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Effects of humic stress on the zooplankton from clear and DOC-rich lakes

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SUMMARY

1. Humic stress is associated with the widespread and ongoing browning of lakes. Natural landscape gradients in dissolved organic carbon (DOC) potentially result in aquatic communities with different tolerances to humic substances and thus expected contrasting responses to further lake browning.
2. If zooplanktonic species are adapted to different background concentrations of DOC, then we expected that the zooplankton from naturally DOC-rich lakes would maintain higher diversity, biomass and overall density in the face of experimental browning than the zooplankton from DOC-poor lakes. We tested this hypothesis in a common-garden experiment by exposing, in enclosures, zooplankton from replicate DOC-rich and DOC-poor source lakes to simulated browning and to clear water.
3. We conducted a 2 × 2 × 3 factorial-design field transplant experiment with zooplankton from replicate DOC-rich (>8.5 mg L^{-1}) and DOC-poor (<3.5 mg L^{-1}) lakes (Québec, Canada) over eight weeks. There were two fixed effects: water treatment (brown or clear water) and zooplankton source (from DOC-rich or DOC-poor lakes). Lake source was included as a random variable in the model for the response of copepod body size in the enclosures. A substance derived from peat, 'Super-Hume', was used as a source of DOC.
4. The diversity, biomass and total density of zooplankton from DOC-rich and DOC-poor lakes did not differ upon experimental addition of further DOC. This was despite the presence of different copepod body size phenotypes between source lakes that could have potentially caused different community responses: several dominant species of copepods (*Cyclops scutifer*, *Leptodiaptomus minutus* and *Tropocyclops prasinus mexicanus*) had a larger mean population body size in DOC-rich source lakes than in DOC-poor source lakes. Our findings suggest that the zooplankton from DOC-rich lakes does neither better nor worse than zooplankton from DOC-poor lakes when faced with browning from a humic stressor.

Keywords: body size, browning, crustacean zooplankton, dissolved organic carbon, intraspecific variation

Introduction

Environmental filtering of species and phenotypes in local communities along landscape gradients can render the biota more or less resistant to an additional local stressor (Urban *et al.*, 2008; Logue *et al.*, 2011). Humic stress is associated with the widespread and ongoing browning of lakes (Monteith *et al.*, 2007; Zhang *et al.*, 2010; Larsen, Andersen & Hessen, 2011). Natural gradients in dissolved organic carbon (DOC) in landscapes may potentially result in aquatic communities with different tolerance and response to lake browning. The zooplankton plays a key role in aquatic ecosystems, transferring carbon and essential compounds from primary producers up through the food web to planktivorous fish (Thorp & Covich, 2010). Dissolved organic carbon is an important regional variable that can drive among-species differences in zooplankton (Beisner *et al.*, 2006; Derry *et al.*, 2009; Shurin *et al.*, 2010) and also possibly intraspecific differences, which are not well understood. Given the important ecological role of zooplankton in food webs, and the potential for further

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lake browning to affect this group of organisms, it is important to understand zooplankton responses to simulated browning from both an interspecific perspective and an intraspecific perspective.

Dissolved organic carbon can be a strong selective agent in lakes at both the community (species sorting) and population (phenotypic variation) levels. First, it can limit the total density of zooplankton by shading the phytoplankton, limiting its growth (Ask, Karlsson & Jansson, 2012) and potentially influencing the phenology of algal blooms that affect the spring emergence of different zooplankton groups (Nicolle *et al.*, 2012). At the same time, DOC can also act as a resource for zooplankton by increasing bacterial productivity (Jansson *et al.*, 2000; Lennon & Pfaff, 2005), and a growing number of studies have shown that allochthonous (terrestrial) DOC can play a role in aquatic food webs as a resource for consumers such as zooplankton (Carpenter *et al.*, 2005; Karlsson *et al.*, 2007; Cole *et al.*, 2011; Berggren *et al.*, 2014). Secondly, DOC can influence the zooplankton via its effect on predation. In waters rich in DOC, the zooplankton may shift to larger species because visually feeding fish forage less efficiently (Brooks & Dodson, 1965; Wissel & Boeing, 2003; Hart & Bychek, 2011). Thirdly, DOC can have direct physiological effects on the zooplankton (Steinberg *et al.*, 2006). Therefore, it is not surprising that DOC is known to be an important determinant of species composition and abundance in zooplankton communities in north temperate lakes (Beisner *et al.*, 2006; Derry *et al.*, 2009; Shurin *et al.*, 2010). However, very few studies have explored patterns of intraspecific variation along DOC gradients, even though it can be as important as interspecific differences in community response to stress (Hersch-Green, Turley & Johnson, 2011).

Body size is important in ecological studies of animals because of its relationship with many other important life-history traits such as survival, metabolic rate, feeding rate, fecundity and population size, which have ramifying effects on communities and ecosystems (Peters, 1983; Jansson *et al.*, 2007). In the zooplankton, body size can be influenced by food quantity and quality, fish predation, acidity and competition (Hart & Bychek, 2011), all of which in turn may be modified by DOC (Jansson *et al.*, 2007). There is evidence that the two dominant taxonomic groups of zooplankton are larger in DOC-rich lakes compared with clear lakes (calanoid copepods: Carter *et al.*, 1983; cladocerans: Yan *et al.*, 2008). Since larger individuals tend to be more robust in face of environmental change and stress, and because of the association of body size with other traits such as fecundity and feeding rate (Peters, 1983; Jansson *et al.*, 2007; Hart & Bychek, 2011), intraspecific variation in body size along gradients in DOC gradients could potentially influence community responses to humic stress associated with lake brownification.

Our hypothesis was that, if the zooplankton from DOC-rich lakes consists of larger-bodied individuals compared with DOC-poor lakes at the intraspecific level, then we should expect that the diversity, biomass and overall density of communities from DOC-rich lakes would be more resilient in face of an additional humic stressor compared with the zooplankton of lakes poor in DOC. We first tested whether dominant copepod species present in both DOC-rich and DOC-poor lakes with similar zooplankton species composition were represented by different phenotypes in body size. Then, in the main part of the study, we performed a field transplant experiment in enclosures with the zooplankton from replicate DOC-rich and DOC-poor lakes. Our study has relevance for the prediction of ecological impacts of climate change, because studies of north temperate lakes suggest a general pattern of browning from increased terrestrial inputs of DOC in association with changing patterns in acidic deposition, precipitation and climate (Monteith *et al.*, 2007; Zhang *et al.*, 2010; Larsen *et al.*, 2011).

Methods

Study site and design of the field experiment

North temperate lakes are characterised by broad natural gradients in dissolved organic carbon (DOC) as a result of variation in catchment properties, the spatial configuration and composition of vegetation, hydrology and within-lake processes (Canham *et al.*, 2004). In the Laurentian region of Québec, which lies within the Canadian Shield, this variation in ambient DOC ranges from 3.0 to 15.5 mg L^{-1}. To test our hypothesis, zooplankton from six circumneutral (pH > 6) lakes either rich (>8.5 mg L^{-1}) or poor (<3.5 mg L^{-1}) in DOC were transferred to a common-garden field experiment (Table 1). The experiment was comprised of 36 *in situ* enclosures that were deployed in a clear, ultra-oligotrophic lake from June to September 2012 (Lac Violon; 45°56′31″N, 74°05′20″W; Table 1). The experiment was a 2 × 2 × 3 factorial design with $n = 3$ replicates. It consisted of two fixed effects that each had two treatment levels: (i) water treatment (decrease or increase in DOC relative to conditions in the lake from which the zooplankton were taken) and (ii) nearby regional sources of the zooplankton [three DOC-poor lakes (<3.5 mg L^{-1})

Table 1 Physical, chemical and biological characteristics of source lakes and incubation lake (CDOM, coloured dissolved organic carbon; TP, total phosphorus; Chl*a*, chlorophyll *a* concentration; BP, bacterial production). Trophic classification is from Wetzel (2001)

Lake name	Zooplankton source category	Lake source	Trophic classification	Lat. (N)	Long. (W)	Max depth (m)	DOC (mg L^{-1})	CDOM (Absorption 440 nm)	TP (μg L^{-1})	Secchi depth (m)	pH	Chl*a* (μg L^{-1})	BP (μg C L^{-1} day^{-1})
Clair	DOC-poor	L1	Ultra-oligotrophic	46°05′	73°47′	24.3	2.96	0.01	4.57	6.00*	8.03	0.69	1.67
Desmarais	DOC-poor	L2	Ultra-oligotrophic	43°10′	74°38′	33.1	2.85	0001	3.77	6.00*	7.63	0.38	0.80
Tracy	DOC-poor	L3	Ultra-oligotrophic	45°55′	74°03′	24.5	3.04	0.00	3.50	5.50	7.72	0.65	5.67
À l'Ours	DOC-rich	L4	Mesotrophic	45°57′	74°03′	16.7	8.9	0.04	12.69	1.75	7.86	2.45	6.17
Pin Rouge	DOC-rich	L5	Mesotrophic	45°57′	74°02′	14.0	8.6	0.04	11.16	1.75	7.29	2.28	11.06
Noir	DOC-rich	L6	Mesotrophic	45°58′	74°01′	–	9.6	0.05	20.68	1.25	7.43	2.73	15.39
Violon	Incubation	–	Ultra-oligotrophic	45°56′	74°05′	22.5	3.5	0.01	3.90	5.50	7.55	0.66	1.17

*Secchi disc reached the lake bottom at the sampling site.

versus three DOC-rich lakes (>8.5 mg L^{-1})]. Clear water conditions (DOC, 3.5 mg L^{-1}) were attained in half of the enclosures with 54-μm-filtered water taken directly from Lac Violon. In the other half of enclosures, DOC enrichment, characterised as brown water (DOC, 15.0 mg L^{-1}), was achieved by addition to Lac Violon water of SuperHume (http://www.earthcrew.com/SuperHume.htm), a naturally occurring humic substance that has recently been used for studying ecological consequences of terrestrial dissolved organic carbon in aquatic food webs (Lennon *et al.*, 2013). Lake source was included as a random variable in the model (three different lakes per zooplankton source category).

The transparent, polyethylene enclosures were suspended in Lac Violon at a depth of 8 m on anchored, floating rafts and were representative of epilimnetic light and temperature conditions within this lake. Each enclosure was 1 m in diameter and 6 m deep, filled with double-screened (100-μm and then 54-μm Nytex mesh) epilimnetic water from Lac Violon, to remove crustacean zooplankton but to allow most algal species to pass through. They were filled up to a volume of 5000 L on 14 and 15 June 2012, and phytoplankton were allowed to equilibrate for one week before stocking with zooplankton. While half of the enclosures received no further water treatment and named as 'clear water' treatment, an increase in DOC in the second half of the enclosures was achieved by two additions of SuperHume® [first addition was at same time as they were filled with water, and the second addition was one week later (23 June 2012)] to reach the desired concentration of 15.0 mg L^{-1} called brown water treatment. The SuperHume was added as a liquid concentrate and well mixed in the enclosures at the time of filling. It raised the DOC concentration from 3.5 ± 0.5 mg L^{-1} to 14.9 ± 1.8 mg L^{-1} without acidifying the water ('brown water').

On 21–22 June 2012, samples of zooplankton were collected from the source lakes for inoculation into the enclosures at similar concentrations to those found in the respective source lake. Live epilimnetic zooplankton was collected from source lakes by vertical tows with 30-cm-diameter 54-μm mesh Nytex nets and transferred into 20-L polyethylene containers (four containers had the equivalent for 5000 L mesocosm stocking) that were stored inside coolers at approximately lake temperature. All zooplankton were gently stocked into randomly selected enclosures (with respect to water treatment) within 5 h of collection from the source lake. We also collected one 5 m vertical haul from each source lake at the time of enclosure stocking. Animals in that sample

were anaesthetised with carbon dioxide and preserved in a buffered 4% sugar-formalin solution for later counting. Week 0 (2 July 2012) of zooplankton sampling occurred one week after the second addition of SuperHume. The first 10 days before week 0 of the experiment were an adjustment period for DOC concentrations in enclosure enclosures with brown water (although zooplankton had already been stocked into the enclosures). In the last two weeks of June, we gradually raised DOC conditions to target concentrations of 15 mg L^{-1}. Therefore, zooplankton in the enclosures were exposed to a gradual increase in DOC concentration over the adjustment period of 10 days prior to week 0 of the experiment, after which time DOC concentrations were held at 15 mg L^{-1} for the 8 week experiment. Sampling of physicochemical conditions, basal resources and zooplankton communities in each enclosure was conducted every second week for 8 weeks in summer 2012 (end of the experiment on 3 September 2012).

A consideration in our experiment was the influence of DOC on the attenuation of ultraviolet (UVB) radiation and the possibility of direct damage to the zooplankton or alteration of their vertical migration (Schindler & Curtis, 1997; Boeing *et al.*, 2004). Epilimnetic zooplankton that were stocked into enclosures were collected from each source lake over a 5-m-depth interval from the surface, and UVB radiation effects extended to a maximum depth of 1.5 m in the clearest lake (DOC 2.85 mg L^{-1}, Table 1; Scully & Lean, 1994; Morris *et al.*, 1995). Furthermore, in the enclosures, calculations of 1% UVB attenuation depths were 0.5–1 m in clear water enclosures and 0.07–0.10 m in the brown water enclosures (Scully & Lean, 1994; Morris *et al.*, 1995). Since the enclosures extended to 6 m depth in Lac Violon, the enclosures were sufficiently deep to provide refuge from UVB. It is, therefore, highly unlikely that UVB influenced our results.

Physicochemical conditions

Water chemistry was sampled from all enclosures to ensure that target concentrations of DOC were sustained and did not drift significantly during the experiment. Water chemistry parameters included total phosphorus (TP), coloured dissolved organic carbon or absorption at 440 nm (CDOM) and dissolved organic carbon (DOC). Physical conditions were characterised with a multiparameter YSI Professional series sonde (model 10102030; Yellow Springs Inc., Yellow Springs, Ohio, USA) and included temperature (T), dissolved oxygen (DO), pH and conductivity (C) at 0.5 m depth in each enclosure. In the laboratory, the DOC concentration was measured in water samples filtered through of 0.45 μm filters (surfactant-free membrane filters) after acidification (phosphoric acid 5%) followed by sodium persulphate oxidation using a 1010 TOC analyser (O.I. Analytical, College Station, TX, U.S.A.). The absorption at 440 nm (CDOM), used as an index of water colour, was measured on water samples with a 2-cm quartz cuvette in a BiochromUltrospec® 2100 pro spectrofluorometer (Cuthbert & del Giorgio, 1992). Total phosphorus was measured spectrophotometrically on the same instrument by the molybdenum blue method after persulphate digestion (Griesbach & Peters, 1991).

Basal resources

To measure responses of basal resources in the presence and absence of DOC enrichment, chlorophyll *a* (chl *a*) was measured as a surrogate of algal biomass and bacterial production (BP) was measured to estimate bacterial activity during the experiment. Chl *a* was quantified by passing samples through glass fibre filters (Whatman GF/F), extraction of the Chl *a* in hot ethanol and measuring the chlorophyll spectrophotometrically on a BiochromUltrospec® 2100 pro with a 10-cm quartz cuvette (Winterman & de Mots, 1965; Sartory & Grobelaar, 1984). Rates of bacterial production (BP) were determined by incorporation of tritiated leucine (40 nM final concentration) in bacterial protein synthesis (Smith & Azam, 1992; Kirchman, 1993). The samples were incubated for 60 min in the brown at ambient water temperature. Incubation was stopped by adding 100% trichloroaceticacid (TCA). Samples were stored at 4 °C prior to counting in a Packard Tri Carb Liquid Scintillation Analyser, model 2800 TR. While the samples were not incubated *in situ* during the measurement of bacterial production, rates reflect the recent history of the conditions in each individual enclosure.

At week 4 of the experiment (the mid-point), we determined the species composition and relative density of the phytoplankton. One replicate of each water treatment × zooplankton source combination was randomly selected for characterisation of phytoplankton in the enclosures. The phytoplankton was sampled with a vertical integrator tube from the 5 m water column present in the enclosures. A subsample of this integrated water was fixed using Lugol's solution (elemental iodine and potassium iodide in water). Identification was performed to genus using an inverted microscope (Olympus 1X71; Olympus, Tokyo, Japan) and following standard protocol (U.S. EPA, 2012). A minimum of 400

natural algal units were counted and identified per sample. Phytoplankton linear dimensions were assessed using a digital camera (Olympus U-T MAD) mounted on the microscope and converted to biovolume using method of Hillebrand *et al.* (1999).

Crustacean zooplankton

Crustacean zooplankton were sampled from each enclosure once every two weeks for 8 weeks from 2 July to 3 September 2012. Two vertical hauls were taken from 5 m depth to the surface with a 15-cm-diameter 54-μm mesh net (9% of enclosure volume). Zooplankton from both hauls were pooled, anaesthetised with carbon dioxide and preserved in a buffered 4% sugar-formalin solution for later counting. Crustacean zooplankton were identified and population densities were counted using a high-resolution dissecting microscope (SZ2-IL-ST; Olympus SZ). Taxonomic keys used included Thorp & Covich (2010) and Witty (2004) for general identification, and Smith & Fernando (1978) for copepods. The key of De Melo & Hebert (1994) was used to identify Bosminidae and Haney *et al.* (2013) as a visual general key. Calanoid and cyclopoid nauplii were combined. Crustacean zooplankton were counted using a protocol that targeted mature individuals that could be identified unambiguously to species, as well as to detect rare species (Girard & Reid, 1990). Subsamples (10 mL) were taken from a standardised 50 mL sample volume, and at least 250 individuals were counted so that no more than 50 copepodids per order and no more than 30 nauplii per order were included in the sum to 250 individuals, even though more were counted. Total zooplankton biomass, the biomass of major taxonomic groups (calanoid and cyclopoid copepods and cladocerans) and species-specific biomass (mass m^{-3}) were calculated using standard regression for biomass calculation from Culver *et al.* (1985).

For intraspecific differences in body size, we focussed on three dominant copepod species: the calanoid *Leptodiaptomus minutus,* and the cyclopoids *Cyclops scutifer* and *Tropocyclops prasinus mexicanus* (because of their high initial density and their presence among both zooplankton sources lakes). A photograph of each adult individual was taken in each sample, up to a maximum of 60 individuals, with a 10× dissecting microscope camera (SZ2-IL-ST, Olympus SZ, Japan Camera system Olympus DP21). Body size measurements were pooled across replicates from each lake, such that the minimum number of adults measured was six and the maximum number of adults was 190 for each source lake. The total length of adult copepods did not include the caudal setae, as recommended by McCauley (1984), and was measured from digital photographs using Olympus camera–microscope software (Olympus DP21-HS).

Statistical analyses

For physicochemical conditions and basal resources in experimental enclosures, the focus was on differences between treatments, and time interactions with these treatments, using repeated-measures (RM) MANOVAs. Square root transformations were carried out for chl *a*, TP, conductivity, DOC and CDOM to improve homogeneity of variance and normality. Bacterial production, dissolved oxygen and pH were not transformed before analyses because those data were normally distributed. If significant interactions between main effects and with time were detected ($P < 0.05$), then we tested these with least square mean contrasts and Greenhouse–Geisser tests (Quinn & Keough, 2002) in JMP 7© 2007 by SAS Institute Inc., Cary, NC, U.S.A. For phytoplankton composition, principal component analysis (PCA) was performed on Hellinger-transformed (Legendre & Gallagher, 2001) biovolume of genera (mg m^{-3}) (R studio version 0.97.248, 2014) to detect differences in the composition between enclosures. Only the 12 most abundant species are shown in biplots to simplify graph interpretation, but all species were included in the PCA.

Community-level metrics included all 31 zooplankton taxa identified in enclosures (20 cladoceran and 11 copepod species). Univariate community response variables included Shannon–Wiener (S-W) diversity, species richness and total community biomass. Multivariate community structure was revealed by Nonmetric Multidimensional Scaling (NMDS) on Bray–Curtis dissimilarity matrices of the communities at week 0 and at week 8. NMDS was performed on all zooplankton species using the ADONIS function in the Vegan package (HStevens@muohio.edu, adapted to vegan by Jari Oksanen.) in R (R Core Team, 2014). Unidentifiable immature copepods (copepodite and nauplii) were excluded from all diversity and density analyses, but were included in analyses of total community biomass. Of the 31 zooplankton taxa, most species had variable presence/absence depending on lake source and occurred in low densities when present. For this reason, we focussed on the density of five species present in all lakes for individual species responses in the enclosures (copepods *Acanthocyclops robustus*, *Cyclops scutifer*, *Leptodiaptomus minutus* and *Tropocyclops prasinus mexicanus,* and the cladoceran *Bosmina longirostris*). Of these five focal taxa, we measured body size for three dominant copepods

(*C. scutifer*, *L. minutus* and *T. prasinus mexicanus)*. Two taxa were excluded from analyses despite their density at certain times during the experiment: *Chydorus* sp. and *Holopedium gibberum*. *Chydorus* sp. is a littoral taxon, whereas our focus was on pelagic species. *Holopedium gibberum* was removed because it disappears from Laurentian lakes by the end of June, and so changes in density during the experiment reflect seasonal rather than treatment effects.

For analysis of the densities of individual species, we focussed on initial and final effects rather than time interactions, because we needed to separate the effects of different stocking concentrations collected from different lakes from our response variables. To do this, we calculated Ln-transformed proportions of final/initial values. For week 0 of the experiment, this was calculated as $\mathrm{Ln}(N_i + 1) - \mathrm{Ln}(N_s + 1)$, where N_i is the initial diversity, richness, total biomass or species-specific density in week 0 in a given enclosure, and N_s is the equivalent response variable (but directly measured from a given source lake immediately prior to mesocosm stocking). For week 8 of the experiment, this was calculated as $\mathrm{Ln}(N_f + 1) - \mathrm{Ln}(N_i + 1)$, where N_f is the final diversity, richness, total biomass or density of a particular species in week 8 in a given enclosure and N_i is the initial diversity, richness, total biomass or species-specific density in week 0 in a given enclosure. For week 8, we used N_i rather than N_s as the initial value, to eliminate the effects of loss associated with stocking the enclosures and the 10-day adjustment period during which the DOC was gradually raised. The proportions were Ln-transformed to bring data distributions as close to normality as possible. These proportions were scaled by +6 to eliminate negative values from statistical models.

To test the community response with the univariate proportions described above, we conducted factorial ANOVAs with two fixed factors: water treatment (WT; clear water versus brown water) and zooplankton source type (ZS; DOC-poor versus DOC-rich lakes). However, since residuals were generally non-normally distributed, despite ln-transformation, we used type III permutational multivariate analysis of variance (PERMANOVA) with 999 permutations of residuals to create distribution-free data (Anderson, Gorley & Clarke, 2008) and calculated a pseudo-*F* that is equivalent to the *F* statistic produced using traditional ANOVA (Anderson, 2001). Significant terms and interactions were investigated using *a posteriori* pairwise comparison ($P < 0.05$). PERMANOVA analyses were performed using PRIMER v. 6.1.11 (Clarke & Gorley, 2006) with PERMANOVA+1.0.1 add-on package) (Anderson *et al.*, 2008).

Copepod population body size among source lake types at the time of enclosure stocking (N_s; DOC-rich lakes versus DOC-poor lakes) was tested by a pairwise univariate nonparametric Wilcoxon test on non-normal data. Final copepod body size in enclosures at week 8 of the experiment (N_f) was analysed with a linear mixed model (LMM), because a random variable (lake source) was introduced into the model and because residuals had a normal or near normal distribution (Bolker *et al.*, 2009). Fixed effects were as described above for overall numbers of zooplankton and of individual species density, but lake source was included as an additional random variable, because body size measurements were pooled across three replicate enclosures for each of six source lakes. Significant overdispersion was not detected in the linear mixed model analysis. A baseline model was constructed with all the possible interactions and main effects, and the best-fitting model was selected as the one minimising the Akaike information criterion (AIC) (Burnham & Anderson, 2002). Linear mixed model analysis was undertaken using the lmer function in the lme4 package (Bates *et al.*, 2014) in R (R Core Team, 2014).

Results

Phenotypic variation of zooplankton in source lakes along DOC gradient

Of the species of zooplankton that were measured for body size, all three copepods (*Leptodiaptomus minutus*, *Cyclops scutifer* and *Tropocyclops prasinus mexicanus*) were consistently larger in DOC-rich source lakes than in DOC-poor lakes (Fig. 1). Individuals of the calanoid *L. minutus* from DOC-rich lakes (mean 923.5 ± SE 10.7 μm) were significantly larger than individuals from DOC-poor lakes (822.6 ± 10.3 μm) (Wilcoxon test, $Z[1, 203] = -5.91$, $P < 0.01$]. Cyclopoid copepods *C. scutifer* (DOC-rich lakes, 1368.0 ± 23.8 μm; DOC-poor lakes, 1167.0 ± 21.1 μm) [Wilcoxon test, $Z(1, 266) = -5.78$, $P < 0.01$] and *T. p. mexicanus* (DOC-rich lakes, 636.5 ± 10.7 μm; DOC-poor source lakes, 531.6 ± 10.3 μm) [Wilcoxon test, $Z(1, 163) = 4.36$, $P < 0.01$] also followed a pattern of larger individuals in DOC-rich lakes compared with clear lakes.

Enclosure experiment: physicochemical conditions

Contrasting concentrations of DOC (mg L^{-1}, means ± SE) were achieved by humic addition to half

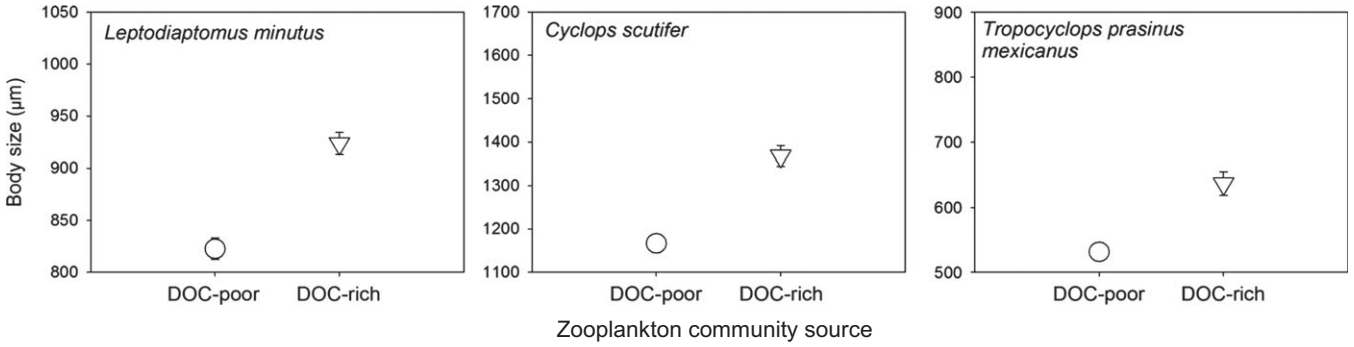

Fig. 1 Mean (±SE) body size (length, µm) of three dominant copepod species (calanoid *Leptodiaptomus minutus*, and the cyclopoids *Cyclops scutifer* and *Tropocyclops prasinus*) from DOC-rich and DOC-poor source lakes at the start of the experiment.

of the enclosures (brown water enclosures, 14.9 ± 1.8: clear water enclosures, 3.5 ± 0.5). These differences were maintained throughout the experiment with no further humic addition after week 0 (water treatment main effect and water treatment × time interaction; Table S1; Fig. 2a). Brown water enclosures had lower dissolved oxygen concentrations compared with clear water enclosures (water treatment main effect; Table S1; Fig. 2b).

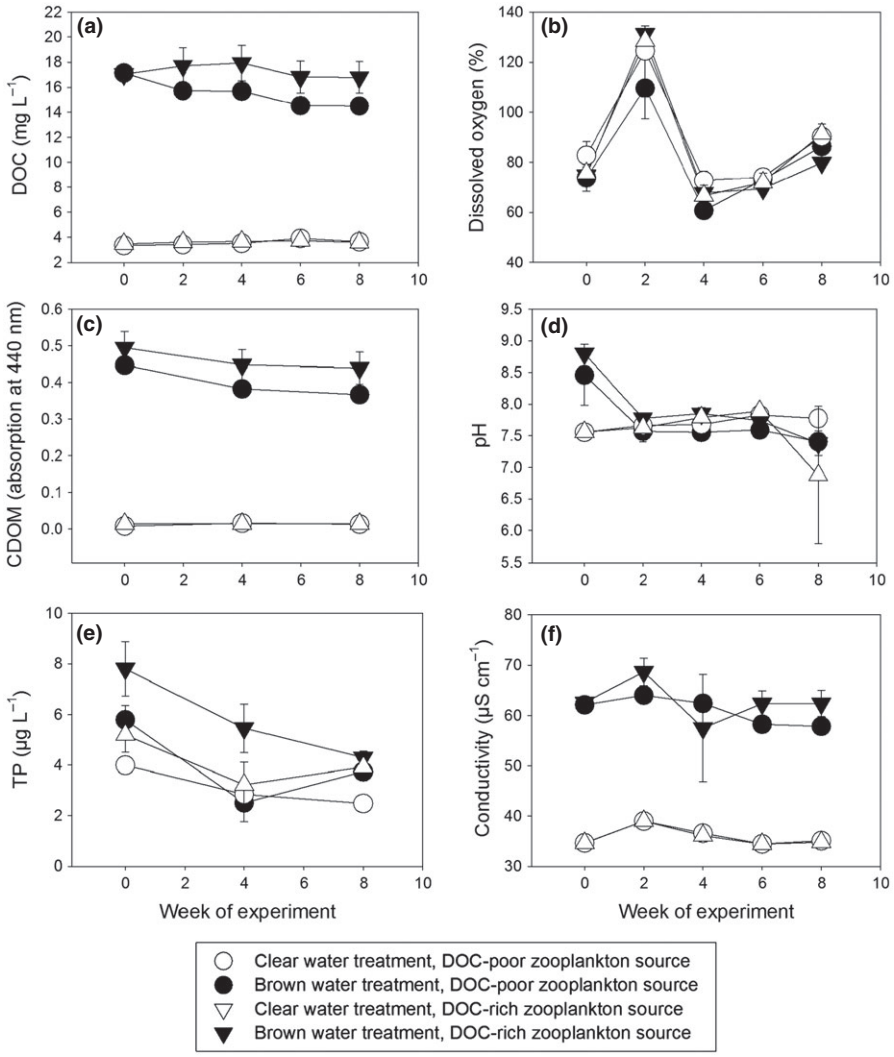

Fig. 2 Physicochemical water conditions in enclosures over the 8 weeks of the experiment (means ± SE) of (a) dissolved organic carbon (DOC, mg L^{-1}), (b) dissolved oxygen concentration (DO, mg L^{-1}), (c) coloured dissolved organic matter (CDOM, absorption at 440 nm), (d) pH, (e) total phosphorus concentration and (f) conductivity (µS cm^{-1}).

Water colour (CDOM) followed DOC concentration and remained higher in the brown water than in clear water enclosures (water treatment main effect and water treatment × time interaction; Table S1; Fig. 2c). The pH did not vary significantly through time or with treatment (Fig. 2d; Table S1). Total phosphorus concentration was slightly higher in brown water enclosures and varied over time, but zooplankton from lakes high in DOC also had a positive significant effect on the TP (water treatment main effect, water treatment × time interaction and zooplankton source main effect; Table S1; Fig. 2e) possibly because of differences in nutrients among source lakes (more TP in DOC-rich than in DOC-poor lakes; Fig. 2e; Table S1) and water that was transferred to enclosures in association with zooplankton stocking. Also, Lac Violon water was probably sensitive to any TP addition given its ultra-oligotrophic status (Table 1). Conductivity was more elevated in DOC-rich enclosures compared to clear water enclosures, but remained sufficiently low as to not be expected to influence community and population responses between water treatments (Fig. 2f; Table S1). No effects of zooplankton source were detected on any of the physicochemical conditions (except for TP) in the enclosures (Table S1).

Enclosure experiment: basal resources

Total algal biomass, as measured by chlorophyll *a* concentration, did not respond to humic addition over the 8-week experiment (Fig. 3a; Table S1). Bacterial production increased initially in response to humic addition and this was maintained in the brown water enclosures for up to 6 weeks, after which it declined in the brown water enclosures and was then similar to the clear water enclosures (Fig. 3b; Table S1). Composition of the phytoplankton responded to water treatment: in particular, brown water favoured a higher density of cyanobacteria (*Microcystis* sp.), compared with clear water enclosures (Fig. 3c). In clear water, enclosures with zooplankton from lakes rich in DOC had a greater density of the non-edible green algae *Mougeotia* sp. (Fig. 3c) and enclosures with zooplankton from DOC-poor lakes had a

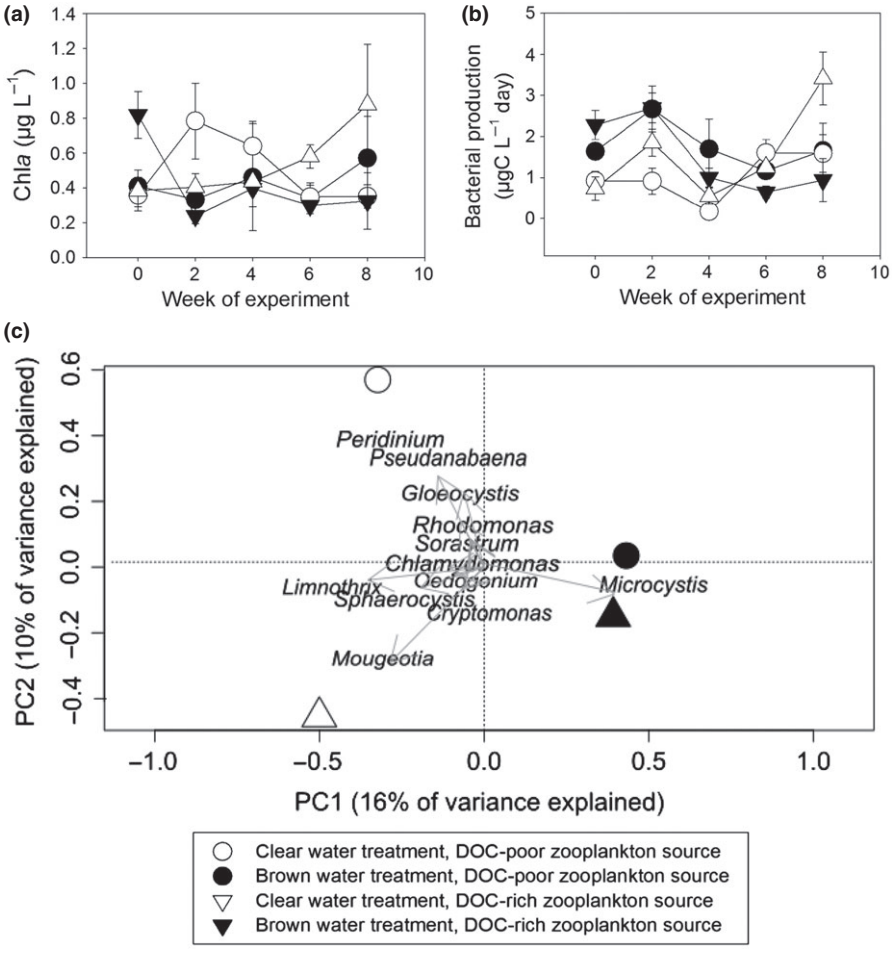

Fig. 3 Basal resources in enclosures over the experiment: (*a*) total chlorophyll *a* concentration (mean ± SE, Chl*a*, µg L^{-1}) over the 8 week duration of the experiment; (b) bacterial production (mean ± SE, µg C L^{-1}·day); (c) phytoplankton composition (PCA of biovolumes of genera) in week 4.

higher density of the dinoflagellate *Peridinium* sp., the filamentous cyanobacterium *Pseudanabaena* sp. and the green alga *Gloeocystis* sp. (Fig. 3c).

Enclosure experiment: the crustacean zooplankton

Humic addition reduced zooplankton species richness and total biomass, but not Shannon–Wiener diversity at week 0, and all community metrics were reduced in the brown water treatment by week 8 (Table 2; Fig. 4). The DOC-rich source zooplankton (with larger copepod body sizes; Fig. 1) had higher total biomass compared with the DOC-poor source zooplankton under the clear water treatment, but not the brown water treatment (after statistically removing the effects of differences in stocking density among source lakes; water treatment × zooplankton source interaction in week 0, $P = 0.001$ pairwise test on PERMANOVA; Table 2; Fig. 4c). There was also an interaction between water treatment and zooplankton source in the NMDS ordination on Bray–Curtis dissimilarity matrices of zooplankton composition in week 0 (water treatment × zooplankton source in week 0, $P = 0.001$ pairwise test on PERMANOVA; Table 2; Fig. 4d). Main effects of water treatment and zooplankton source persisted in the NMDS of Bray–Curtis similarity of the zooplankton in week 8 (Table 2; Fig. 4e), but no univariate community source effects were detected by the end of the experiment (Table 2; Fig. 4a,b,c).

There were differences in the response of individual species densities in response to humic addition. First, the density of the cyclopoid copepods *Cyclops scutifer* and *Tropocyclops prasinus mexicanus* immediately declined in brown water compared with clear water in week 0 and remained at lower densities in brown water enclosures until week 8, irrespective of zooplankton source (Table 2; Fig. 5b,d). Second, the small cladoceran *Bosmina longirostris* had also declined in brown water enclosures by week 8, but this reduction in density depended on zooplankton source: *Bosmina* from lakes rich in DOC declined more

Table 2 Initial (week 0) and final (week 8) results for community metrics Shannon–Wiener (S-W) diversity, species richness, total biomass and Bray–Curtis dissimilarity, and for individual species responses in density [$\mathrm{Ln}(N_f + 1) - \mathrm{Ln}(N_i + 1) + 6$] in week 0 and 8. The five focal species were *Acanthocyclops robustus, Bosmina longirostris, Cyclops scutifer, Leptodiaptomus minutus* and *Tropocyclops prasinus mexicanus*. Results are from a 2 × 2 × 3 factorial PERMANOVA with water treatment and zooplankton source as fixed effects and lake source as a random effect (lake-specific *P-values* not shown here) under 999 permutations

	Community structure				Individual species				
PERMANOVA	S-W diversity	Species richness	Total biomass	Bray–Curtis dissimilarity	*T. prasinus mexicanus*	*C. scutifer*	*A. robustus*	*L. minutus*	*B. longirostris*
Week 0									
d.f. effect	1	1	1	1	1	1	1	1	1
d.f. total	29	29	29	32	29	29	29	29	29
Water treatment									
Pseudo-F	–	32.58	32.08	16.48	6.39	5.16	–	–	–
P(perm)	–	**<0.01**	**<0.01**	**<0.01**	**0.01**	**0.02**	–	–	–
Zooplankton source									
Pseudo-F	–	–	8.58	3.05	–	14.51	19.08	42.33	18.37
P(perm)	–	–	**<0.01**	**0.01**	–	**<0.01**	**<0.01**	**<0.01**	**<0.01**
WT × ZS									
Pseudo-F	–	–	6.22	3.47	–	–	–	–	–
P(perm)	–	–	**0.01**	**0.01**	–	–	–	–	–
Week 8									
d.f. effect	1	1	1	1	1	1	1	1	1
d.f. error	29	29	29	32	29	29	29	29	29
Water treatment									
Pseudo-F	8.05	4.38	6.22	6.87	4.60	4.07	–	27.39	18.27
P(perm)	**<0.01**	**0.05**	**<0.01**	**<0.01**	**0.03**	**<0.01**	–	**<0.01**	**<0.01**
Zooplankton source									
Pseudo-F	–	–	–	1.88	–	–	–	7.49	–
P(perm)	–	–	–	**0.05**	–	–	–	**<0.01**	–
WT × ZS									
Pseudo-F	–	–	–	1.69	–	–	–	–	8.70
P(perm)	–	–	–	0.08	–	–	–	–	**<0.01**

Significant *P*-values ≤ 0.05 are indicated in bold.

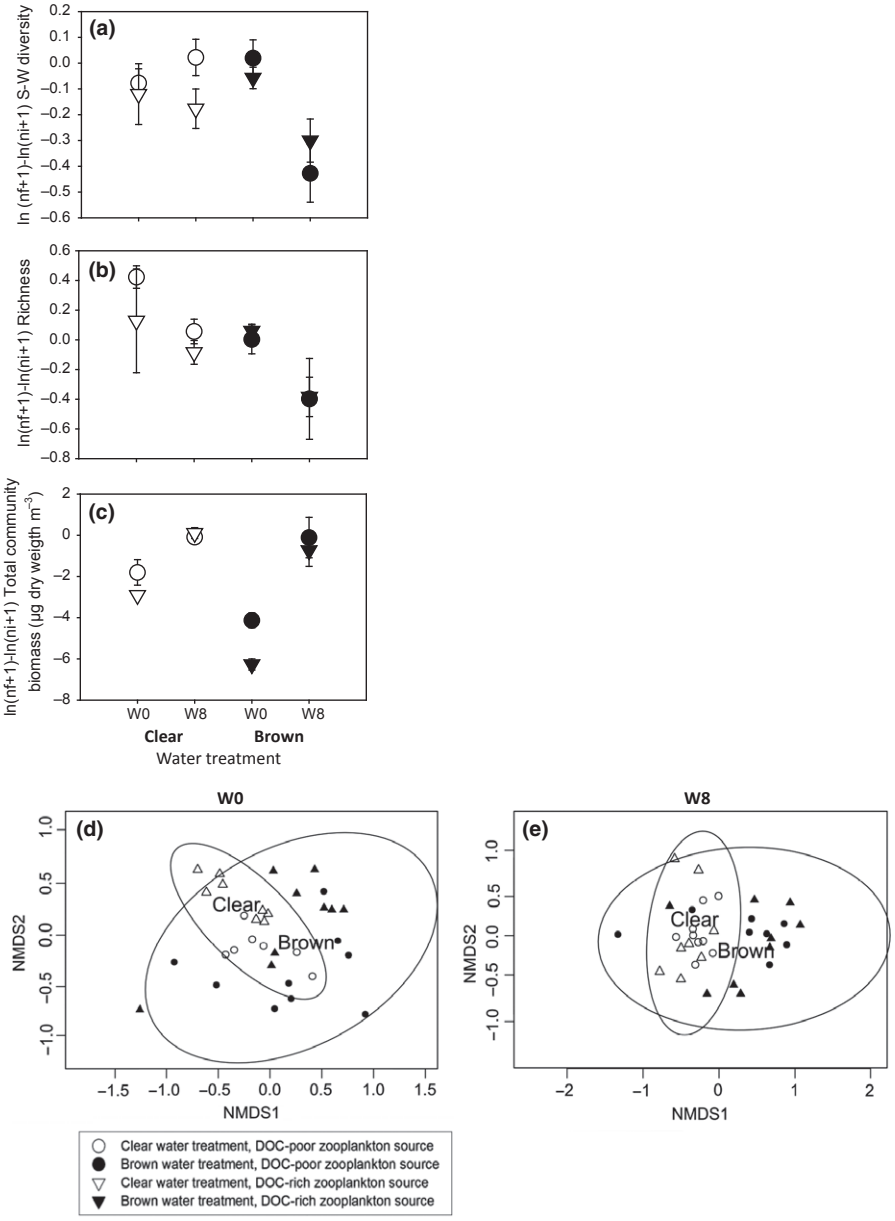

Fig. 4 Zooplankton response to water treatment (clear water versus brown water) and zooplankton source (DOC-poor lakes versus DOC-rich lakes) between week 0 and week 8. For univariate metrics, this was calculated as [Ln (N_i + 1) − Ln(N_s + 1) + 6] in week 0 (N_i is the initial diversity, richness, biomass or species-specific density in week 0 in a given enclosure, and N_s is the equivalent response variable but directly measured from a given source lake immediately prior to mesocosm stocking). In week 8, univariate metrics were calculated as [Ln (N_f + 1) − Ln(N_i + 1) + 6] (N_f is the final diversity, richness, biomass or species-specific density in week 8 in a given enclosure and N_i is the initial diversity, richness, biomass or species-specific density in week 0 in a given enclosure). For week 8, we used N_i rather than N_s as the initial value to eliminate the effects of loss associated with stocking the enclosures and the 10-day adjustment period during which the DOC was gradually raised in the brown water treatment. Figure (a) represents Shannon–Wiener (S-W) diversity, (b) species richness, (c) total community biomass, (d) NMDS ordination of global community structure at week 0 and (e) NMDS ordination of global community structure at week 8 of the experiment. For univariate data (a–c), plots show mean ± SE and scales differ between panels.

than those from clear water lakes (water treatment × zooplankton source in week 0, $P = 0.003$ pairwise test on PERMANOVA; Table 2; Fig. 5e). Third, there was no detectable effect of DOC addition on the density of the cyclopoid *Acanthocyclops robustus* (Table 2; Fig. 5a). Fourth, the calanoid copepod *Leptodiaptomus minutus* was the only species to increase in density in the brown water treatment after 8 weeks (Table 2; Fig. 5c).

Zooplankton source effects in species-specific densities were detected in four of five focal species in week 0, but these effects persisted to week 8 in only one species (Table 2). In week 0, the densities of *C. scutifer*, *L. minutus* and *B. longirostris* from DOC-poor lakes were greater than from DOC-rich lakes across water treatments (Fig. 5b,c,e), but this pattern held until week 8 only for *L. minutus* (Table 2; Fig. 5c). By contrast, the density of *A. robustus* from DOC-rich lakes was greater than that from DOC-poor lakes across water treatments in week 0, but this pattern disappeared by week 8 (Table 2; Fig. 5a).

The pattern of larger individuals in the zooplankton sourced from DOC-rich compared to DOC-poor lakes, observed at the beginning of the experiment and persisted to 8 week in *L. minutus and C. scutifer* (Table 3; zooplankton source effect, LMM; Fig. 6). No body size differences were observed among treatments in *T. p. mexicanus* over the course of the experiment (Table 3, Fig. 6).

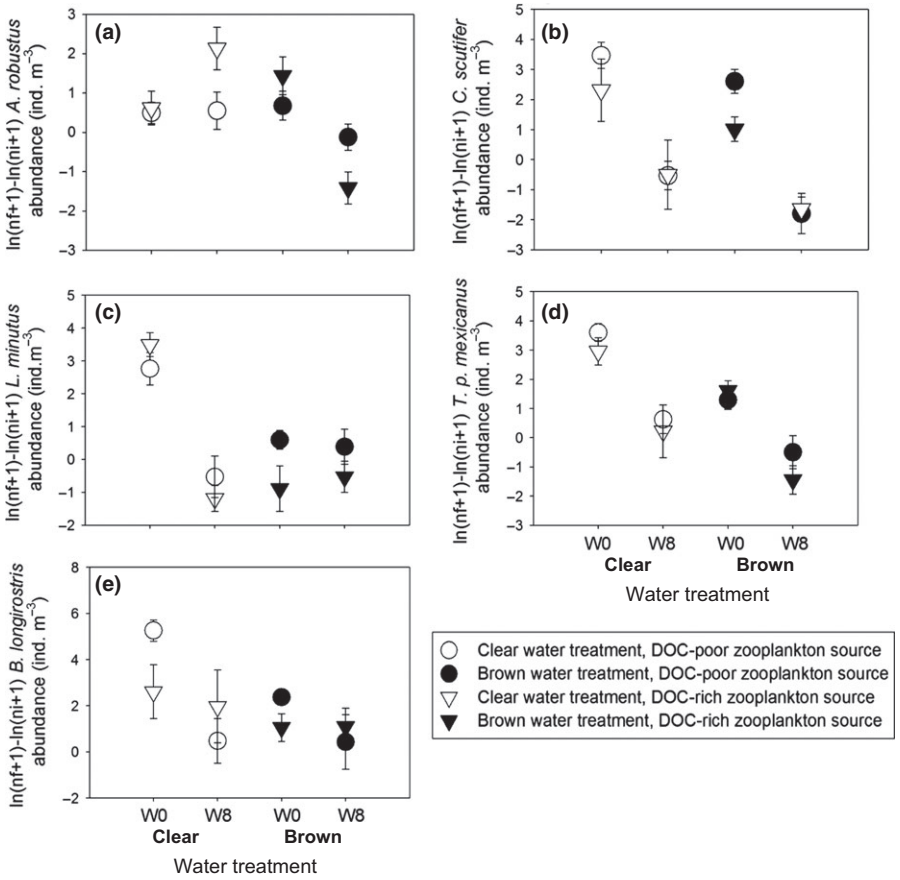

Fig. 5 Individual species responses in density to water treatment (clear water versus brown water) and zooplankton source type (DOC-poor versus DOC-rich lakes) for the five focal taxa that were abundant across zooplankton sources and water treatments between week 0 [$\mathrm{Ln}(N_i + 1) - \mathrm{Ln}(N_s + 1) + 6$] and week 8 [$\mathrm{Ln}(N_f + 1) - \mathrm{Ln}(N_i + 1) + 6$], in which N_i is the initial diversity, richness, biomass or species-specific density in week 0 in a given enclosure, N_s is the equivalent response variable but directly measured from a given source lake immediately prior to mesocosm stocking, and N_f is the final diversity, richness, biomass or species-specific density in week 8 in a given enclosure. For week 8, we used N_i rather than N_s as the initial value to eliminate the effects of loss associated with stocking the enclosures and the 10-day adjustment period during which the DOC was gradually raised in the brown water treatment. (a) *Acanthocyclops robustus*, (b) *Cyclops scutifer*, (c) *Leptodiaptomus minutus*, (d) *Tropocyclops prasinus mexicanus*, (e) *Bosmina longirostris*. Scales differ from one panel to another.

Discussion

The zooplankton from DOC-rich lakes was not more resistant to the addition of a humic stressor than the communities from DOC-poor lakes, in terms of species diversity, richness, total community biomass, and species composition and relative abundance as measured by NMDS (Fig. 4), as well as individual species responses in density (Fig. 5). This is despite phenotypic variation in body size between DOC-rich and DOC-poor source lakes in at least three of the dominant copepod species (Fig. 1), which could have potentially caused divergent responses between these community types to humic addition in the enclosure experiment. The pattern of larger intraspecific individuals from DOC-rich source lakes persisted in two copepod species by the end of the eight-week experiment (*Leptodiaptomus minutus* and *Cyclops scutifer*; Table 3, Fig. 6). Body size of zooplankton is thought to have ramifying effects on communities because of its relationship with survival, metabolic rate and fecundity (Peters, 1983; Hart & Bychek, 2011). Being bigger can be adaptive in stressful environments, because of greater competitive ability, starvation resistance, greater energy reserves and higher fecundity (Peters, 1983; Hart & Bychek, 2011). However, we saw no such effects, associated with zooplankton body size, in response humic addition. The small differences in copepod body size between source lakes (≅0.1–0.2 mm) may have been

Table 3 Result of the linear mixed models (LMM) in which the body sizes of *Leptodiaptomus minutus*, *Cyclops scutifer* and *Tropocyclops prasinus mexicanus* in week 8 of the experiment were the response variables in a model with two fixed factors (water treatment and zooplankton source) and one random factor (lake source)

Fixed effects	d.f.	*t*	*P*
Leptodiaptomus minutus			
Water treatment	23.33	27.82	0.07
Zooplankton source	23.88	2.58	**0.02**
Water treatment × Zooplankton source	24.87	−0.37	0.71
Cyclops scutifer			
Water treatment	19.66	2.08	0.33
Zooplankton source	16.32	1.01	**0.05**
Water treatment × Zooplankton source	25.23	−0.40	0.69
Tropocyclops prasinus mexicanus			
Water treatment	6.60	1.87	0.11
Zooplankton source	4.23	0.38	0.72
Water treatment × Zooplankton source	16.58	0.71	0.49

Significant *P*-values ≤ 0.05 are indicated in bold.

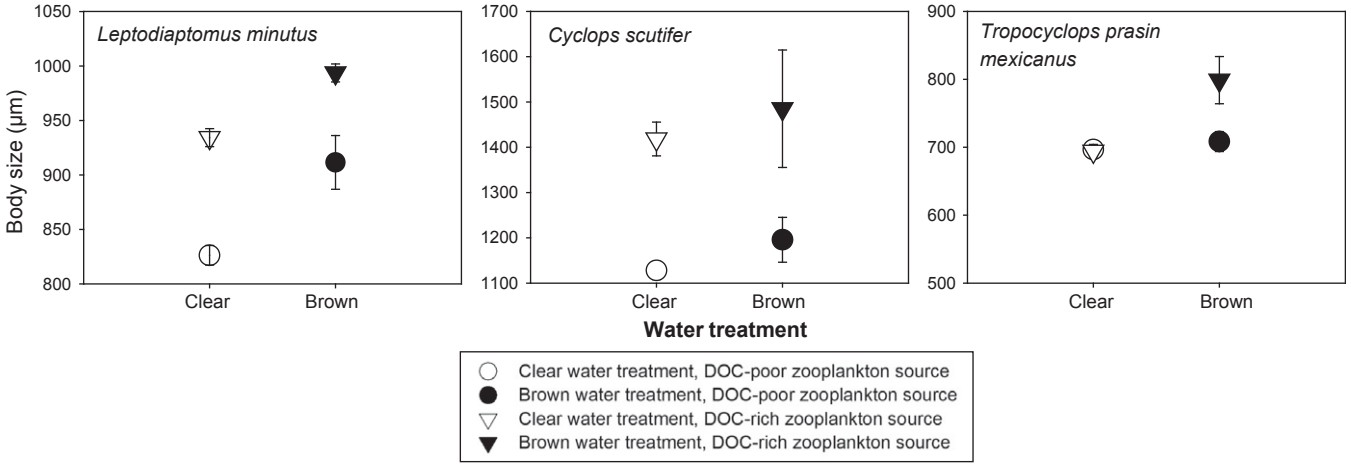

Fig. 6 Mean (n = 3) (±SE) body length (µm) of the three dominant copepod species (calanoid *Leptodiaptomus minutus,* and the cyclopoids *Cyclops scutifer* and *Tropocyclops prasinus*). Mean body size was calculated on copepods pooled from the three lakes within each zooplankton source category (DOC-rich versus DOC-poor lakes) under a brown or a clear water treatment in week 8 of the experiment.

insufficient to alter resource depletion or cause differences in survival or reproduction over the relatively brief time period of the experiment. Our study suggests that, as north temperate lakes become browner (Monteith *et al*., 2007; Zhang *et al*., 2010; Larsen *et al*., 2011), zooplankton in DOC-rich and DOC-poor lakes will respond similarly in terms of diversity, total biomass and density in the short term.

We detected phenotypic variation in the body size of three copepod species between DOC-rich and DOC-poor source lakes. *Cyclops scutifer*, *Leptodiaptomus minutus* and *Tropocyclops prasinus mexicanus* all had populations consisting of larger individuals in DOC-rich than in DOC-poor lakes (Fig. 1). This finding suggests a general pattern of modest gains in size for both calanoid and cyclopoid copepods in waters with low transparency. Carter *et al*. (1983) compared calanoid copepod body size across a freshwater DOC gradient involving 58 lakes and also found larger individuals of *L. minutus*, but not *Skistodiaptomus oregonensis*, in DOC-rich waters. The lake sources chosen for this experiment were relatively small relative to other Laurentian lakes and contained a similar zooplankton. In particular, there was an abundance of *T. prasinus mexicanus*, which is most numerous in small Laurentian lakes, and an absence of large calanoids (*Leptodiaptomus sicilis* and *Leptodiaptomus ashlandi*), that occupy large Laurentian lakes (Pinel-Alloul *et al*., 1990). The intraspecific differences in copepod body size between DOC-rich and DOC-poor lakes could result from: (i) differences in the quality and quantity of basal resources (Karlsson *et al*., 2007; Ask *et al*., 2012; Faithfull *et al*., 2012); (ii) differences in foraging efficiency of visual fish predators (Wissel & Boeing, 2003); and (iii) mild physiological stress (Steinberg *et al*., 2006), or a mixture of these possibilities. The maintenance of copepod body size differences between DOC-rich and DOC-poor lakes could potentially have indirect, extended effects on communities and ecosystems over much longer time periods than what we measured in our study.

The humic stressor unexpectedly negatively influenced the diversity, richness, total biomass, global community structure and density of crustacean zooplankton. This is because DOC can act as a resource in ultra-oligotrophic lakes such as Lac Violon, because of its association with phosphorus (Jones, Solomon & Weidel, 2012) and high bacterial productivity (Jansson *et al*., 2000; Lennon & Pfaff, 2005). There was no decline in pH after the humic addition (Fig. 2d). It is possible that zooplankton in the brown water treatment were limited by algal resources, but we did not find any differences in algal biomass between brown water and clear water enclosures (chlorophyll *a*, Fig. 3a). However, brown water enclosures were more dominated by cyanobacteria (Fig. 3c; *Microcystis* sp.), and cyanobacteria are known to be a less nutritious (and even toxic) to zooplankton (Wilson Sarnelle & Tillmanns, 2006). Further, bacterial productivity was higher in the brown water treatment up to at least the mid-point of the experiment (Fig. 3b). Pelagic bacteria do not contain essential algal-derived compounds, such as polyunsaturated fatty acids (PUFAs), that are necessary for zooplankton reproduction (Arts, Brett & Kainz, 2009). In addition to the indirect effects of humic addition on zooplankton via basal resources, it is also possible that DOC acted as a direct physiological stressor. Zooplankton survival and biomass were

already reduced by week 0 of the experiment in brown water enclosures compared with the clear water enclosures. That is, reductions occurred during the 10-day period before week 0 (when DOC was raised to experimental treatment concentrations), a short time period in which acute rather than chronic effects related to diet are likely to be detected.

Species densities were higher from DOC-poor source lakes than from DOC-rich lakes in almost every species in week 0, but persisted to week 8 in only two species: the calanoid copepod *L. minutus* and the small cladoceran *B. longirostris*. *L. minutus* was denser when sourced from DOC-poor than from DOC-rich lakes, across water treatment (Fig. 5c). For *B. longirostris*, however, the response of density to water treatment depended on zooplankton source: from DOC-rich lakes, density declined in brown water, compared to clear water, but from DOC-poor lakes, it did not (Fig. 5e), the opposite of what we had predicted. One possible explanation for zooplankton source effects in both *L. minutus* and *B. longirostris* is that animals from DOC-rich lakes were in poorer condition (because of a lack of algal food rich in PUFAs despite being larger in body size) than those from DOC-poor lakes at the beginning of the experiment. This explanation would require testing with additional research. Certainly, many studies have found evidence of a varying subsidy of allochthonous (terrestrial) carbon in zooplankton (Rautio, Mariash & Forsstrom, 2011; Berggren *et al*., 2014), although cladocerans are more likely than copepods to ingest bacteria in DOC-enriched lakes (Karlsson *et al*., 2007; Faithfull *et al*., 2012).

The nature of the DOC addition in experiments can be important for the response of zooplankton to simulated browning. Results from other studies that have manipulated DOC in different ways are conflicting. For instance, the density of calanoid copepods has been shown to decrease (transplant of natural DOC-rich surface water; Nicolle *et al*., 2012), increase (glucose addition; Faithfull *et al*., 2012) or stay the same (soil humic addition; Lefébure *et al*., 2013). We manipulated brown water by adding a naturally occurring humic substance for which a laboratory study found no toxic effects on *Daphnia* sp. and similar chemical properties to DOC in aquatic ecosystems (Lennon *et al*., 2013). SuperHume is an organic carbon slurry derived from leonardite, a mineraloid associated with shallow deposits of lignite, generally of peat origin. The organic matter in leonardite is extremely rich in humic acids, and it is for this reason that the substance is commercially mined and used widely as a soil conditioner (Lennon *et al*., 2013). The addition of SuperHume to the enclosures effectively caused 'browning', because the organic carbon added has a high specific absorbance and increased water colour even at low concentrations. In addition, although SuperHume is composed of peat-derived fossil carbon, which should in theory be mostly recalcitrant, our measurements of bacterial production suggest that SuperHume also delivered some labile C that was readily used by bacteria. SuperHume thus resembled terrestrial inputs in terms of delivering highly coloured compounds, as well as carbon that could be used by aquatic bacteria. However, SuperHume did have negative effects on zooplankton survival, in contrast to previous reports by Lennon *et al*. (2013). We have analysed the chemical composition of SuperHume, and it differs in some key aspects from the organic carbon dominant in surface waters, which is derived from either fresh terrestrial inputs or *in situ* primary production (Berggren *et al*., 2010), especially in terms of the dominance of complex, humic-like molecular structures. SuperHume delivers other compounds, in addition to organic carbon, such as nutrients and metals (i.e. iron), and it is possible that these, and not the organic carbon *per se*, caused the negative zooplankton response. The contrasting effects observed between our study and that of Lennon *et al*. (2013) are unclear and may potentially be related to inconsistencies in the composition of SuperHume during the mining process. Regardless of the effectiveness of SuperHume as a proxy of recent terrestrial carbon loading, our results do not support the hypothesis that pre-acclimation/pre-adaptation to high DOC conditions enables zooplankton to cope differently with a DOC-linked stress.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Repeated-measures analysis of variance (RM-ANOVA) by simple factor and interaction effects of time for physicochemical (dissolved organic carbon (DOC), coloured dissolved organic matter (CDOM), total phosphorus (TP), dissolved oxygen (DO), pH, conductivity), and basal resources (total chlorophyll *a* (chl *a*) and bacterial production (BP)).

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