PURE HUMIC SUBSTANCES HAVE THE POTENTIAL TO ACT AS XENOBIOTIC CHEMICALS – A REVIEW

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SUMMARY

In this review we argue against two paradigms: (1) humic substances (HS) are not taken up by aquatic organisms; (2) HS are inert in aquatic systems, except for the release of reactive oxygen species after irradiation. In fact, these paradigms are recycled and do not apply any longer. We show that HS-like substances, such as caffeic acid oxidation products, are taken up by all aquatic organisms studied so far. Furthermore, we present phenomenological as well as mechanistic evidence that HS have direct effects on aquatic plants and animals. The effects may be categorized as non-specific, such as expression of heat shock proteins (hsp) and modulation of biotransformation enzymes, or specific, such as inhibition of photosynthetic oxygen release in plants. Basic ecotoxicological requirements are fulfilled: several mechanisms apply to a variety of aquatic organisms, dose-response relationships and quantitative structure effect relationships may be established where applicable. We conclude that HS are natural xenobiotics that exert a chemical stress and, thus, are able to structure aquatic guilds by various modes of action.

KEYWORDS: Humic substances, [^{14}C]KOP, uptake, direct effects, heat shock proteins, biotransformation, inhibition of photosynthetic oxygen release.

1 INTRODUCTION

In all aquatic ecosystems, humic substances (HS) are the major component of dissolved organic matter (DOM) and exceed carbon in all living organisms by one order of magnitude or more [1–4]. Depending on the source of organic material involved in the humification process, HS comprise a variety of molecular structures and differ with respect to their origin. Microbial and abiotic degradation yields in a variety of molecular structures, such as alkylaromatic, quinoide, and aliphatic structures in the core, and amino acid or carbohydrate structures, and carbonyl-, carboxyl-, phenyl-, and hydroxyl groups in the periphery [4–6].

The ecochemical relevance of HS is mostly discussed with respect to their capability to bind or integrate pollutants like organic xenobiotics and heavy metals, consequently decreasing their bioavailability and toxicity [7–12]. Direct adverse effects are seldomly discussed. The functional groups responsible for the interaction with pollutants may also directly interact with biological systems, such as membrane affection by either accumulation [13] or tenside-like action of HS [14, 15]; alteration of reproduction of the nematode *Caenorhabditis elegans* [16]; effect on survival of the waterflea *Daphnia magna* [17]; influence on growth of terrestrial plants [18, 19]; modulation of enzyme activities [3, 19, 20]; and antimicrobial and antimycotic activity [22, 23], leading to suppression of pathogens. In addition, photochemical reactions can initiate reactions leading to reactive oxygen species, such as $^1O_2$, $O_2^{\bullet-}$, and H_2O_2 [24–26], which are able to induce oxidative damage to biomembranes. Alterations of oxygen-stress enzymes are caused by HS in the aquatic macrophyte *Ceratophyllum demersum* [27].

2 UPTAKE OF A HUMIC ACID LIKE SUBSTANCE

The question as to whether or not HS are taken up by organisms, has been argued intensively in the literature. One cannot deny that beneficial, as well as adverse effects can be observed when organisms come into contact with HS in their various forms: soil water solutions, peats, bogs, or HS-rich surface waters. Most soil scientists, for instance, attribute any effect on plants to indirect modes

of action, such as modulations of the bioavailability of key nutrients. So do many freshwater ecologists. In addition to altered bioavailability of nutrients, further indirect modes of action are discussed, such as decreased light climates which affect primary producers and optical foragers alike, altered food web structures, and decreasing space for water breathing organisms due to increasing anoxia in the lower strata of the water column [28, 29]. Most freshwater ecologists exclude direct interactions between HS and aquatic organisms, because uptake of HS is not considered to be feasible. In contrast, biomedical scientists accept uptake of HS up to approximately 1.0 kDa [30, 31]. Furthermore, they report interactions of HS with several receptors [30] and with blood coagulation [32]. Investigations with radiolabeled humic acid-like substances show that 1-5% of topically applied humic polymers penetrate from an 1% W/O emulsion into human skin [33].

Few earlier studies [32, 34] discuss the uptake as a decisive basic mechanism to explain surprising results, such as modulation of photosynthesis in macrophytes and algae, induction of heat shock proteins (hsp) in fish and invertebrates, modulation of transformation enzymes, and alteration of the endoplasmic reticulum [35]. In one key study, Ziechmann [20] writes that humic acid precursors, namely, from aqueous peat extracts and compressed peat, pass through the skin of pigs and mice and can accumulate subcutaneously. Recent studies [36] also show that HS or at least parts thereof can be taken up by cell cultures and, at least, parts of these molecules can be found even in the DNA. Nardi [19] showed that the physiological effects of HS on terrestrial plants depends on the source, concentration, and molecular mass of the HS. The authors present evidence that HS <3.5 kDa easily reach the plasmalemma of higher plant cells and, in part, are taken up. Thus, the uptake of HS by organisms can be counted as a direct effect, although mechanistic studies are rare.

In an unpublished study, we present evidence that ^{14}C-labeled humic-like substances prepared by enzymatic oxidation of caffeic acid with tyrosinase (EC 1.14.18.1) [37] are taken up and bioconcentrated by several aquatic organisms. The peak molecular mass of the caffeic acid oxidation product (KOP) detected from a non-radioactive sample was found to be 11,600 Da [38]. Figure 1 shows that, after 24 hours of exposure, a macrophyte (*C. demersum*), an invertebrate (*Gammarus pulex*), and a vertebrate (tadpoles of the moor frog *Rana arvalis*) are able to bioconcentrate ^{14}C in their bodies. The percentage uptake of the exposed [^{14}C]KOP is: *C. demersum* 7.3 $\pm$ 1.4%, *G. pulex* 6.9 $\pm$ 1.0%, and *R. arvalis* 11.7 $\pm$ 2.7%. It may still be argued that it is not the intact oxidation product of caffeic acid, but also smaller photodegradation products, which are bioconcentrated. Nevertheless, it is evident that at least low-molecular mass (photodegradation) products of the humic-like substances are taken up and bioconcentrated by aquatic organisms, and that the bioconcentrated substances are responsible for several effects addressed below, which are feasible only if HS are themselves taken up by the aquatic organisms. If parts of the broad molecular mass distribution of caffeic oxidation products are in the molecular mass range of 1.0 kDa, they cover well the molecular masses of most fulvic acids (FA) in aquatic ecosystems [2].

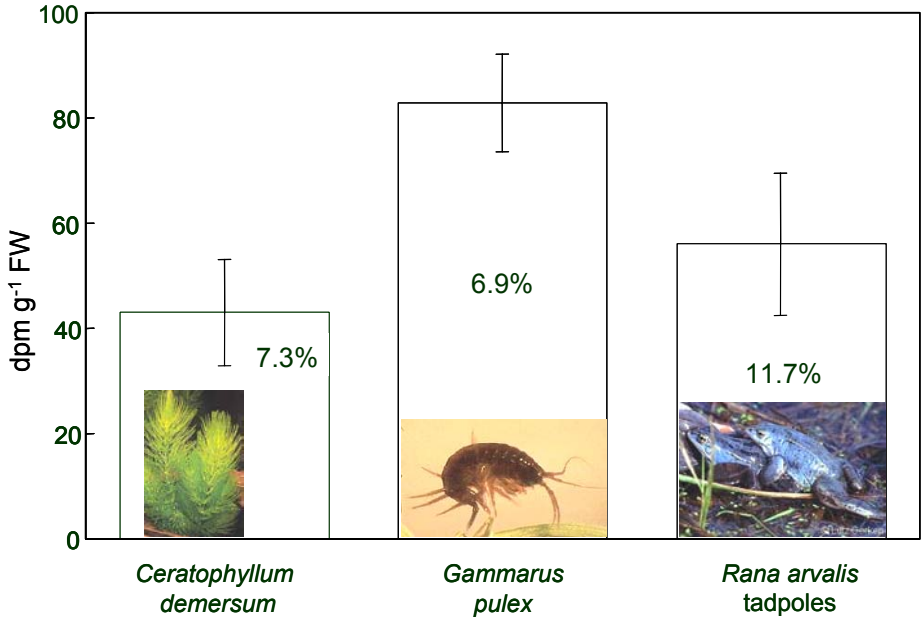

FIGURE 1 - Uptake of [^{14}C]-labeled caffeic acid oxidation product ([^{14}C]KOP) by three aquatic organisms within 24 hours (means $\pm$ standard deviation) (unpublished).

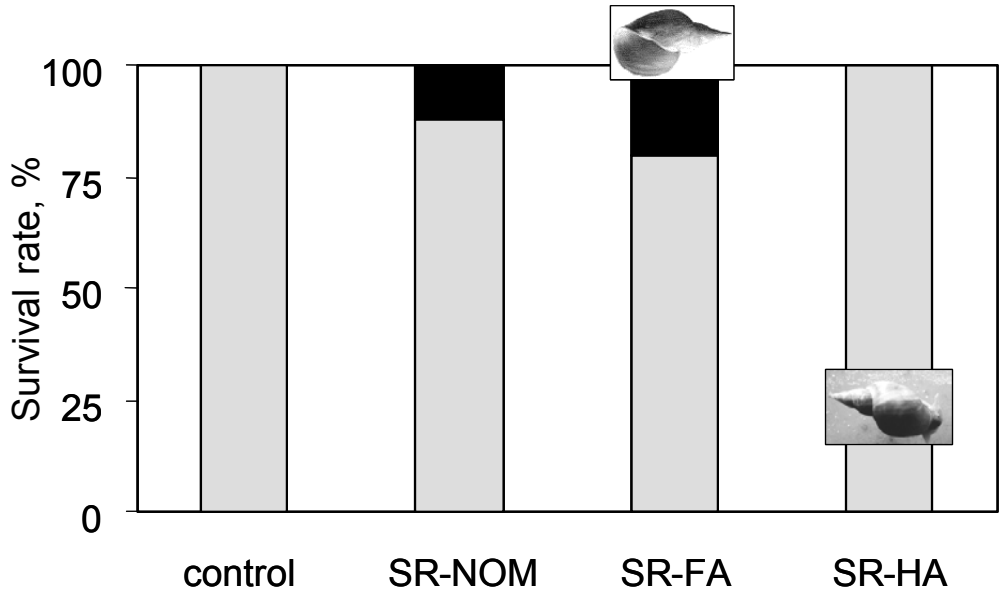

FIGURE 2
Survival rates of the common European freshwater snail, *Lymnea stagnalis*, exposed to 0.5 mg L^{-1} DOC of various HS from the Suwannee River (SR) for 24 hours. Grey columns: living snails; black columns: dead snails (unpublished).

3 TOXIC EFFECTS

A. PHENOMENOLOGICAL EVIDENCE

Invertebrates: Snails

From recent cross experiments in which the European freshwater snail, *Lymnea stagnalis*, is exposed to different HS from the Suwannee River, clear evidence exists that under circum-neutral conditions the snails respond to HS exposure, as if exposed to man-made chemicals (xenobiotics). After a 24-h exposure to 0.5 mg L^{-1} DOC the activity of the transformation enzyme systems is extremely elevated, and Suwannee River natural organic matter (NOM) and FA, but not humic acids (HA), cause death of 10–20% of the animals (Figure 2). This finding means that HS appear to have a toxic potential per se.

Future studies may determine whether the toxic potential of HS pertains only to exotic species or also to indigenous species. Probably, the indigenous flora and fauna are better adapted to 'their' HS, than are exotic species.

Fish

In a recent study, Meinelt et al. [39] tested the survival of embryos of an *r*-strategist (zebrafish, *Danio rerio*), which were exposed to the synthetic HS1500 over a period of six weeks. Figure 3 shows that a concentration of 500 mg L^{-1} HS1500 (a clearly higher concentration than commonly found under natural conditions) significantly reduces the survival rate of the exposed embryos. On the other hand, low concentrations of 5 to 50 mg L^{-1} increased the survival rate relative to the control. But, since even under natural conditions a great proportion of embryos dies during ontogenesis, it cannot be judged whether the obvious decrease in the embryo mortality rate is a really beneficial effect or within natural ranges. Typically the offspring of *r*-strategists does not contain large amounts of energy reserves, whereas the number of the gametes produced by the parents is huge. Environmental changes will thus immediately lead to increased losses in the brood stock. Such high HS concentrations like 500 mg L^{-1} might be toxic to the weak juveniles.

On the other hand, the offspring of the so-called *K*-strategists is energetically better provided than that of the *r*-strategists. The „life-bearing" swordtails (*Xiphophorus helleri*) are typical *K*-strategists. Exposure of swordtail to HS1500 concentrations of up to 180 mg L^{-1} did not significantly increase the mortality of the brood stock over a five-month period (Fig. 4). In a long-term study, Meinelt et al. [39] did find a better growth of juveniles (length, mass, condition factor) as well as a better compensation of long-term stress (handling) in the HS-exposed groups. This means that, particularly at low HS concentrations, fish have more advantages than disadvantages. It can be speculated that this might be due to the inhibition of fish-pathogenic microbes like bacteria, fungi, and viruses. At the end, the question of whether or not HS are beneficial to fish is a function of the HS concentration, and may be also of the HS qualities, the energy content of the fish, and the inhibition of pathogens as well.

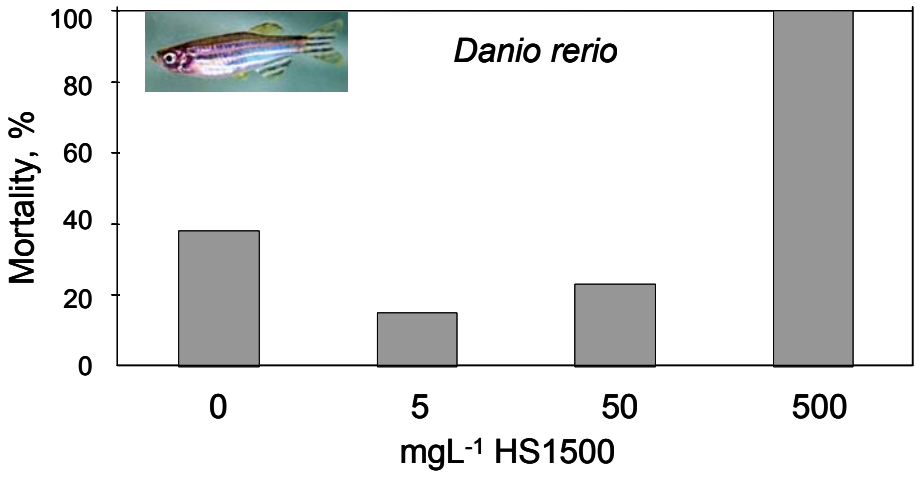

FIGURE 3 - Modulation of the mortality rate of embryos of a *r*-strategist (zebrafish, *Danio rerio*) exposed to different concentrations of the synthetic HS1500 [unpublished].

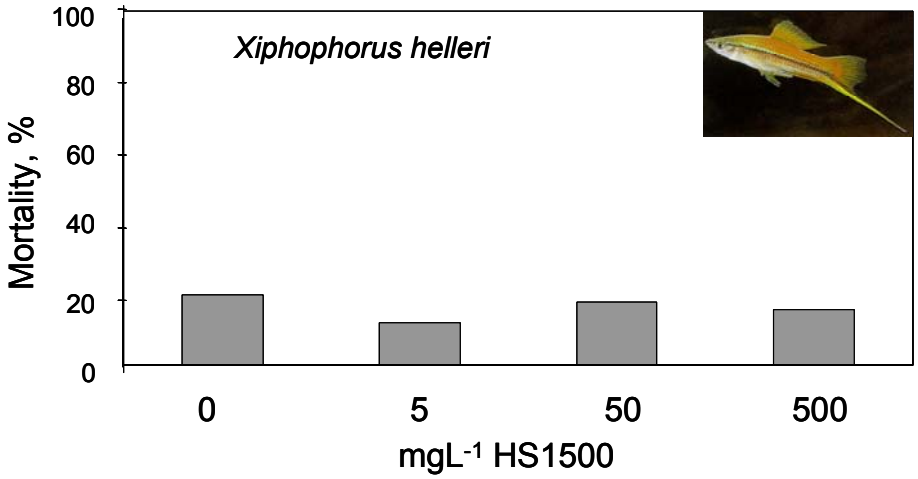

FIGURE 4 - Modulation of the mortality rate of embryos of a *K*-strategist (swordtail, *Xiphophorus helleri*) exposed to different concentrations of the synthetic HS1500 [39].

B. NON-SPECIFIC MECHANISMS

Non-specific mechanisms were studied by analyzing heat shock proteins (hsp) and biotransformation enzyme activity. Hsps are a chaperon family, protecting proteins in the cells during stress phases. Their cellular content increases after stress as caused by heat, heavy metals, and xenobiotics, with denaturated proteins being the causing principle [41]. Heat shock protein 70 (hsp70) binds to *de novo* synthesized proteins to prevent mistakes in folding, and in this form the protein is transported to its target place, where it achieves its final form and function. Hsp 70 also binds to partly denatured proteins to either refold them correctly and retain their function, or, if damage is too severe, guide them to places of controlled lysis.

Modulation of biotransformation enzymes, such as glutathione *S*-transferases (GSTs) is another unspecific physiological reaction of stressed organisms. GSTs are ubiquitous conjugation enzymes of the biotransformation pathway. They react with moderate hydrophilic xenobiotics, which include electrophilic groups. Activation by other enzyme systems like cytochrome P-450 monooxygenases is not required, if the substance already comprises

a functional group for the GSTs. Conjugation to glutathione increases water solubility of the substances and supports their excretion. A broad substrate specificity is attained by several soluble GST isoenzymes, and one microsomal form [40].

Heat shock protein 70

The expression of hsp 70 in carp shows different background intensities in the control fish, depending on the tissue: High expression in the liver, low to very weak expression in the gills, or the muscles, respectively. In the gills, which are the main contact organs during exposure *via* the medium, the moderate expression of the hsp70 is highly increased by all fractions of Suwannee River HS (Fig. 5A). The muscles show nearly no reaction on the HS (not shown). The different Suwannee River HS fractions (FA, HA, and NOM), caused almost no different reactions, only FA induced a slightly stronger reaction in the gills. Expression of hsp 70 after exposure of the carp to Svartberget, Humex A, Humex B, and Nordic Reference NOMs are investigated in gills only. All control carps show weak or no hsp 70 expression; hsp 70 are slightly increased by Svartberget and Nordic Reference NOMs and clearly increased by Humex A and Humex B NOMs (Fig. 5B).

The displayed mechanism does not only apply to fish, but seems to be more common. For instance, also gammarids show similar reactions upon exposure to HS isolates. In a screening test we exposed gammarids from Lake Müggelsee (Berlin, Germany) and Lake Baikal (Siberia, Russia) to Sanctuary Pond NOM (Ontario, Canada). Exposed individuals of *Gammarus tigrinus* and *Gammarus ischnus* expressed hsp 70 more strongly than control individuals (Fig. 5C). The induction of hsps appears to be a general reaction of aquatic organisms to exposure of HS and NOM.

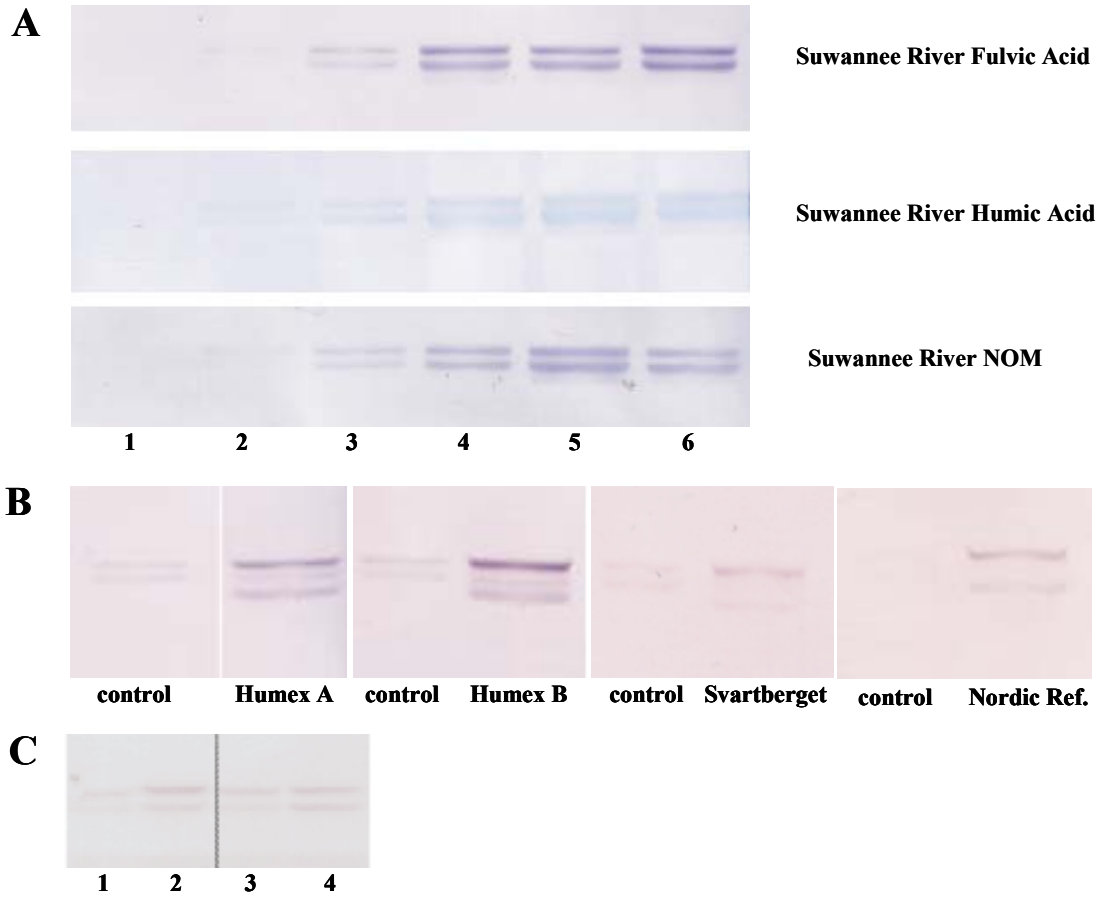

FIGURE 5 - Western Blot detection of expression of the heat shock protein 70 in gills of carp, and in gammarids after exposure to HS. A: Carp gills, lane 1-3: control carp, lane 4-6 exposure to 5 mg L^{-1} isolated Suwannee River HS fractions, as indicated. Each line represents a sample of one single carp to show individual differences. B: Carp gills of control versus exposed carp. Exposure to 0.5 mg L^{-1} of the named NOMs from the Nordic Countries, from left to right: HUMEX A, HUMEX B, Nordic Reference, Svartberget, all NOM. C: Amphipods, exposed to 50 mg L^{-1} of Sanctuary Pond NOM lines 1, *Gammarus tigrinus* control, 2, *G. tigrinus* exposed, 3, *G. ischnus* control, 4, *G. ischnus* exposed. A detection of hsp 70 in the Baikalian *Eulimnogammarus cyaneus* failed (not shown) (from [42]).

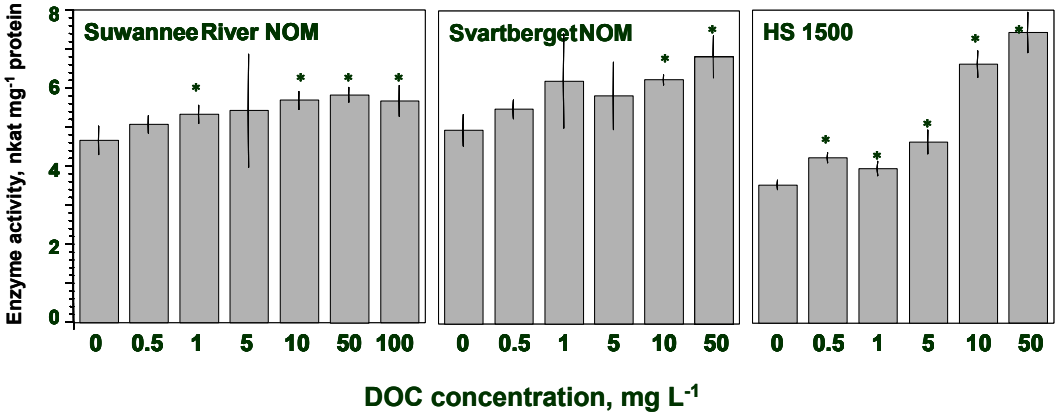

FIGURE 6
Glutathione-*S* transferase response in *Daphnia magna* with increasing concentrations of two NOM isolates and of the synthetic HS1500 [42] (means, ± standard deviation) * significantly different from the control.

For these physiological interactions, obviously caused by HS, a fundamental requirement is that the HS come into contact with cell surface or cell internal structures. Expression of hsp 70 increases, if denaturated proteins occur in the cell, no matter, if physical stress, like heat, or chemical stress, like xenobiotics, lead to their denaturation [43]. Thus, the used HS apparently cause protein denaturation in the gills of the carp and in the amphipods. A further possibility is the HS mediated generation of reactive compounds, which then will cause damages to the proteins. HS from different origin cause similar effects, but vary in intensity.

Dose-response: biotransformation enzymes (glutathione S-transferases)

In a study on *D. magna* Wiegand et al. [42] found that under pH neutral conditions HS isolates from the Suwannee River activate biotransformation enzymes such as glutathione-*S* transferases (GST). There is a dose-response relationship between HS and NOM exposure, and biotransformation enzyme activity (Fig. 6). It is evident that *D. magna* responds significantly to increasing exposure concentrations, with increases in GST activity. This is most pronounced with the synthetic HS1500 and least pronounced with Suwannee River NOM. Since all three isolates activate the phase II enzyme, a common mechanism might be assumed.

C. SPECIFIC MECHANISMS

Very recently, we tried to elucidate in more detail, the mechanism behind the HS- and NOM-induced modulation of photosynthetic oxygen release. The test alga was *Scenedesmus armatus*. Prior to photosynthesis measurement, the algae are exposed to HS and, in order to avoid light quenching during measurement, are transferred into a HS-free synthetic medium. Suwannee River NOM, a forest soil leachate FA, and a synthetic HS (HS1500) significantly reduce the photosynthetic oxygen release (Fig. 7).[1] The reduction must be due to internal cell mechanisms, probably to interference of HS or their low-molecular mass fractions within the photosynthetic electron chain.

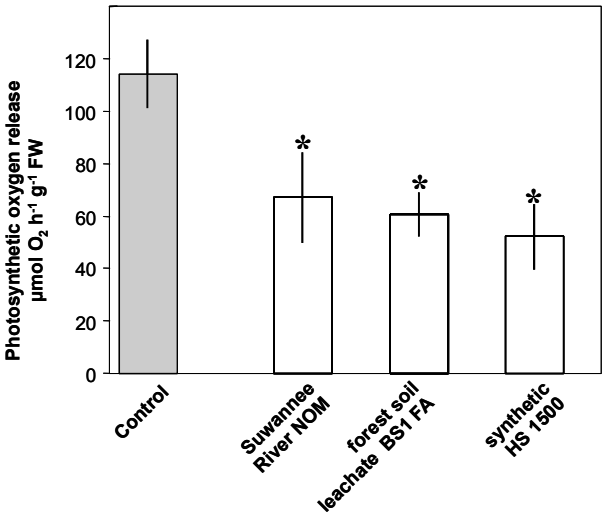

FIGURE 7 - Reduction of photosynthetic oxygen release in the coccal green alga *Scenedesmus armatus* after 18-h pre-exposure to 0.5 mg L^{-1} DOC of three HS and NOM isolates [47]. Data are means of three replicates, ± standard deviation; * significantly different from control; FW = fresh weight

[1] Perminova et al. [44] did not find any apparent effect of HS on the green algal species *Chlorella vulgaris*, as determined by chlorophyll fluorescence measurements. This finding, however, does not contradict the statement above for two reasons: first, the applied toxicological endpoints are of different susceptibility (according to our experience with *S. armatus*, chlorophyll concentration is not a very sensitive toxicity endpoint); second, 1 h of exposure time may generally be too short to provoke detectable changes.

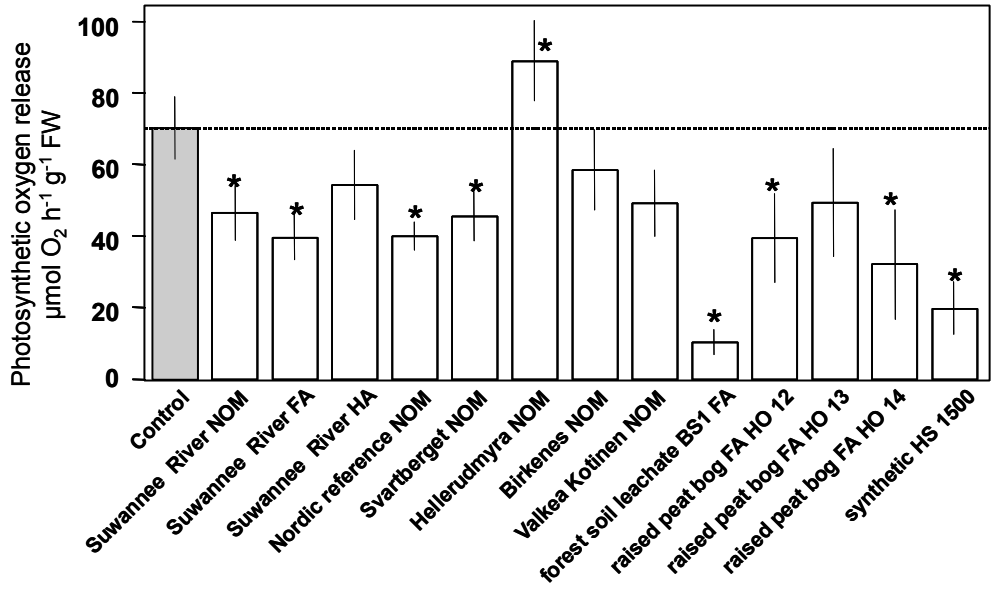

FIGURE 8 - Photosynthetic oxygen release in the coontail *Ceratophyllum demersum* after 24 h exposure to different HS and NOM, 0.5 mg L^{-1} C each. Prior to photosynthesis measurements, the plants are transferred into HS-free solutions. Most HS and NOM isolates significantly reduce the oxygen production, only one isolate significantly enhances it. Data are means of three replicates, ± standard deviation; * significantly different from the control; FW = fresh weight (modified from [47]).

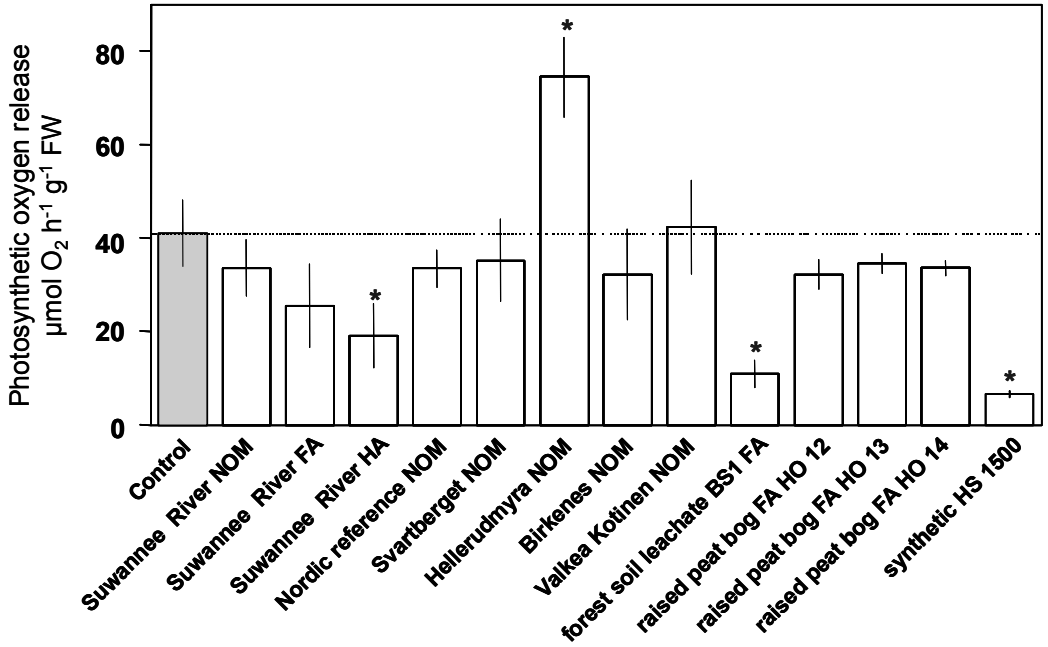

FIGURE 9 - Photosynthetic oxygen release in the tropical water moss *Vesicularia dubyana* after 24 h exposure to different HS and NOM, 0.5 mg L^{-1} C each. Prior to photosynthesis measurements, the plants are transferred into HS-free solutions. Three HS isolates significantly reduce the oxygen production, only one isolate significantly enhances it. Data are means of three replicates, ± standard deviation; * significantly different from the control; FW = fresh weight (modified from [47]).

Similar results, including hints on potential modes of action are obtained with two macrophytes, the hornwort *C. demersum* (Fig. 8) and the tropical water moss *Vesicularia dubyana* (Fig. 9). Recent microbiological studies show that HS have the potential to act as electron acceptors [45, 46], thus it appears most likely that this ability also applies to the effect described for algae.

Pflugmacher et al. [27, 47] describe the modulation of various physiological and biochemical parameters of *C. demersum* in the presence or absence of HS. In *C. demersum*, the photosynthetic oxygen release is significantly reduced by 8 out of 13 HS and NOM isolates, whereas in *V. dubyana* only 3 of 13 isolates reduce the oxygen release (Figs. 8, 9). The adverse effect of HS and NOM exposure can be seen even with the naked eye, for instance, *C. demersum* eventually turns yellow upon exposure to the forest soil leachate (BSI).

So far, the evidence that HS or NOM are the causative agents of direct effects on aquatic organisms remains somewhat circumstantial, because one cannot exclude that the observed effects may be due to contaminants sorbed onto the tested isolates. However, evidence would strongly increase, if the observed effects could be related to structural features of HS themselves, for instance by quantitative structure activity/effect relationships (QSAR). In fact, a QSAR can be established for the inhibition of photosynthetic oxygen release in aquatic plants. The electron trapping property can be attributed to the quinoide fraction of HS and NOM. Quinoide structures can act as electron acceptors, and thus interfere with the electron flow in photosystem I. Taking semiquinone radicals, which can be determined by electron spin resonance, as an indirect, but significant measure for quinoide structures [48–50], it is evident that the reduction of photosynthetic oxygen release can be significantly related to quinoide structural units in the HS materials (Fig. 10). The spin content of the HS and NOM predicts approximately 90% of the reduction of photosynthetic oxygen release in both macrophytes tested so far [51]. To date there is no mechanistic explanation for the different behavior of Hellerudmyra NOM (Figs. 8 and 9).

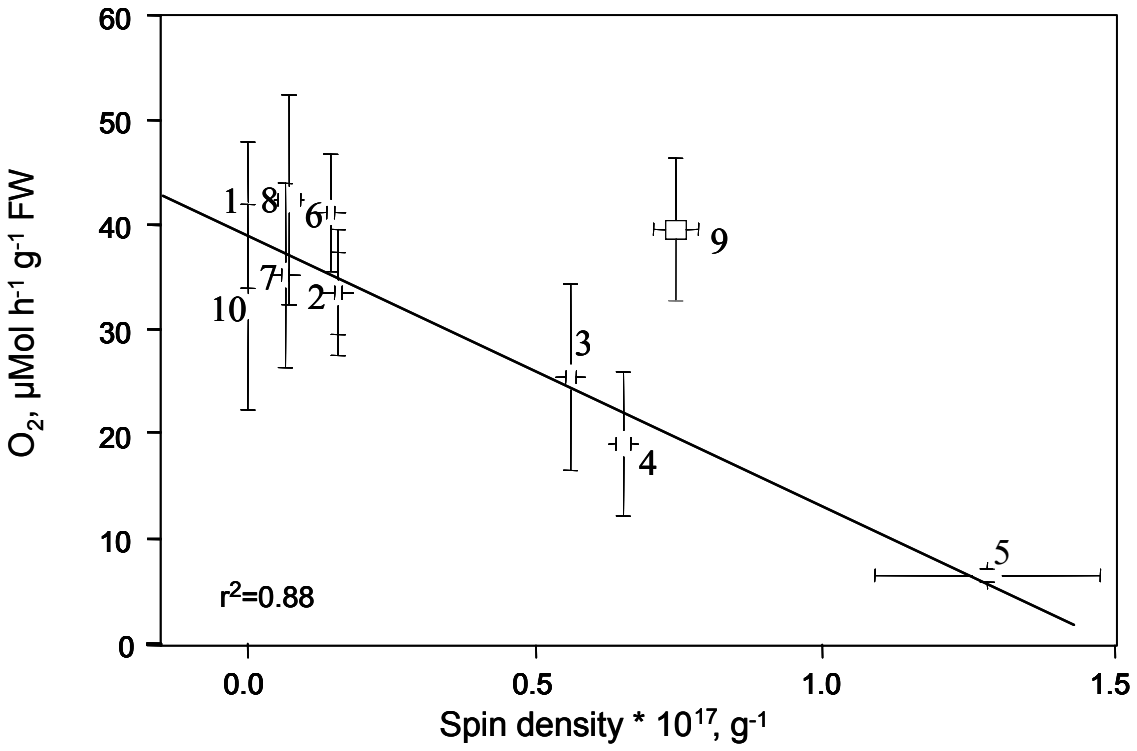

FIGURE 10 - Spin density of HS and NOM as a predictor for the reduction in photosynthetic oxygen release in *Vesicularia dubyana* [51]. 1: control; 2: Suwannee River NOM, 3: Suwannee River FA, 4: Suwannee River HA, 5: synthetic HS1500, 6: Hellerudmyra NOM, 7: Svartberget NOM, 8: Valkea-Kotinen NOM, 9: Hietajärvi NOM, 10: Birkenes NOM. Note that several soil and peat HS and NOM isolates which have not been tested with aquatic plants so far, possess even a higher spin content than the synthetic HS1500. Soil and peat HS have even higher spin contents than the displayed HS and NOM isolates. Hietajärvi NOM has been excluded from the regression.

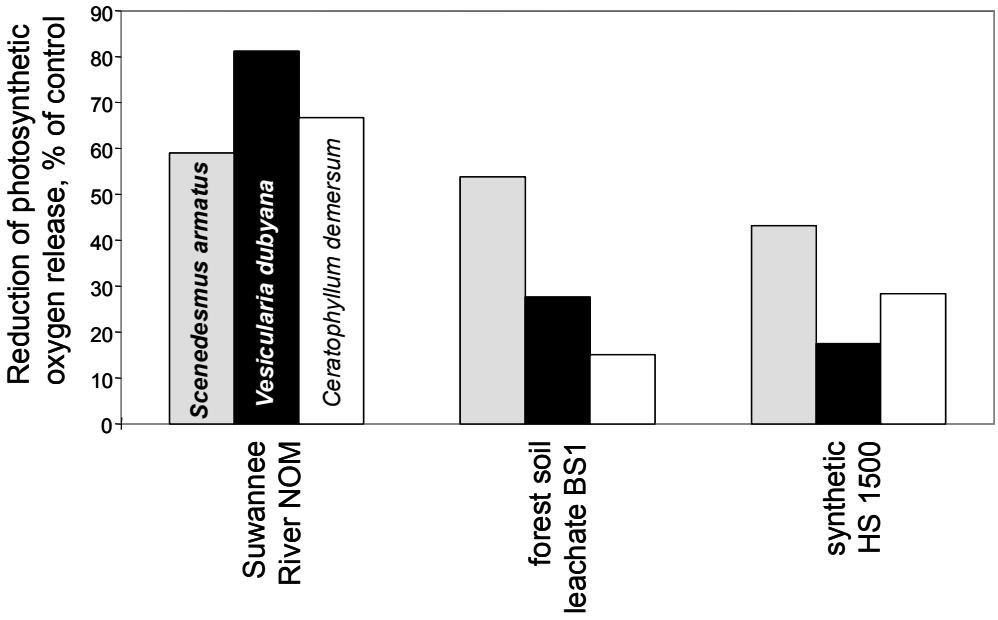

FIGURE 11 - Different susceptibilities of the three species of aquatic plants towards three different HS or NOM, as indicated by reduction of photosynthetic oxygen release [11].

When comparing all three aquatic plants tested so far (Fig. 11), it is evident that there is no 'most sensitive' species. With Suwannee River NOM, the most sensitive species is the coccal green alga *S. armatus*; with the soil FA it is the angiosperm *C. demersum*; and with the synthetic HS1500 it is the water moss *V. dubyana*. That means that a specific region, with specific terrestrial plant cover resulting in HS with specific chemical features, will produce a specific aquatic community under non-eutrophicated conditions. Some general rules may apply, such as water mosses gain dominance in humic waters. But, even his statement has to be confirmed in future studies. At present we are still clearly in the realm of information gathering, particularly since such effects of HS on photosynthetic oxygen release of aquatic plants are unexpected and contradict conventional paradigms on the inability of plants to take up HS or NOM.

CONCLUSION

The knowledge, of how HS affect exposed aquatic organisms, has been rather limited. In many instances, only indirect, external action like modulations of bioavailability of nutrients, bioavailability of metals, and xenobiotics, or the suppression of pathogens have been studied. In this review, we show that HS and NOM are able to directly affect physiological precesses of aquatic organisms comparable to xenobiotic chemicals. Upon HS exposure, aquatic organisms display induced hsp concentrations or altered activities of biotransformation enzymes, leading mainly to reduced life times or survival. Furthermore, the numbers of offsprings of the nematode *C. elegans* may be altered, mainly increased [15]. With aquatic plants, HS and NOM may reduce photosynthetic oxygen release. The quantitative expression of these effects depends on the concentrations of quinoide structures in the humic materials. All the effects mean that populations, consortia, and communities (guilds) of aquatic organisms will be structured according to their susceptibility towards ambient HS. We may assume that with increasing numbers of ecological/ecochemical studies on HS, an increasing number of to date unexpected effects in freshwater ecosystems will be quantitatively related to specific structures in HS.

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