that acute exposure of rainbow trout *Oncorhynchus mykiss* (Walbaum) to HS induce increased ion loss, probably because of a surfactant effect (Thurman, 1985; Visser, 1985) induced at the gill membrane level, or because Ca^{2+} is stripped out of the paracellular tight junctions. However, when the tetra *Paracheirodon axelrodi* (Schultz) was chronically exposed to HS, the fish displayed increased branchial ion uptake rates and also reduced ion loss from the gills (Matsuo *et al.*, unpublished). The reduced ion loss may be facilitated by the down-regulation of genes encoding for cation-transporting ATPases as shown with *C. elegans* (Menzel *et al.* 2005). In addition to this protection from ionoregulatory damage, there are some more 'beneficial' effects of HS to fish in extremely soft waters. For instance, in these waters, metal toxicity in

fish is expected to be most severe, because of the low availability of hardness cations, particularly Ca^{2+} , to out-compete the metal ions for gill binding sites (Playle *et al.*, 1993). Recently, Matsuo *et al.* (2005) showed that previous acclimation of the fishes to concentrations of HS ranging from 20 to 80 mg L⁻¹ C prevented Cu and Cd accumulation at the gills of *Colossoma macropomum* (Cuvier). This suggests that long-term exposure of fish to HS induces changes in gill binding properties, resulting in a branchial physiological conditioning effect that may be beneficial in protecting against water-borne metals. Exposure of *O. mykiss* (Matsuo *et al.*, 2004) and *C. macropomum* (Matsuo *et al.*, 2005) to Cu in the presence of HS also proved to be very effective in protecting against acute exposure in soft water, given the known complexation effects that occur between metals and HS in the water column.

Chemical stress defence reactions

As a clearly non-specific response to chemical stress, chemical defence proteins (stress proteins, such as HSP70, better known as *heat shock proteins*) have been expressed in all the freshwater animals studied so far (fish and invertebrates; Wiegand *et al.*, 2004; Timofeyev *et al.*, 2004). Environmentally realistic, or even lower, exposure concentrations of NOM induce increased HSP70 concentration in the organisms. Figure 2 shows the increased HSP70 concentrations in two gammarid species upon exposure to a Canadian NOM source at low concentrations.

All organisms have the means to rid themselves of chemical burdens (exotic food chemicals, xenobiotics etc.), i.e. they have developed so-called biotransformation pathways. Also HS behave like chemical clues in the biotransformation pathway.

Since HS possess a variety of functional groups, we assume that the Phase II enzymes of the biotransformation system, in particular, are subject to modulation upon HS exposure (Steinberg *et al.*, 2003; Timofeyev *et al.*, 2004; Wiegand *et al.*, 2004). However, biotransformation capacity may become exhausted upon exposure to HS, with fatal consequences. Among others, molluscs appear to be very sensitive. For instance, a relatively high proportion (10–20%) of a sample of European freshwater snails, *Lymnea stagnalis* L., died after exposure at 0.5 mg L⁻¹ DOC of Suwannee River FA (Steinberg, 2003). These FA are known to contain a high moiety of aromatic building blocks (<http://www.ihss.gatech.edu/>). Also, one of the European freshwater pearl mussels (*Margaritifera margaritifera* L.) declined significantly after forestry in the catchment changed from deciduous to coniferous trees (Björk, 2004). We assume that the changes to the forest led to HS with higher aromatic moieties that supported less microbial growth, and also probably had a greater direct impact on the mussels, than HS from deciduous or mixed forest. From a biochemical perspective, these field studies were substantially supported by the fact that in *M. margaritifera* specimens exposed at circum-neutral pH, biotransformation enzymes, such as glutathione S-transferase and antioxidant enzymes, were extremely high (Steinberg, unpublished) when compared to other animals tested so far: *Lymnea stagnalis* (unpublished), gammarids (Timofeyev *et al.*, 2004, in press, b), *Daphnia* (Meems *et al.*, 2004), common carp (*Cyprinus carpio* L.) yearlings (Wiegand *et al.*, 2004), and zebra fish (*Danio rerio* Hamilton) embryos (Cazenave *et al.*, 2006). Due to the lower food quality of the HS from coniferous forest, the energy spent on its biotransformation could not be compensated for and the animals died. This assumption gets very recent support, because Cazenave *et al.* (2006) showed that the physiological response to NOM incurred energetic costs, which reduced the energy resources of the yolk of the zebra fish embryos, which in turn might affect their normal development: Exposure to non-contaminated HS (NOM from Lake Schwarzer See) also led to teratogenic effects within 48 h of exposure (Cazenave *et al.*, 2006). These observations are in agreement with a previous study of carbohydrate metabolism in perch in humic waters, which found that the adaptation of fishes to these environments was accompanied by a slight decrease in their lipid content (Meshcheryakova *et al.*, 2004).

Beside Phase II, Phase I enzymes are also induced and modulated. For instance, ongoing studies (Matsuo *et al.*, in press) find induction of CYP1A by HS. The induction was

concentration-dependent. These findings suggest that there may be as yet unknown CYP1A inducing components (aryl hydrocarbon receptor agonists) in HS.

In addition to the biotransformation pathway, a new principle for handling toxins and xenobiotic chemicals has arisen. Organisms possess the so-called multi-xenobiotic resistance transporter (MXR). This is a membrane-bound group of P-glycoproteins, which act as a pump against xenobiotics and/or their metabolites (Kurelec, 1992; Smital *et al.*, 2004). The MXR pump can be blocked or even inhibited by so-called chemosensors. Chemosensitisation of the MXR defence system could cause increases in intracellular accumulation and subsequent toxic effects of xenobiotic chemicals and heavy metals otherwise effluxed by MXR transport proteins. So far, many xenobiotics and also some natural products, particularly biomolecules from marine algae, have been found to act as chemosensors. In several studies, we tested a NOM isolate (Lake Schwarzer See, Brandenburg State, Germany) with respect to its capability to act as chemosensor, as exemplified with *Eulimnogammarus cyaneus* (Dyb.) (Fig. 3). It is evident that the presence of HS increased the residual concentration of rhodamine B in this amphipod (rhodamine B is an easily detectable model xenobiotic compound used in MXR studies). The results indicate that the presence of HS modifies the MXR activity. This chemosensitisation depends on both exposure concentration and exposure time.

The result above may also answer other questions in ecochemistry. In their review, Haitzer *et al.* (1998) found that seven out of 27 studies reported an increase in bioconcentration of xenobiotics when the animals were co-exposed to HS at relatively low DOC concentrations. Although several hypotheses were discussed by Haitzer *et al.* (1998) and Steinberg (2003), none appears to be convincing and a subsequent experimental verification failed (Haitzer *et al.*, 2001). The chemosensitisation of the MXR pump by dissolved HS has not been discussed, however, and seems to be a plausible mechanism.

The same mechanism may also apply to metal bioconcentration. For instance, John *et al.* (1987) showed that the Cd uptake by Atlantic salmon (*Salmo salar* L.) is strongly dependent on the concentration of HS with a pronounced maximum at low (2.7 mg L^{-1}) DOC concentrations. High HS concentrations reduce the bioconcentration of Cd. Reduction in bioconcentration has also been observed with zebrafish eggs by Burnison *et al.* (in press). Again, chemosensitisation of the MXR efflux by lower HS concentrations, which leads to elevated internal Cd concentrations, may be one potential mechanism. Furthermore, it is also likely that the prevention of ionoregulatory disturbance in extremely soft waters (mentioned above) is also facilitated by chemosensitisation of ion channels.

Most exposures of freshwater organisms to HS are mild, sometimes even beneficial, chemical stresses rather than lethal to the freshwater organisms exposed. For instance, exposure of young swordtails (*Xiphophorus helleri* Heckel) to synthetic HS significantly enhanced their growth as compared to the controls (Meinelt *et al.*, 2004). Furthermore, after two-weeks of very stressful handling, the HS-exposed fishes recovered quickly whereas growth stagnated in the controls. **The feminising potential is not restricted to the artificial HS1500 and the swordtail, because this potential has also been proven with fertilised eggs of the common carp and various HS isolates in the meantime (Meinelt, unpublished).**

It is essential to note that there is increasing awareness that mild chemical stresses (in the so-called hormetic dose or concentration range) may even be beneficial at both the individual (Calabrese, 2005; Calabrese & Baldwin, 2003; Le Bourg, 2003) and population levels (Rattan, 2004). In general, mild chemical stress to individuals means training of the chemical defence system (biotransformation enzymes, anti-oxidant enzymes, stress proteins). Humic substance-related hormetic effects have been observed with the activity of Phase I and II enzymes (Timofeyev *et al.*, 2004; Meems *et al.*, 2004), *Daphnia* behaviour (Baganz *et al.*, 2004) and the number of offspring of *C. elegans* (Höss *et al.*, 2001, Steinberg *et al.*, 2002). This means that exposure to low concentrations of HS may contribute to the “training” of the defence system, and even more complex individual reactions. This notion becomes more attractive, and opens novel opportunities for ecological research, if one puts it into the wider context of stress research. In a pioneering review, Minois (2000) stated that the training of the defence system may lead to stress resistance which may be related to longevity. This has so far been proven effective in many animals (*e.g.*, rotifers, *C. elegans*, spiders, fishes, rodents, and the fruit fly

Drosophila melanogaster Meigen). More recently, Murphy *et al.* (2003), Morrow *et al.* (2004), Gems & McElwee (2005) and others identified key molecules involved in longevity. Pro-longevity genes include some that encode antioxidant enzymes and biotransformation enzymes, and others encoding heat-shock proteins, particularly of low molecular weight (HSP22). With the exception of the small-weighted HSP, genes of all enzyme families have been found upregulated in *C. elegans* after exposure to natural and artificial HS (Menzel *et al.*, 2005).

In addition to the chemical stress-hypothesis, another mechanism for life-span expansion may apply: the activation of the sirtuin family of deacetylases by small molecules (Howitz *et al.*, 2003; Lamming *et al.*, 2004). The activation of these deacetylases mimics caloric restriction which itself is known to slow down ageing in yeast and metazoans. Interestingly, a class of polyphenolic molecules produced by plants in response to stress, such as resveratrol, butein, piceatannol, quercin, can activate the sirtuins (Howitz *et al.*, 2003). These polyphenols are very similar to lignin constituents and, even more, to tannins which are major compounds in HS (Leenheer & Rostad, 2004).

Furthermore, it is interesting to note and discuss that the antioxidant response upon HS exposure is similar to the response of many invertebrates to parasite infections: development of a kind of oxidative stress (Wilson *et al.*, 2001; Mucklow *et al.*, 2004; Newton *et al.*, 2004). Since parasite infestation may induce micro-evolution in the host species (Capaul & Ebert, 2003; De Bruin *et al.*, 2004), it is open to question whether or not even the more subtle chemical stress of HS exposure also has the potential to initiate micro-evolution in freshwater plants and animals.

Interference within photosynthetic oxygen production in macrophytes and algae

From recent environmental and microbial studies, it is evident that HS have the potential to act as electron acceptors for microbial respiration (Lovley *et al.*, 1996; Scott *et al.*, 1998). Having studied a variety of bacteria with completely diverse phylogeny, Coates (pers. comm., Boston Humic Substances Seminar VII, March 2004) concluded that the ability of bacteria to utilise HS as a redox catalyst must be a very ancient feature in evolution. This statement supports our view of HS as a chemical clue for the evolution of certain biotransformation properties in organisms.

One may hypothesise that HS, once taken up, should interfere within all electron transport reactions in organisms or cells. To test this hypothesis, we exposed several freshwater plants and cyanobacteria to different HS sources and measured the photosynthetic oxygen release. Prior to photosynthesis measurement, the plants were transferred into HS-free media to exclude light quenching effects. Suwannee River NOM, a forest soil leachate FA, and the synthetic HS1500, all significantly reduced photosynthetic oxygen release by the green coccal alga *Scenedesmus armatus* (Chodat) GM Smith (Fig. 4). All other photoautotrophic organisms tested responded similarly.

An ongoing comparison of terrestrial and macrophyte-derived leachate revealed that leachate of *Phragmites communis* (Cav.) Trin. ex Steud has a high potential to reduce the photosynthetic oxygen release in the coontail, *C. demersum* (Fig. 5). To the best of our knowledge, this is the first evidence that, during decay, *Phragmites* releases toxic compounds which interfere within the photosynthesis of other macrophytes. The adverse effect of *Phragmites* leachate is even stronger than that of oak (*Quercus rubor* L.), a plant well known for its high tannin content.

By applying a Quantitative-Structure-Activity-Relationship (QSAR) technique, it has been shown (Paul *et al.*, 2003) that stable radicals, which are indicative for quinone structures in HS, may statistically count for approximately 83 % of the reduction in oxygen release (Fig. 6). HS may directly quench electrons or bind to the bioquinones in photosystem II (PS II) and thereby block electron transfer. Both mechanisms may be valid, although the latter appears more likely, since Pflugmacher *et al.* (2006) found that PS II was most affected. PS II is responsible for the cleavage of water and the subsequent release of molecular oxygen. Furthermore, tellimagrandin II, a hydrolysable polyphenol (tannin) produced and released by *Myriophyllum* spp. (Gross, 1999), inhibits oxygen release in microalgae. Leu *et al.* (2002)

showed that the target site of this inhibitor is different from the Q_B-binding site, a common target of commercial herbicides such as chlorinated urea compounds. It is very likely, but requires confirmation, that dissolved humic substances, which have several similarities to tannins, provoke the same mode of action as tellimagrandin II.

Another mode of action, internal oxidative stress, may also apply. Like any xenobiotic chemical or heavy metal, internal HS may produce an oxidative stress (for a review, see Blokhina *et al.*, 2003). This has already been proven with various *Gammarus* species (Timofeyev *et al.*, 2006, in press). In plants, increasing exposure concentrations of HS leads to increasing oxidative stress and the subsequent reduction of photosynthetic efficiency (Blokhina, 2000). Future studies will reveal which mode of action is prevalent and whether or not the two mechanisms are interrelated.

Internal H₂O₂ could be derived from biotransformation activity, and by uptake of externally produced H₂O₂ after irradiation of dissolved HS (Scully *et al.*, 1996; Paul *et al.*, 2004). For instance, UV-irradiation on leachates of *P. australis* and *Hydrocharis morsus-ranae* L. show that external H₂O₂ production can be up to 160 nM h⁻¹ (with *P. australis* leachate) (Farjalla *et al.*, 2001). H₂O₂ is relatively stable and easily penetrates biomembranes (Blokhina, 2000) and, hence, the site of its production is relatively unimportant.

When comparing the oxygen release of all three freshwater plants tested so far (Fig. 7), it is evident that there was no 'most sensitive' species: With Suwannee River NOM, the most sensitive species was the coccal green alga *S. armatus*; with the soil FA it was the angiosperm *C. demersum*; and with the synthetic HS1500 it was the tropical water moss *Vesicularia dubyana* (C. Müller). Consequently, the dictum of the absence of a 'most sensitive species' (Cairns, 1986) does not only apply to man-made chemicals (ecotoxicology), but applies more generally to other environmental stressors and species tolerance.

From an ecological point of view, this means that a given terrestrial plant cover results in HS with particular chemical properties (such as a characteristic ratio of aromatic to aliphatic moieties and content of stable radicals), and will, under oligotrophic conditions, induce a specific guild of primary producers which are tolerant to the exposure to these HS qualities and quantities. This aspect of HS ecology should be tested in field studies.

As initial support for this assumption that HS have the capacity to structure primary producers guilds, we found clear differences in sensitivity to HS between one coccal green alga and one coccal cyanobacterium. We compared *Chroococcus minutus* (Kützing) Nägeli and *Desmodesmus communis* (Hegewald). The hypothesis was that cells that lack internal, membrane-bound compartments would be generally more sensitive to HS than cells possessing internal, membrane-bound compartments. Both species were exposed to a variety of HS from Scandinavian sources under identical conditions (for HS sources, see Vogt *et al.*, 2004) with exposure concentrations of □0.3 and □1.5 mg L⁻¹ DOC. The cyanobacterium was more sensitive to all HS material than the coccal green alga. One representative example is presented in Figure 8, displaying the responses of both species upon exposure to Hietajärvi NOM. Even the lowest exposure concentration (□0.3 mg L⁻¹ DOC) reduced photosynthetic activity and population growth in the cyanobacterium.

There have been several attempts to combat cyanobacterial growth by adding, to the culture medium or into enclosures, cheap natural phenolic compounds, such as leachates from barley straw, rice straw and leaf litter, and many have been successful (Welch *et al.*, 1990; Ridge & Pillinger, 1996; Ridge *et al.*, 1999; Imai *et al.*, 1999; Dr H.-M. Oh, Korean Research Institute of Bioscience & Biotechnology, Daejeon, South Korea, pers. comm.). Interestingly, with 2 mg L⁻¹ fulvic acid, approximately 1 mg L⁻¹ DOC, Imai *et al.* (1999) also applied relatively low concentrations of the water-soluble HS very successfully. **Very recently, Sun *et al.* (2005)**

reported that even 0.01 mg L⁻¹ C of a commercial HA has been effective in slightly reducing the cell yield of *Anabaena circinalis*. A complete suppression of cell growth, however, was achieved with HA concentrations ≥1mg L⁻¹ C. In a subsequent paper, Sun *et al.* (in press) elucidated the underlying mechanism. The authors found that light is an important factor for the cell lysis, whereas intracellular H₂O₂ might be the chemical factor for this process. Furthermore, the results indicated that HA or its possible photodegradation product(s) can

induce damage to intracellular catalase, which leads to intracellular H_2O_2 to be accumulated to an abnormally high concentration, eventually resulting in cell death.

The sensitivity of cyanobacteria to HS may also explain patterns in the field. Thus, eutrophic humic-rich lakes do not support the cyanobacterial blooms characteristic of eutrophic but humic-poor lakes. In the humic-rich systems, raphidophytes are more common, such as *Gonyostomum semen* (Ehrenberg) Diesing (Cronberg *et al.*, 1988; Hehmann *et al.*, 2001; Willén, 2003; Findlay *et al.*, 2005). Thus, cyanobacteria appear to be unable to use their accessory pigments (particularly phycocyanin) to exploit the reddish light prevailing in humic-rich lakes (Eloranta, 1999).

There are not only significant differences in the sensitivities of prokaryotes and eukaryotes, when exposed to the same humic source, but also between closely related algae. Very recently, Karasyova *et al.* (in press) detected different sensitivities of *Monoraphidium minutum* (Nageli) Kom.-Legn. and *M. convolutum* (Corda) Kom.-Legn. Both of these coccal greens were exposed to identical concentrations of Suwannee River NOM. Comparing control and exposed algae, *M. minutum* showed a significant decrease in cell yield, whereas in *M. convolutum*, there was a growth promotion at day 4 (Fig. 9). These results confirm our statement above that HS have the potential to structure guilds, here the primary producer guild.

All these macrophytes, algae and cyanobacteria were exposed to exotic HS. One might expect that the effects would be less adverse if the primary producers were exposed to their local NOM, because the organisms would have been able to develop adaptations to compensate for the potential adverse effects. In an unpublished study, we exposed *M. convolutum*, isolated from the humic-rich part of the artificially divided Lake Große Fuchskuhle (Brandenburg State, Germany), to NOM isolated from the same habitat by reverse osmosis. Lipid peroxidation was chosen as a response parameter in *M. convolutum*. Up to the ambient DOC concentration (12 to 14 mg L⁻¹ DOC; Sachse *et al.*, 2001), the anti-oxidant systems in *M. convolutum* eliminated the ROS, and the lipid peroxidation was low. Higher than ambient concentrations significantly increased lipid peroxidation. This is another indication that allochthonous humic material controls the composition of the primary producer guild.

Chemical attraction in *Caenorhabditis elegans* (Maupas)

In addition to all these non-specific and specific effects, HS may function as environmental signalling chemicals. With the soil- and sediment-dwelling nematode *C. elegans*, oligonucleotide-based whole genome DNA microarray experiments were performed with control and HS treated worms. Notable transcriptional changes were identified in chemosensors and olfactory receptors. Humic substances from different sources have the potential to act as environmental signals. The adult worms migrated towards the humic material. This appears to be a genetically fixed property of *C. elegans*; although bred in humic-free laboratory cultures for decades, *C. elegans* still prefers humic-containing rather than humic-free environments. This finding definitely points to the ability of organisms, strains or even species not only to tolerate the potential mild chemical stress of HS, but actively to select their habitat on the basis of its HS content and probably also HS quality (Menzel *et al.*, 2005). An effect of HS on habitat choice would also infer a possible role for HS in determining community composition.

Potential strategies against the adverse effects of HS

At present, there are only few examples of how organisms may overcome the adverse effects of HS. Spores of the water mould, *Saprolegnia parasitica* Coker, were exposed to several NOM isolates. Artificial and natural humic material significantly reduced the colony growth of the parasitic fungus in most but not all cases, NOM from Lake Schwarzer See being an exception (Fig. 10). Furthermore, the reduction in colony growth was concentration-dependent with higher concentrations being most effective. However, the parasitic fungus did not die, but developed sporangia, which may survive the HS exposure.

Larger-scale ecological significance of HS and some remaining questions

Compared to our knowledge of the direct effects of HS on freshwater organisms, much more is known about the more indirect effects of HS. One of the most pronounced indirect effects is the input of biochemically fixed energy from allochthonous sources. This provides much of the energy to aquatic communities from their terrestrial surroundings. This can be seen most clearly in running waters but also evident in oligotrophic freshwater lakes. Organic carbon from terrestrial sources delivers energy and nutrients to freshwater bacteria. This energy is then transferred to zooplankton and fish. The freshwater microorganisms therefore demonstrate the link between the terrestrial primary production and freshwater secondary production (Jones 1992; Cole, 1999; Cole *et al.*, 2001). Fixed C is made available to microorganisms in freshwaters through the action of light on the chromophoric part of HS. The first clear evidence of oligotrophic lakes being net-heterotrophic (respiration exceeds primary production by algae and macrophytes) was presented by Salonen *et al.* (1992) and del Giorgio & Peters (1993). In nutrient-rich lakes, the autochthonous production dominates. In his recent review, Jones (2005) discussed the hydrological and climatic conditions and also the food web structures, that lead to the net-heterotrophy prevalent in humic lakes. Furthermore, he showed a high reliance by zooplankton on allochthonous carbon in a wide range of lakes (Jones 2005 and references therein).

It is well accepted that the exploitation of dissolved carbon from terrestrial sources may involve photolysis and the subsequent microbial uptake of the short-chained mono- and dicarboxylic and amino acids that are released (*e.g.*, Geller, 1985; 1986; Tranvik & Höfle, 1987; Tranvik 1988, 1992; Wetzel *et al.*, 1995). Microbial enzymes may also attack on the carbon skeleton of the HS directly. Münster *et al.* (1998) were the first to test for a non-specific peroxidase in a humic lake and emphasise that enzymes which directly attack the C skeleton of HS, rather than the side chains, evidently play a fundamental role in making the carbon in HS accessible. Similar results were reported from Lake Fuchskuhle (Fig. 11). This lake has artificially been divided into four, and one quarter (the SW basin) receives seepage water from an oligotrophic bog. It is evident that peroxidase activity was elevated in the latter basin. Interestingly, the highest activities were found during autumn and winter, when there is the highest inflow of surface and seepage waters. Because the search for freshwater microorganisms responsible for this peroxidase activity has so far failed, Burkert *et al.* (2003, 2005) concluded that it is possible that the peroxidase is washed in from the catchment during the high flow periods, as were the HS. This would mean that oligotrophic lakes and their metabolism depend on their catchments in two ways: (1) C and energy is washed in as HS and (2) the enzymes necessary to degrade these substances are also imported from the terrestrial environment.

Most of the bioavailable organic carbon is released after photolytic degradation of dissolved organic material rich in conjugated double bonds (humic material). However, there is accumulating evidence that microbial consortia have the capability to utilise directly humic material in laboratory cultures of algae and bacteria (Steinberg & Bach 1996), in tropical waters (Amado *et al.* 2006; Farjalla *et al.*, in press b), and in leachates from macrophytes from tropical lagoons (Farjalla *et al.*, 2001). The last study shows that microbial production in the dark may be quantitatively comparable to, or even exceed, the bacterial growth on photochemically modified leachate. Further comparative studies will indicate which pathway prevails under which conditions.

In his review, Jones (2005) points out the rapid growth of interest in ecological stoichiometry in relation to the regulation of key processes in food webs. Where the productivity of organisms is now invariably assessed in terms of carbon (as a surrogate for energy), sustained growth of organisms requires more than carbon and may be regulated by the supply of nutrients such as P and N. For example, due to the different stoichiometric composition of phytoplankton (carbon-rich) and bacterioplankton (phosphorus-rich), biomass production in Lake Öträsket was higher when the edible plankton was dominated by phytoplankton than by bacterioplankton (Drakare *et al.*, 2002). As the productivity of bacterioplankton and phytoplankton was nutrient limited in Lake Öträsket, a probable explanation was that the cell

C:N:P ratio permitted phytoplankton to produce more biomass than bacteria per unit available N or P.

Also, the quality of HS plays a crucial role in the nutrition of planktonic microorganisms. Farjalla *et al.* (2002) concluded that low phosphorus concentration and low quality of the bulk DOC were the main factors limiting bacterial growth in a set of tropical coastal lagoons. More recently, Farjalla *et al.* (in press a) showed that, in an artificial lagoon, low DOC quality was due a high C:N ratio (low amino acid moiety), as has also been shown for other climatic regions and laboratory cultures (Sun *et al.*, 1997; Hunt *et al.*, 2000). Consequently, the C:N ratio may serve as a universal predictor of the microbial carrying capacity.

Furthermore, adverse effects of HS on microorganisms either directly, or after irradiation, have to be taken into account. For instance, Anesio *et al.* (1999) observed that UV-irradiated detritus from *P. australis* had a negative effect on the growth of free-living bacteria. Obviously, the phenolic lignin compounds of the supporting tissue are transformed into potentially toxic forms after being released into the environment.

Hitherto we have dealt with the control of assemblages by HS via the provision of nutrients and energy. However, we have several times pointed at the chemical stress imposed on organisms by exposure to HS. In general, net-heterotrophy may be caused by the promotion of heterotrophic, and/or the suppression of autotrophic, processes. Consequently, the resulting community response is a trade-off of at least two competing processes (for a mathematical model of this competition, see Steinberg & Brüggemann, 2001). Many, but not all, of the HS isolates tested so far potentially suppressed photosynthetic oxygen release (Pflugmacher *et al.*, 2006). Also *Quercus*, and particularly *Phragmites*, leachates possess this potential (Fig. 5). Obviously, some phenolic compounds are effective, since from studies on composting it is well understood that several phenolic intermediates released during the early stages are particularly phytotoxic (Helfrich *et al.*, 1998; Blewett *et al.*, 2005).

Many authors who have studied primary production and community respiration in non-eutrophic humic lakes, find net-heterotrophy of the plankton. However, in oligotrophic Canadian Shield lakes, Carignan *et al.* (2000) found that gross photosynthesis was almost always larger than community respiration (i.e. the lakes were net-autotrophic). DOC appeared to suppress both photosynthesis and respiration. The authors consider that their results argue against the importance of exogenous organic carbon supply as a significant energy source to the freshwater plankton. Unfortunately, Carignan *et al.* (2000) do not provide any DOC quality parameters, and therefore one can only speculate that it might have been DOC quality (specific compounds or structures) which inhibited microbial growth more than photosynthesis by the algae. This view gets support from very recent findings of a moderately antibiotic activity of humic matter in hydroponic systems (Gryndler *et al.*, 2005).

Conclusion

Due to their ubiquity, and the variety of functional groups, dissolved HS have the potential to affect almost any biochemical and biogeochemical pathway in both freshwater organisms and ecosystems. Their role in indirectly fuelling the heterotrophic components of freshwater ecosystems is well known. However, HS are also active environmental chemicals. They allow fish to survive in extremely soft waters and, by external or even internal modes of action, prevent ionoregulatory disturbance. Due to the low molecular mass of their building blocks (~0.5 kDa), they appear capable of passing biomembranes. Inside the organism, while being natural environmental chemicals, HS are metabolised like xenobiotics. They provoke a variety of non-specific and specific reactions in the organisms, starting from stress responses, such as the induction of stress proteins, to a more or less herbicide-like mode of action towards primary producers. According to recent paradigms of ageing and evolution, mild chemical stresses have the potential to increase life-span and probably trigger micro-evolution. Yet it is an open question, whether or not the stress caused by HS falls into this category. Humic substances may modulate the activity of membrane-bound pumps for xenobiotic chemicals, and also probably of ion-channels, thus moderating the bioconcentration of xenobiotics and (heavy) metals. However, there may be intra- and inter-specific differences in susceptibility to dissolved HS. The overall effect of the direct interactions of dissolved humic materials with

organisms must structure freshwater communities, as shown with primary producers. The interference with primary producers can be attributed to quinones in the HS. We assume that most the other effects can also be attributed to specific structures. Consequently, the DOC has to be chemically characterised, rather than simply measured as bulk concentration. In general, it could be argued that HS are controlling factors in aquatic ecology as important, for instance, nutrients, temperature or even light. We are just at the beginning to understand the complete function of HS in freshwater ecosystems. Wetzel (1995) was right, when writing ‘*population fluxes are not representative of the material and energy fluxes of either the composite pelagic region or the lake ecosystem.*’

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GLOSSARY

bioconcentration diffuse uptake from the environment and internal storage of xenobiotic chemicals and/or heavy metals by aquatic organisms

chemosensitiser originally from oncology: any of several compounds that reduce or even inhibit the functioning of a particular cellular glycoprotein, which is the basis of the MXR

chemosensitisation reduction of the activity of the glycoprotein pump

cyp cytochrome P 450 gene

DOC, TOC dissolved organic carbon, total organic carbon

FA fulvic acid, soluble in the whole pH-range

GST glutathione *S*-transferase

guild group of organisms living in the same type of environment and sharing a similar function, such as primary production, filter feeding or decomposition.

HA humic acid, soluble in alkaline, but not in acidic solutions

hormesis from (eco)-toxicology: dose response phenomenon characterized by a low dose stimulation, high dose inhibition

HS humic substances, include HA and FA

HSP heat shock protein, nowadays called stress protein, a chaperone induced by a variety of physical and chemical stresses. The figure behind HSP indicates the molecular mass in kDa; for instance, HSP70 has a molecular mass of 70,000 Da

HS1500 artificial humic substance with a mean molecular mass of 1.5 kDa; autoxidation product of polyphenols with alkyl bridges; similar to lignin building blocks

IR infra-red irradiation

molecular weight must be more stringently called molecular mass, in Da.

MXR multiple xenobiotic resistance, a membrane-bound P-glycoprotein which pumps chemicals out of cells

NOM natural organic matter, isolated by reverse osmosis (or ultrafiltration)

OC organic carbon

PS photosystem

QSAR quantitative structure-activity-relationship, a regression analysis relating effects to effective structures in chemicals

ROS reactive oxygen species, such as singlet oxygen, hydrogen super oxide, super oxide anions

UGT UDP-glucuronosyltransferases are responsible for the enzymatic addition of sugars to fat-soluble chemicals

UV ultraviolet irradiation

xenobiotic chemicals organic chemicals, alien to biological systems, often halogenated, produced by man; however, consideration of the natural halogen chemistry suggests that the separation between natural and xenobiotic potentially toxic chemicals is fictitious (cf. Steinberg, 2003)