



## DISSOLVED HUMIC SUBSTANCES FACILITATE FISH LIFE IN EXTREME AQUATIC ENVIRONMENTS AND HAVE THE POTENTIAL TO EXTEND THE LIFESPAN OF *CAENORHABDITIS ELEGANS*

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### ABSTRACT

Dissolved humic substances (HSs) are taken up by organisms and interact on various molecular and biochemical levels. With two examples, we show that HSs facilitate fish life in low pH and low calcium waters and promote longevity by mild chemical stress. We re-visit recent papers on fish adaptation in the Amazonian Rio Negro and re-interpret the results on the basis of the recent finding of gene control in the nematode *Caenorhabditis elegans*. In the nematode, several genes are up-regulated. This may also account for the maintenance of fish life in the Rio Negro. Exposure to HSs exerts mild chemical stress on the exposed organisms and deprives them of energy; however, the nematode *C. elegans* actively looks for such environments. With life-table and DNA gene filter studies we show that HSs may cause longevity and multiple stress resistance. Furthermore, we propose likely structures of the HSs responsible for these effects.

**Keywords:** Natural organic matter, humic substances, lifespan extension, ion regulation, transcriptional control

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### 1. INTRODUCTION

A classical but still recycled paradigm states that dissolved humic substances (HSs), mainly fulvic acids (FA), are too large to be taken up by aquatic organisms and consequently do not interact with their metabolism. However, recent eco-chemical and ecological studies show that FA building blocks are about 0.5 kDa in molar mass [1-4]. Similar results apply to natural organic matter (NOM) [5, 6]. This molar mass is clearly in or even below the range of bioactive molecules such as cyanotoxins (molar mass >1 kDa), for which the uptake by aquatic organisms is well proven [7]. Consequently, one may argue that the above cited paradigm might be based on the lack of appropriate experiments. In fact, recent studies with radioactively labeled HSs and NOM show that all exposed organisms take up these materials easily [8-12]. Since HSs are non-endogenous substrates and the aquatic organisms try to get rid of them after being taken up, it is not surprising that HSs provoke all kinds of anti-stress reactions which are well known in ecotoxicology from organismal response to xenobiotic exposure [13]:

- Electrochemical membrane irritation as shown with giant cells of the Charophyte *Nitellopsis obtusa* [14]
- Induction of stress proteins as shown with algae [15], amphipods [16], and fish [17]
- Development of oxidative stress symptoms, including lipid peroxidation as a likely antioxidant defense. This has been shown with red blood cells [18] and amphipods [19, 20]
- Induction and modulation of biotransformation enzymes [16-26].

Because all of these reactions consume energy, which has to be compensated for at the expense of energy resources or regular metabolism, as clearly shown with zebrafish embryos [22], the impacts of HSs on aquatic organisms have to be classified as adverse. But does this classification really hold true if one studies the effects of HSs exposure in more detail or in a broader context? If doing so, the experiments sometimes reveal unexpected results that are not consistent with some current paradigms of ecotoxicology. For instance, when the nematode *Caenorhabditis elegans* Maupas had been exposed to HSs, several genes encoding for chemo- and olfactory receptors were up-regulated (Table 1, [21]), meaning that the nematode should be attracted by these substances. Indeed, a simple attractant test showed that the majority of adult nematodes actively move to

bacteria heaps mixed with HSs. From the studies referred to above, it is obvious that the nematode would suffer from chemical stress and should be deprived of energy. Despite this adverse impact, there also must be beneficial effects to the nematodes and

the overall result of this trade-off must be positive, otherwise this species would have become extinct in the real world since it inhabits HS-rich saprophytic environments [27].

**Table 1** Excerpt of the whole genomic DNA microarray of *C. elegans* exposed to two humic materials, synthetic HS1500 and Lake Fuchskuhle NOM [21]

Gene code	Protein	Fuchskuhle (IF) <sup>a</sup>	HS1500 (IF) <sup>a</sup>
Transporters			
C04B4.4	Chloride channel protein	7.7	25.5
T02C5.4	Calcium channel alpha subunit	2.1	9.2
M04B2.5	Potassium channel protein	3.9	5.4
C13C4.5	Sugar (and other) transporter	2.8	21.7
Chemoreceptors			
Y57A10B.5	G protein-coupled chemoreceptor	2.9	51.1
R03H4.3	Olfactory receptor (srx-3)	3.9	26.8
C54F6.11	Olfactory receptor	16.7	4.5
B0454.3	Chemoreceptor (sri-28)	2.0	2.1
Stress defense			
H10D18.6	UDP-Glucuronosyltransferase	2.0	51.6
D2045.9	Glycosyltransferase	3.9	4.5
F02C12.5	Cytochrome P450	2.1	4.6
F13A7.10	Glutathione S-transferase	2.1	2.6
F32A5.2	Peroxidase	49.7	17.4

<sup>a</sup> IF = induction factor as compared to the controls

This striking example indicates that the interaction of aquatic and soil organisms with the biochemical matrix of their habitats, the HSs, must be more than a simple adverse chemical stress. Instead, this paper will show that exposure to HSs appears to be crucial for life in general. This will be demonstrated with fish in extreme aquatic environments. Furthermore, HSs have the potential to expand the individual lifespan of nematodes. To achieve these goals, we will re-visit papers on fish life in the Amazonian Rio Negro and try to superimpose the findings there with recent results of a whole-genome microarray study with the nematode *C. elegans*. This approach is only an analogy, but it may generate robust hypotheses on which way to direct future studies.

The second approach is an in-depth study of the effects of chemical stress induced by dissolved HSs and a likely building block. In-depth means that we include parameters that surpass classical ecotoxicological ones and which are based on the main

hypotheses of the *Green Theory of Aging* (see below for details). Furthermore, we try to relate the described effects to structural features of the HSs. In sum, this paper is intended to widen ecological perspective on control by HSs in the environment. These substances are integral parts of any ecosystem, and we are only at the very beginning of understanding their role there. Several of these ideas have already been indicated in a previous review [28]; in this contribution we continue reviewing and furthermore refer to new experimental data that are consistent with the previous hypotheses.

## 2. FISH LIFE IN EXTREME AQUATIC ENVIRONMENTS

In a recent series of papers, the essential control by HSs of fish life in the Amazonian Rio Negro system has been demonstrated experimentally [29-34]. Numerous slow-moving blackwaters associated with flooded forest along the Rio Negro have pH as low as

3.0-4.0 [35], which should render them devoid of fish. However, the great diversity of fish in the Rio Negro (>1000 species [36]) indicates that the fish of these blackwaters are highly adapted to extreme conditions, in particular to low pH and low  $\text{Ca}^{2+}$  and  $\text{Na}^+$  concentrations. Gonzalez & Preest [29] showed that two major physiological mechanisms apply to maintain homeostasis: (1) effective uptake of cations from waters with very low concentrations and (2) effective retention of internalized cations. It is feasible that HSs may interfere with both processes. Furthermore, when fish were exposed to low pH in Rio Negro water instead of deionized water (with the same concentrations of major ions), the effects of low pH were reduced. This suggests that high concentrations of HSs in the water may interact with the branchial epithelium in some protective manner [30]. Wood et al. [31] pointed at the different HSs qualities and acclimations of the fish to the HSs source when they reported that HSs from the Rio Negro prevent ionoregulatory disturbance in freshwater stingrays (*Potamotrygon* spp. Garmann) exposed to pH 4.0. But Aldrich humic acid (HA) did not prevent disturbance, probably due to the fact that the fish never came in contact with these substances and could not acclimate to them. In fact, there seem to be major differences in freshwater fish from contrasting habitats. For example, Matsuo et al. [33] noticed that acute exposure of rainbow trout *Oncorhynchus mykiss* (Walbaum), an inhabitant of HSs-poor waters, to HSs induces increased ion loss, probably because of a surfactant effect [37] induced at the gill membrane level, or because  $\text{Ca}^{2+}$  is stripped out of the paracellular tight junctions. However, when the tetra *Paracheirodon axelrodi* (Schultz), an inhabitant of Amazonian blackwaters, was chronically exposed to HSs, the fish displayed increased branchial ion uptake rates and also reduced ion loss from the gills [34]. It is suggested that the basis for this tolerance is an increased branchial affinity for  $\text{Ca}^{2+}$  at the paracellular tight junctions in the gills, which in turn limits branchial permeability and prevents ion leakage under dilute, acidic conditions [29, 30, 32]. As a specification of this mechanism, we offer the following likely explanations. Based on a whole-genome microarray with *C. elegans* (excerpts in Table 1), we show that HSs directly interact with genes of this nematode and thus hypothesize that HSs also interact with the genome of Rio Negro fish in comparable modes of action. In particular:

- $\text{Ca}^{2+}$ -transporters are induced (T02C5.4 in *C. elegans*), which may be active against a strong gradient; and
- Sugar and other transporters are induced

(C13C4.5 in *C. elegans*). Since this transporter is not very specific, it may even facilitate the uptake of HS-complexed  $\text{Ca}^{2+}$ .

Furthermore, the maintenance of the extremely high gradient between internal and external  $\text{Ca}^{2+}$  concentrations may not only be due to an effective uptake mechanism, but also to reduced or controlled losses of ions. The latter may be facilitated by two different mechanisms:

- Down-regulation of two genes that encode for cation-transporting ATPases, as shown with *C. elegans* [21], and
- Blockage of non-specific membrane-bound export pumps, as shown with multi-drug transporters (so-called multi-xenobiotic resistance): for details, see [38] in amphipods [39]. We assume that HSs also may internally block ion channels if they are not already down-regulated by HS interactions.

### 3. LIFE SPAN EXTENSION BY HS-MEDIATED CHEMICAL STRESS

In the introduction we have pointed out the fact that exposure to HSs leads to a chemical stress in the organisms and that the nematode, *C. elegans* actively looks for such stressful environments. According to the *Green Theory of Aging* [40-45] as one very plausible of many hypotheses on the mechanisms of aging, 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 [41, 42]. In general, mild chemical stress to individuals means training of the chemical defense system (biotransformation enzymes, anti-oxidant enzymes, stress proteins), which can also lead to multiple stress resistance. HS-related hormetic effects have already been observed, for instance, with the activity of phase I and II enzymes [16, 17], *Daphnia* behavior [46], and the number of offspring of *C. elegans* [47]. This means that exposure to low concentrations of HSs and the subsequent organismal response may contribute to the training of the defense system and may provoke even more complex 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 [42] stated that training of the defense system may lead to stress resistance, which may be related to longevity. Multiple stress resistance has so far been proven effective in many animals (e.g. rotifers, *C. elegans*, spiders, fish, rodents

and the fruit fly *Drosophila melanogaster* Meigen). More recently, several key molecules involved in longevity have been identified [43-45]. Pro-longevity genes include some that encode anti-oxidant enzymes and biotransformation enzymes and others encoding heat-shock proteins, particularly of low molar mass (the Hsp22 family).

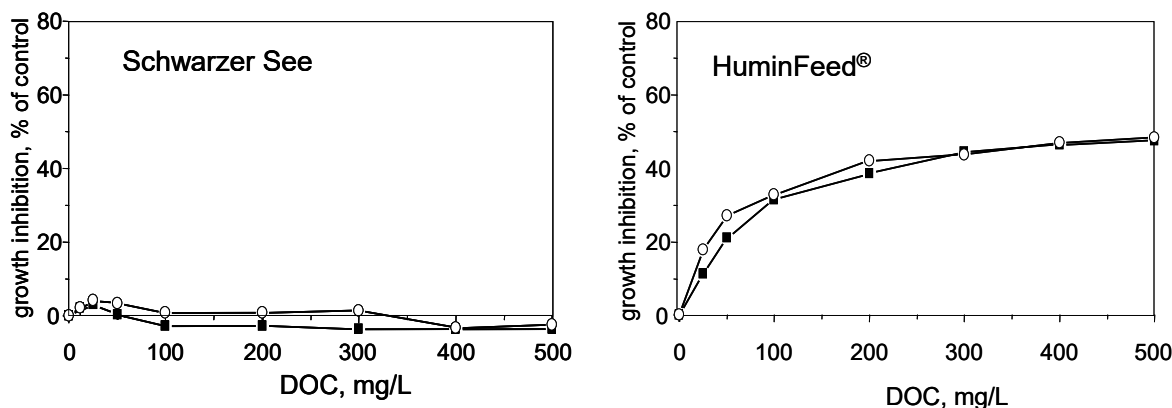
With the exception of low molar mass Hsp, genes of all enzyme families have been found to be up-regulated in *C. elegans* after exposure to one natural and one artificial HSs [21]. In the meantime, the small heat-shock protein gene also has been found up-regulated upon HS exposure (see below).

In a study with 20 HSs, NOM, and the synthetic HS1500, Meinelt et al. [48] have analyzed the potential of these substances to modulate the vegetative growth of the fish pathogenic water mold, *Saprolegnia parasitica* Coker. Some of the isolates supported the vegetative growth (e.g. Schwarzer See NOM, Figure 1, left graph, negative values of growth inhibition), but the majority reduced it, e.g. HuminFeed<sup>®</sup>, a product derived from leonardite (Fig. 1, right graph). A quantitative structure-activity relationship analysis revealed that it is the HSs material with high spin concentration (which equals a high content of organic radicals, indicative of high reactivity), high C:CH<sub>2</sub> and C:H ratios, and high specific UV absorption, which statistically account for vegetative growth reduction, whereas isolates with high polysaccharide contents supported growth (Fig. 2).

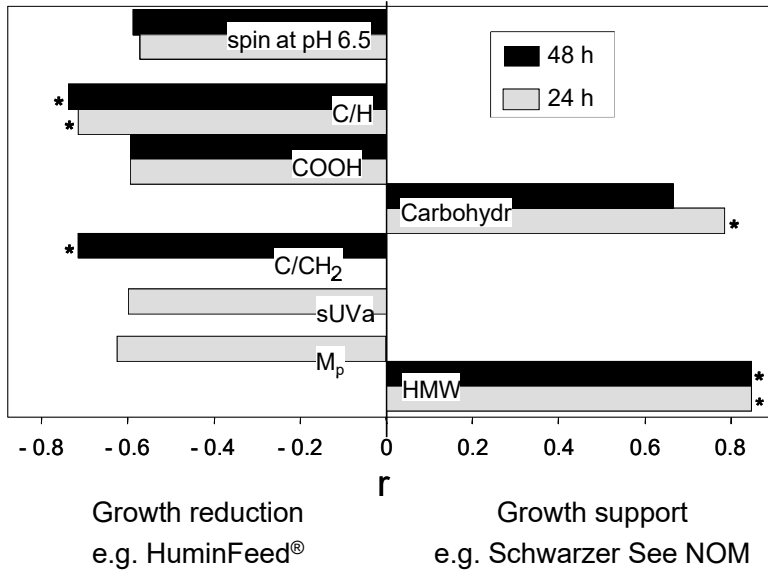
However, reduction of vegetative growth also

includes another aspect which deserves attention: how sustainable is a growth reduction? Are HSs fungicidal or only fungistatic? Actually, the fungus is able to cope with temporarily adverse situations, because it develops spores which may produce hyphae after the HSs exposure is gone. This means that HSs are fungistatic. It is open to future experiment whether or not sporangia or viable zoospores occur more frequently upon exposure to aged HSs than with NOM. In terms of longevity, the production of sporangia/zoospores clearly indicates lifespan extension.

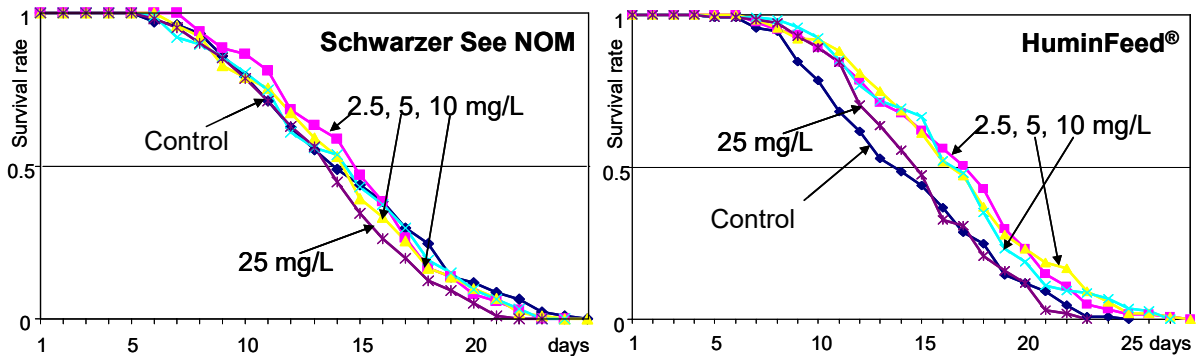
In order to evaluate if a lifespan extension of HS-exposed organisms in fact occurs, *C. elegans* was exposed in a first test to the same two HSs in increasing concentrations as *S. parasitica*. Indeed, Schwarzer See NOM did not modulate the lifespan of *C. elegans* (Fig. 3). In contrast to the NOM isolate, the processed leonardite HuminFeed<sup>®</sup> significantly prolonged the life of the nematode in the low to medium concentration range (2.5, 5, and 10 mg/L DOC), whereas the 25 mg/L exposure was in or even below the range of the control animals (Fig. 3). According to an inventory of DOC-porewater concentrations [49], even 25 mg/L lies well within in the environmentally realistic range. In sum, these findings mean that the modulation of the lifespan by exposure to HuminFeed<sup>®</sup> appears to follow the hormesis principle. The low to medium exposures expand the lifespan<sub>50</sub> (lifespan at the 50% survival rate) as much as approximately 3 days, a lot for an animal that usually only lives about 20 days.



**Figure 1** Growth inhibition of *S. parasitica* plotted vs. DOC of a NOM (left part) and a humic substance (right part) for 24h of exposure (squares) and 48h of exposure (open circles) for two contrasting HSs (from [48], for HuminFeed<sup>®</sup>, the reader is referred to <http://www.humintech.com/001/environment/information/general.html>, accessed August, 2007).



**Figure 2** Correlations of the 24 h and 48 h EC<sub>50</sub> values with structural features. Specific UV absorption (sUVa) and peak molar mass (M<sub>p</sub>) were derived from HPSEC and point to aromatic humic compounds with higher molar mass. COOH and CH<sub>2</sub> are relative measures of these functional groups derived from FTIR spectra, C and H were derived from elemental analysis and spin at pH 6.5 denotes spin concentrations of stable organic radicals in HSs measured by EPR spectroscopy. HMW = high-molar mass. Negative r-values mean reduction of vegetative growth, positive r-value mean growth support as exemplified with two HSs of contrasting effects in Figure 2 (from [48]).



**Figure 3** Survival rates and lifespans of *C. elegans* exposed to Schwarzer See NOM and HuminFeed<sup>®</sup> at increasing concentrations, mg/L DOC. Controls were without any NOM/HS addition. With Schwarzer See NOM, no exposure was significantly different from the controls. With HuminFeed<sup>®</sup>, the differences between control and 2.5, 5 and 20 mg/L exposures were significant at the  $p < 0.005$  level (log-rank test,  $n = 94 - 120$ ). There were no significant differences between the control and the 25 mg/L exposure.

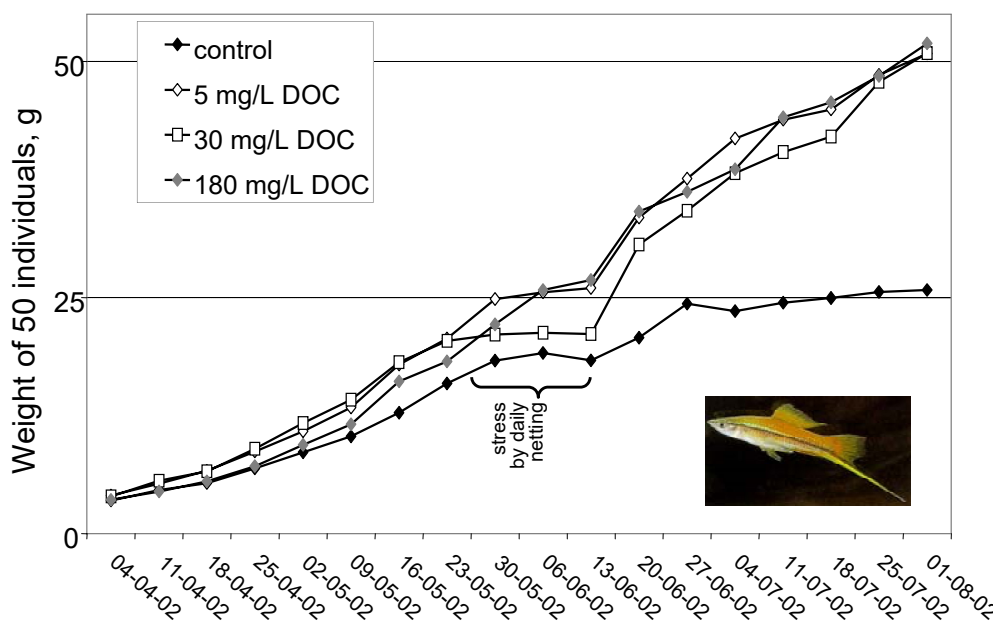
As mentioned above, training of the chemical defense system may lead to multiple stress resistance [42], whereby the inducing stresses may be chemical. The acquired stress resistance must not be chemical but may also be physical, such as heat or cold stress. With respect to HS exposure, multiple stress resistance on the phenotypic level has been shown with the swordtail, an ornamental 'viviparous' fish species (Fig. 4 [50]). Newly born juvenile swordtails were exposed to increasing concentration of the synthetic HS1500 and their development was monitored until sexual differentiation of the individuals. Any addition of HSs, even as low as 5 mg/L DOC, led to a weight gain by the young animals

and to an obvious multiple stress resistance, because even strong daily physical stresses by catching and transport to other aquaria with fresh water containing the analogous amount of HSs caused only a small delay of the growth, whereas the control animals did not really recover from the stressful 14 days. In the meantime, it has been shown with carp that symptoms of density stress (e.g. cortisol levels) can be reduced if the fish are exposed to HSs [51].

In addition to the chemical stress hypothesis, another mechanism for life-span expansion may apply: activation of sirtuins (a family of deacetylases) by small molecules [52]. This mimics caloric restriction which itself is known to slow down aging

in yeast and metazoans. Interestingly, a class of polyphenolic molecules produced by plants in response to stress can activate the sirtuins [52]. These polyphenols are very similar to lignin constituents and, moreover, to tannins, which are major compounds in some and perhaps all HSs [53]. In fact, quercetin significantly prolonged the lifespan and

induced an increased thermal tolerance (unpublished results). Several exposure scenarios have been tested, and all increased the thermal tolerance of the nematodes. This finding is another piece of evidence for the acquisition of a multiple stress resistance upon HSs exposure in general and supports the interpretation of the swordtail experiment above.



**Figure 4** Increases of the survival rate of embryos and juveniles of the swordtail, *Xiphophorus helleri*, exposed to elevated concentrations of the synthetic HS1500. The differences between treatments and control were significant at the  $p < 0.001$  level (from [50]).

**Table 2** Major anti-stress and longevity genes up- and down-regulated in young adult *C. elegans* upon exposure to two humic substances preparations; n = 3, from 42 genes, with at least a two-fold change as compared to control worms [unpublished results]

	Genes up-regulated	Genes down-regulated
Schwarzer See NOM	<i>hsp-16.41</i> (small heat shock protein) <i>F32A5.2</i> (peroxidase) <i>sod-3</i> (Fe/Mn superoxide dismutase) <i>dhs-4</i> (short-chain dehydrogenase) <i>gst-1</i> (glutathione S-transferase) <i>gst-44</i> (glutathione S-transferase) <i>vit-1/2</i> (vitellogenin)	<i>clk-1</i> (clock gene) <i>ctl-1</i> (catalase) <i>dhs-23</i> (short-chain dehydrogenase) <i>sod-5</i> (Cu/Zn superoxide dismutase)
HuminFeed®	<i>hsp-16.41</i> (small heat shock protein) <i>sod-3</i> (superoxide dismutase) <i>cyp-35B1</i> (cytochrome P450) <i>cyp-37B1</i> (cytochrome P450) <i>gst-44</i> (glutathione S-transferase) <i>vit-1/2</i> (vitellogenin)	<i>sir-2.3</i> (sirtuin) <i>gst-10</i> (glutathione S-transferase) <i>ctl-1</i> (catalase) <i>cyp-34A9</i> (cytochrome P450)

Plants also benefit from being exposed to HSs. Since most plants do not age in the strict gerontological sense [54], it can be expected that mild stresses do not necessarily lead to life-span extension of the complete plants, but of certain organs, which means that these organs have developed a multiple stress resistance. With respect to HSs, there are first indications that HS-exposed wheat and apricot seedlings develop a water deficiency and salt resistance [55, 56].

The next step has been to identify major genes involved in lifespan expansion upon HSs exposure. This has been carried out in tests with *C. elegans* exposed to various isolates and preparations, among them Schwarzer See NOM and HuminFeed<sup>®</sup>, and a gene microarray with selected genes (DNA gene filter). Both HSs preparations have an estrogenic potential and induce vitellogenin (compare [57]). The exposed biogeochemicals interacted with genes encoding for biotransformation (particularly, glutathione *S*-transferase, cytochrome-P450), anti-oxidant defense (particularly, superoxide dismutase), or stress proteins (Hsp16) (Table 2). This finding is consistent with the genes and gene products on which the *Green Theory of Aging* is based (see [42]). Particularly, *sod-3* encodes an iron/manganese superoxide dismutase, predicted to be mitochondrial, that might defend against oxidative stress and promote normal lifespan [58]. At present, the different patterns of gene up- and down-regulation cannot yet be sufficiently explained. Nevertheless, it is interesting to note that in young adult *C. elegans* Schwarzer See NOM down-regulates one clock gene (*clk-1*), which directly controls the individual lifespan. Consequently, lifespan control may be a trade-off of at least two competing pathways: lifespan extension by up-regulation of *sod-3* and lifespan decline by down-regulation of *clk-1*. Hence, it is not surprising that exposure to this humic material does not lead to lifespan extension (Fig. 3).

#### 4. CONCLUSIONS

The above results and hypotheses have shown that HSs are more than a biogeochemical matrix that interacts with organisms only in indirect ways, for instance by increasing the bioavailability of trace nutrients. Instead, dissolved HSs facilitate fish life in the Amazonian Rio Negro system, which appears to be hostile when regarding only the inorganic chemical composition of the water. By analogy to the nematode *C. elegans*, we have provided some robust hypotheses of mechanisms of how HSs may interfere on the molecular and the biochemical level to establish and

maintain the extreme gradient of essential cations inside the fish as compared to the almost ion-free outside. These hypotheses may be subject to future experimental validation.

Furthermore, we have presented evidence that HSs may transcriptionally control biotransformation, anti-oxidant and anti-stress defense systems and modulate the respective enzyme activities. According to the *Green Theory of Aging*, these interactions are thought to be a means of lifespan expansion. In addition to these interactions, one clock gene also is down-regulated and no lifespan extension occurs. This indicates that HS-mediated longevity is controlled by more than one pathway. There are also indications that lifespan extension follows the hormetic dose-response principle with only low to medium concentrations being effective in this respect. Putting this thought into an ecological framework, it could mean that lifespan extension may occur under field conditions, but that it may be restricted to low HSs concentrations.

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