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Research paper

Humic dissolved organic carbon drives oxidative stress and severe fitness impairments in *Daphnia*

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ABSTRACT

Increases in dissolved organic carbon (DOC) in the form of humic substances, causing browning of surface water, have been reported worldwide. Field surveys indicate that higher DOC levels can influence primary production and thus plankton composition. Experimental studies on the direct effects of humic DOC on aquatic organisms have shown varying results depending on concentration and additional environmental factors. Moreover, changes in life-histories and stress responses have usually been tested separately, rather than in combination. We experimentally tested the impact of a sudden increase in humic DOC on two species of the zooplankton cladoceran *Daphnia*, across several levels of biological organisation, from cellular to population responses. In *D. magna*, strong impacts on reproduction (delayed maturity and reduced number of offspring) were coupled with overall stress induction (increases in antioxidant capacity and oxidative damage, combined with a reduced amount of available energy). In *D. longispina*, increased mortality and lowered fecundity were observed. We conclude that a strong input of humic DOC into aquatic systems can have severe negative impacts on zooplankton species, and has the potential to alter zooplankton community structures.

1. Introduction

In natural waters humic substances (HS) are a major component of dissolved organic material (Thurman, 1985). They are involved in many physical, chemical and biological processes such as photodegradation, changing the toxicity of metals and xenobiotics, or interacting with metabolic pathways (Steinberg, 2003). Due to their diverse composition, HS are difficult to characterise, thus the concentration of dissolved organic carbon (DOC) is often reported as a bulk measure (Suffet and MacCarthy, 1989). The majority of natural freshwater bodies worldwide show DOC concentrations of 1–30 mg L^{-1}, but in rare cases concentrations can exceed 100 mg and in extreme situations even 300 mg DOC L^{-1} (Sobek et al., 2007; Farjalla et al., 2009). Increases in humic DOC concentrations, resulting in darker surface water, have been observed in many lakes and rivers; first recorded in the 1980s (Evans et al., 2005), the phenomenon is likely to have been underway in the 1960s or even earlier (Andersson et al., 1991; Worrall et al., 2003). Mean annual increases of up to 0.5 mg DOC L^{-1} have been reported over a period of 15 years across 22 lake and stream sites in the UK (Evans et al., 2005), exemplifying similar trends elsewhere in Europe and North America (Andersson et al., 1991; Bouchard, 1997; Worrall et al., 2004). Underlying causes of increasing concentrations are generally associated with environmental change. These include release of DOC from peatlands in response to rising temperatures (Freeman et al., 2001), increasing atmospheric carbon dioxide (Freeman et al., 2004), changes in sulphate deposition (Monteith et al., 2007), altered precipitation patterns (Andersson et al., 1991), and land-use change (Worrall et al., 2003). All of these affect either the availability of DOC in the catchment or the transport of DOC from the catchment to surface waters.

Most studies have reported a rather gradual trend of slowly rising DOC concentrations, however, in rare cases this increase can

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be sudden and extreme. Brothers et al. (2014) reported a fivefold increase, from about 10 to 50 mg L^{-1}, in mean DOC concentrations in a shallow lake in Germany after a period of high precipitation. Elevated water levels flooded adjacent degraded peatlands, thus increasing DOC input into the lake. As a result, hypolimnetic anoxia occurred and benthic primary production was almost fully stopped, leading to a nearly complete loss of macroinvertebrate and fish populations. Effects of humic DOC on freshwater communities have also been investigated in enclosure, pond, or whole-lake experiments, demonstrating a negative impact on primary production through changes in the light regime (Arvola et al., 1996; Jones and Lennon, 2015) and in phyto- and zooplankton community composition (Blomqvist et al., 2001; Shurin et al., 2010).

Nevertheless, field community studies cannot distinguish between cascading indirect effects through food web interactions and the direct toxic effects of these substances. Using experimental approaches, a range of direct effects of humic DOC on physiological and behavioural traits, both positive and negative, have been reported for various aquatic and terrestrial organisms (reviewed in Steinberg et al., 2006). A positive consequence is the potential for humic DOC to activate stress-defense mechanisms and thus contribute to a multiple-stress resistance (Meinelt et al., 2004; Steinberg et al., 2007). However, many reported effects are detrimental. These include, for instance, feminisation in the fish *Xiphophorus helleri* (Meinelt et al., 2004) and the clawed frog *Xenopus laevis* (Lutz et al., 2005), reduction in colony growth of the aquatic oomycete *Saprolegnia parasitica* (Meinelt et al., 2007), and induction of lethality in the freshwater snail *Lymnea stagnalis* (Steinberg et al., 2003).

One organism well-suited to study the effects of humic DOC at different levels of biological organisation is the water flea *Daphnia*. Members of the genus play key roles in lakes and ponds due to their dual function as effective grazers of phytoplankton and as an essential food source for planktivorous fish (Lampert and Sommer, 1997). In addition, *Daphnia magna* and related species have been widely established as model species in a variety of biological disciplines (Lampert, 2011; Seda and Petrusek, 2011), one of which is ecotoxicology. The ecotoxicological assays, focusing on molecular and cellular endpoints, revealed stress-induced responses in *D. magna* toward humic DOC, including increased activity of the antioxidant enzyme glutathione S-transferase (Meems et al., 2004), reduction of antioxidant capacity (Steinberg et al., 2010a), as well as increased DNA methylation (Menzel et al., 2011). Lifespan, a key life-history trait, was extended in *Daphnia galeata* while offspring number decreased in a concentration-dependent manner when exposed to humic DOC (Bouchnak and Steinberg, 2013). Overall there is no clear pattern regarding the impact of elevated levels of humic DOC on *Daphnia*. Moreover, since stress related and life history responses have usually been studied independently, the sequential series of biological events leading to an adverse effect on *Daphnia* populations is not well defined.

Here, we studied the impact of five- and tenfold increases in humic DOC concentrations on *Daphnia* in a controlled laboratory experiment, assessing endpoints measured at the cellular, physiological, organism and population level of biological organisation. We hypothesised that strong, but environmentally realistic, pulses of humic DOC induce a direct stress in *Daphnia*, and thus negatively influence life history traits and population growth.

2. Material and methods

2.1. Daphnia cultures

Two *Daphnia* species were used in the experiment. A *D. magna* clone (E17:07) was isolated from a temporary pond near Oxford, England, and a *D. longispina* clone (Stech12a) from Lake Stechlin, a deep clear-water lake in northeastern Germany. Both clones were exposed to general experimental conditions for four weeks prior to the experiment; five individuals per clone were kept in jars in 550 mL (*D. magna*) or 200 mL (*D. longispina*) of synthetic medium (SSS-medium; Supplementary data Appendix A.1) at a constant temperature of 20 ± 1 °C, a 12:12 h dark:light cycle and fed daily ad libitum with the green unicellular algae *Scenedesmus obliquus* (1 mg L^{-1} C).

2.2. Humic DOC

Humic dissolved organic carbon was added in the form of the commercial humic substance HuminFeed® (HuminTech GmbH, Grevenbroich, Germany). HuminFeed® (HF) is marketed as a food supplement in animal breeding. It is obtained through alkaline extraction of oxidised lignites (leonardite) (http://www.humintech.com). HF consists of 82% humic substances, 18% low-molecular weight compounds and 0% polysaccharides (Meinelt et al., 2007). HF was applied in two concentrations: 50 mg L^{-1} (corresponding to a DOC concentration of about 15 mg L^{-1}, DOC1 treatment) and 100 mg L^{-1} (corresponding to about 30 mg DOC L^{-1}, DOC2 treatment). No HF was added to the control treatment. The day before usage HF was pre-dissolved in deionised (DI) water to a concentration of 1000 mg L^{-1}, heated to 60 °C for an hour to prevent quick precipitation in the experimental medium, and refrigerated. A concentrated SSS-medium was prepared (containing water to 90% of the final volume). Depending on the treatment, different amounts of DI water and/or HF solution were added to the concentrated SSS-medium (control: DI water to 10% of the final volume, DOC1: 5% DI water + 5% HF solution, DOC2: 10% HF solution). All media were exchanged every 2 days. Chemical analyses on selected key parameters were performed on one batch each of all solutions (DOC1, DOC2 and control): (i) freshly prepared and (ii) after 2 days under experimental conditions (i.e. the time span between media exchanges).

2.3. Experimental setup

The experiment was carried out for 21 days under constant temperature, light cycle and food conditions (as above). Neonates of the 3rd generation, all born within 12 h, were transferred to a 50-mL vial containing 40 mL of experimental medium. For *D. longispina*, 15 vials were set up per treatment, each containing two neonates. After 4 days the neonates were placed in separate vials if both were still alive. For the larger *D. magna*, 51 vials were set up per treatment, each containing a single neonate (15 individuals for the assessment of fitness parameters and 36 for oxidative stress parameters and energy reserves). For practical reasons biochemical and physiological parameters were only measured on *D. magna*, since the performance of the same analyses on the smaller *D. longispina* (about one-third the body length) would require dozens of individuals to be pooled together (Borgeraas and Hessen, 2002). After 2 days of exposure, 30 *D. magna* individuals per treatment were selected randomly, pooled by fives into Eppendorf tubes ($n = 6$ tubes per treatment) and stored at −80 °C for subsequent analyses of oxidative stress-related parameters. As *D. magna* reaches maturity after 7–8 days at 20 °C, releasing their parthenogenetic eggs into the brood chamber, after 6 days of exposure six further individuals were randomly taken out to quantify the amount of energy available for reproduction. They were placed individually into Eppendorf tubes ($n = 6$ per treatment), and stored at −80 °C for later analysis. Upon termination of the exposure (day 21), six individuals were again selected randomly, placed individually into