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# Terrestrial dissolved organic matter supports growth and reproduction of *Daphnia magna* when algae are limiting

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We experimentally show that *Daphnia magna* can use terrestrial-derived dissolved organic matter (t-DOM) to support growth and reproduction when alternative food sources are limiting or absent. Unlike previous studies, we restricted available food to limiting algae (0.1 mg C L<sup>-1</sup> of *Scenedesmus obliquus*, provided to all treatments) and bacteria (conditions were not sterile) by excluding heterotrophic protists and running our experiment in darkness to prevent algal growth. *Daphnia* receiving 10 mg t-DOC L<sup>-1</sup> leached from either beech (*Fagus sylvatica*, t-DOM<sub>beech</sub>) or hazel leaves (*Corylus maxima*, t-DOM<sub>hazel</sub>) had significantly higher juvenile growth rates and deposited larger clutch sizes across a range of realistic temperatures (15, 20, 25°C) than *Daphnia* receiving no t-DOM, which failed to deposit eggs. Growth rates of t-DOM-supplied *Daphnia* were similar (0.10 ± 0.01 d<sup>-1</sup>) and clutch sizes were low (<2 eggs female<sup>-1</sup>) across temperatures. Our experimental leachate additions mimic fresh t-DOM inputs in natural systems (e.g. during runoff or precipitation) and suggest that t-DOM and t-DOM-supported bacteria can supplement *Daphnia* growth and reproduction across a range of temperatures even when algae are strongly limiting. Low growth and reproduction rates, however, indicate that t-DOM based resources are unlikely to sustain *Daphnia* populations independently from sufficient algal contributions.

**KEYWORDS:** zooplankton; allochthonous; leaf leachate; growth; algae

## INTRODUCTION

Terrestrial dissolved organic matter (t-DOM) is a major source of energy and nutrients for microbes in freshwater ecosystems (Lennon *et al.*, 2006; Forsström *et al.*, 2013). However, the extent and conditions under which upper trophic level consumers, e.g. zooplankton, use t-DOM as a resource, either directly or indirectly via bacteria or heterotrophic protists (Jansson *et al.*, 2007), remains contentious (Cole *et al.*, 2006; Brett *et al.*, 2009).

While one body of literature has focused on the potential for t-DOM to exert oxidative stress on zooplankton (Euent *et al.*, 2008; Steinberg *et al.*, 2010; but see Hofmann *et al.*, 2012), another has shown that the common cladoceran *Daphnia* can take up dissolved organic carbon directly from ambient water based on radiolabelled tracers (Hessen *et al.*, 1990; Speas and Duffy, 1998). *Daphnia* can also grow on experimentally provided bacterial diets as long as some algae are present, at a 20:80 phytoplankton:bacteria ratio, for example (Taipale *et al.*, 2012). Mesocosm studies have further shown that zooplankton can incorporate some of their biomass from glucose- or t-DOM-supported bacterial production (Salonen & Hammar, 1986; Daniel *et al.*, 2005; Karlsson *et al.*, 2007; Faithfull *et al.*, 2011) and stable isotope studies support the existence of a t-DOM to zooplankton or a t-DOM to bacteria to zooplankton trophic pathway in natural lake ecosystems (Grey *et al.*, 2001; Jones *et al.*, 2001; Karlsson *et al.*, 2003, 2004). The extent of allochthony by zooplankton, however, appears to be context dependent and could be related to the relative availability of algae and t-DOM-supported bacterial production based on recent studies that combine multiple tracers (Berggren *et al.*, 2014; Tanentzap *et al.*, 2014).

In light of the above studies, the conditions under which t-DOM acts as a resource for zooplankton remain to be clarified. No study to date, for example, has explored if and how t-DOM and bacteria, which usually lack n-3 and n-6 fatty acids and are of low biochemical quality (Taipale *et al.*, 2012), can support zooplankton growth and fitness in the absence of sufficient higher quality food sources (e.g. algae or heterotrophic protists). This is important because when food quantity is sufficient, zooplankton growth may be limited by food quality (Bukovinszky *et al.*, 2012), such that any increase in the availability of high quality algal or protist resources could dominate the zooplankton growth response and obscure the actual contribution and importance of lower quality terrestrial resources (Brett *et al.*, 2009). Additional studies are therefore warranted to explore if and how t-DOM resources may support zooplankton growth and reproduction when the availability of higher quality resources is controlled and maintained at a limiting level.

Theoretically, the amount of low quality resources such as t-DOM and t-DOM-supported bacteria should have their largest impact on zooplankton growth under conditions of: (i) very low algal food quantities, those that are below saturation, when quantity is more growth limiting than nutritional quality (Bukovinszky *et al.*, 2012), or (ii) warmer temperatures that increase metabolic need for food quantity. For example, *Daphnia magna* growth rate at food concentrations of 0.1 mg C L<sup>-1</sup> is the same at 15 and 20°C, but increases above 20°C as long as food quantity also increases (Giebelhausen and Lampert, 2001). Additions of t-DOM and bacteria may therefore act to increase food quantity and support growth when the concentration of algal food sources is strongly limiting, and the relative increase in fitness might be larger at higher temperatures.

To examine the role of low algal food concentrations in the potential use of t-DOM as a dietary resource, we conducted a series of controlled life table experiments and tested the effects of t-DOM additions on the growth and reproduction of *Daphnia magna* (“*Daphnia*” from this point forward). We constructed a specific set of conditions under which t-DOM is theoretically the most important for increasing *Daphnia* fitness parameters to specifically determine the capacity for *Daphnia* to use t-DOM as a resource under conditions of low algal food availability. Our experiment differs from previous studies by: (i) employing controlled experiments that use a *Daphnia* clone (to eliminate effects of genetic variability), (ii) exploring fitness responses across a range of realistic but controlled temperatures and (iii) providing only a known and controlled amount of t-DOM and algae as food sources (i.e. protists were excluded and experiments run in darkness to prevent algal growth). We tested the null hypotheses, under an algal limiting scenario, that: (i) *Daphnia* growth rates do not differ between treatments containing high, but not unreasonable, concentrations of t-DOM compared with treatments without t-DOM, and, (ii) that there is no effect of temperature on t-DOM use, and consequently, growth rates of *Daphnia*. While our experimental setup (i.e. low and controlled availability of algae combined with an absence of protists as food sources for zooplankton) might not reflect a typical lake scenario, it enables us to separate the potential growth effect of dietary t-DOM (a presumably poor quality diet) from higher diet quality sources (algae and protists).

## METHOD

### *Daphnia* and algal cultures

Laboratory experiments were performed with a clone of *Daphnia magna* that had been cultured in ADaM medium

(Klüttgen *et al.*, 1994) and fed daily with *Scenedesmus obliquus* ( $\sim 1 \text{ mg C L}^{-1}$ ) at room temperature for many generations. *Scenedesmus obliquus* was cultured semi-continuously in SAG (Sammlung von Algenkulturen Göttingen) medium (Guillard and Lorenzen, 1972) at room temperature ( $20^\circ\text{C}$ ) under a 14:10 h light:dark cycle. To achieve the desired quantity of *S. obliquus* in the experimental food suspensions ( $0.1 \text{ mg C L}^{-1}$ , see Experimental design section below), light extinction at 800 nm of the stock culture was measured photometrically (Shimadzu UV-1700 UV-VIS spectrophotometer, Kyoto, Japan) and the appropriate dilution was obtained using previously established calibration curves for optical density versus carbon concentration.

### DOM preparation

Senescent leaves from common beech (*Fagus sylvatica*) and hazel (*Corylus maxima*) were collected in late autumn near the shore of pre-alpine Lake Lunz, Austria (47:51 N, 15:04 E). As per previous studies (Lush and Hynes, 1973; Camilleri and Ribi, 1986), leachates were prepared by drying leaves overnight ( $50^\circ\text{C}$ ) to facilitate subsequent homogenization. Ground leaves (33.6 g of beech and 35.2 g of hazel) were added to  $0.2 \mu\text{m}$ -filtered lake water (3.9 L) in glass flasks. The leaf-water mixtures were stored, undisturbed and uncovered for 48 h (most DOC leaches from leaves within 40 h, Lush and Hynes, 1973) at room temperature and in the dark to prevent algal growth. The leaf-water mixtures were first pre-filtered through mesh screens (to remove large leaf particles) then through GF/C filters before finally being filtered to remove bacteria (Teflon<sup>TM</sup>  $0.2 \mu\text{m}$ ). Therefore, t-DOM is operationally defined here as organic matter  $< 0.2 \mu\text{m}$ . The resultant t-DOM stock solutions (beech leaf, t-DOM<sub>beech</sub>; hazelnut leaf, t-DOM<sub>hazel</sub>) were immediately analyzed for [DOC] (total organic carbon analyzer, SIEVERS<sup>TM</sup> Ionics Instruments) to determine the dilution needed for the experiment and stored frozen ( $-20^\circ\text{C}$  in 50 mL Falcon tubes) until use. The [DOC] of the t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> stock solutions were 570 and  $257 \text{ mg C L}^{-1}$ , respectively.

### Experimental design

The experiment was designed to establish how additions of t-DOM ( $10 \text{ mg DOC L}^{-1}$ ) from two different sources (beech and hazel leaves) and across three temperatures ( $15^\circ\text{C}$ ,  $20^\circ\text{C}$  and  $25^\circ\text{C}$ ) impacted the growth and reproduction of *Daphnia* (Fig. 1). The experiment was performed in temperature-controlled chambers in the dark to prevent experimentally-added algae from growing. To commence the experiment, ten neonates ( $< 24 \text{ h}$  old) were randomly allocated into each experimental vessel (250 mL glass jars

		t-DOM		
		No t-DOM	t-DOM <sub>beech</sub>	t-DOM <sub>hazel</sub>
Temperature	15°C	○ ○ ○	○ ○ ○	○ ○ ○
	20°C	○ ○ ○	○ ○ ○	○ ○ ○
	25°C	○ ○ ○	○ ○ ○	○ ○ ○
Algae ( $\text{mg C L}^{-1}$ )		0.1	0.1	0.1
DOC ( $\text{mg C L}^{-1}$ )		0	10	10

**Fig. 1.** Experimental design for the present study. Ten neonate *Daphnia* were allocated to each replicate at each combination of t-DOM and temperature to commence the experiment.

containing 200 mL ADaM medium). Three replicates of ten neonates each were also allocated into pre-weighed tin cups and dried overnight for determination of initial body weights. Each temperature and t-DOM treatment was run in triplicate (Fig. 1). During the experiments, *Daphnia* were transferred to clean jars containing new food suspensions that were prepared daily, and were monitored for maturity. The t-DOM treatments at each temperature were stopped simultaneously when 50% of daphnids in any one replicate (glass jar) deposited eggs into the brood pouch. On this day, the number of eggs were counted for each individual *Daphnia* and final dry weights were determined by pooling all individuals from a single replicate into a pre-weighed tin cup (see Supplementary data, Table SI for n of each pooled sample). Additionally, zero, one or two *Daphnia* from each combination of t-DOM and temperature were randomly selected and maintained at the experimental conditions to determine the viability of released neonates (see Supplementary data, Table SI for numbers of *Daphnia* retained from each replicate). *Daphnia* growth rates were calculated as  $\ln(\text{Wt}) - \ln(\text{W0})/t$  and where; Wt = final dry weight, W0 = initial dry weight and t = length of the experiment in days. The mean number of eggs per female was calculated for each replicate of each t-DOM and temperature treatment.

Algae (*S. obliquus*) were provided to all treatments at a concentration of  $0.1 \text{ mg C L}^{-1}$  because *D. magna* do not survive well when no algae is available (Brett *et al.*, 2009; Taipale *et al.*, 2012; Wenzel *et al.*, 2012). We chose to provide an algal concentration of  $0.1 \text{ mg C L}^{-1}$ , which is below the incipient limiting level for *Daphnia magna* ( $0.3 - 0.5 \text{ mg C L}^{-1}$ , Giebelhausen and Lampert, 2001), to ensure that algae were indeed limiting (sufficient algae would likely have overwhelmed the growth response) and to mimic plausible scenarios of low algal concentration in some lakes (e.g. Karlsson *et al.*, 2003; Taipale *et al.*, 2009). A concentration of  $10 \text{ mg DOC L}^{-1}$  was chosen as the target concentration for t-DOM leachate additions in our experiment because it is close to the mean [DOC] calculated from a survey of 7514 lakes from the Northern

hemisphere ( $7.58 \pm 0.19$  mg DOC L<sup>-1</sup>, Sobek *et al.*, 2007).

### Dissolved and particulate nutrient content of food suspensions

Dissolved (DOC; SRP, soluble reactive phosphorus) and particulate (POC, particulate organic carbon; PP, particulate phosphorus) nutrients were measured to explore the quantity and quality of potential resources available to *Daphnia* among the different treatments. Food suspensions (5 mL for DOC and 25 mL for SRP) were filtered (0.2  $\mu\text{m}$ ) into acid-washed vials. For POC and PP, 200 mL of the food suspension was collected onto filters (pre-combusted and acid washed GF/C). DOC was analyzed using a total organic carbon analyzer (SIEVERS<sup>TM</sup> Ionics Instruments), TOC was measured with an Elemental Analyzer (CE Instruments, Milan, Italy), SRP was quantified using persulfate digestion followed by molybdate reaction and PP was measured after sulphuric acid digestion followed by molybdate reaction (Wetzel and Likens, 2003).

Additions of t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> were anticipated to supply additional energy and nutrients for bacterial growth compared with algae only treatments. Thus, bacterial cells were counted in food suspensions that were made exactly as per the experimental protocol, but without *Daphnia*, and incubated at the experimental temperatures for 24 h. This was to explore the possible quantity of bacteria available to *Daphnia* for consumption each day, before the food suspensions were replaced, and in the absence of a grazing effect. Bacterial cell counts were measured via flow cytometry (Beckman Cell Lab Quanta SC, California, USA) following staining (SYTOX green nucleic acid stain) of fixed bacteria (37% formaldehyde). Fluorescence microscopy of the 24 h incubated food suspensions supported cytometer results and ruled out the presence of alternative food sources (e.g. protists).

### Fatty acid analysis of t-DOM and alga

Lipids were extracted from freeze-dried (96 h) t-DOM leachates and the *Scenedesmus* culture ( $n = 3$  for each, 45 mL and 100 mL, respectively, filtered onto pre-combusted and pre-weighed GF/C filters) using chloroform:methanol (2:1) as described elsewhere (Heissenberger *et al.*, 2010). Sample dry weights were  $31.5 \pm 0.3$ ,  $17.9 \pm 3.2$  and  $13.5 \pm 2.0$  mg for *Scenedesmus*, t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub>, respectively. Total lipid extracts were esterified to obtain fatty acid methyl esters (FAME) using toluene (1 mL) and H<sub>2</sub>SO<sub>4</sub>-methanol (2 mL; 1% v/v). FAME were analyzed using a gas chromatograph (TRACE GC THERMO) equipped with flame-ionization detection, a temperature-programmable injector and an autosampler. A Supelco<sup>TM</sup>

SP-2560 column (100 m, 25 mm i.d., 0.2  $\mu\text{m}$  film thickness) was used for FAME separation. Fatty acid concentrations were calculated using calibration curves based on known standard concentrations and were expressed as mass fractions (i.e.  $\mu\text{g FAME} \cdot \text{mg dry weight sample}^{-1}$ ).

### Data analysis

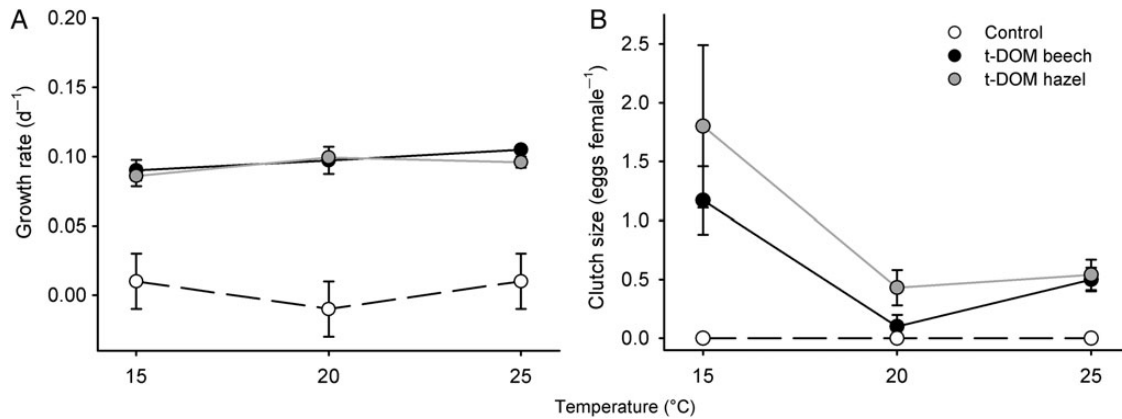
The assumptions of normality and homogeneity of variance of response variables and error terms were explored via Shapiro–Wilk and Levene’s tests, respectively. Bacterial cell counts and eggs female<sup>-1</sup> were log transformed prior to analysis. We used two-way ANOVA to determine the effect of t-DOM, temperature and the t-DOM\*temperature interaction on *Daphnia* growth rates and eggs female<sup>-1</sup>. One-way ANOVA was used to compare DOC, SRP, POC and PP concentrations among the algae only, t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> initial food suspensions (which were made and sampled immediately). For the food suspensions that were made and incubated for 24 h at the experimental temperatures, two-way ANOVA was used to determine the effect of t-DOM and temperature on bacterial cell counts. All statistical analyses were performed in R (R Development Core Team, 2012) and the significance level was set at 0.05. All values are reported as means  $\pm 1$  SD.

## RESULTS

### *Daphnia* maturity, growth and reproduction

Mortality was higher at 15°C than in the other temperature treatments, but did not exceed 20% in the t-DOM<sub>beech</sub> or t-DOM<sub>hazel</sub> treatments (Supplementary data, Table SII). All replicates of t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> in a single temperature treatment were stopped simultaneously and final weights collected on Day 18, 20 and 16 for 15, 20 and 25°C, respectively (Supplementary data, Table SI) when 50% of the *Daphnia* (i.e. five individuals) in any single replicate of either t-DOM<sub>beech</sub> or t-DOM<sub>hazel</sub> deposited eggs. Both t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> treatments were stopped concurrently at each temperature to determine how differences in t-DOM source affected the ability of *Daphnia* to grow and reproduce following the same time duration under the experimental conditions. Due to high mortality (50%), the treatments containing algae only (no t-DOM) had to be stopped before *Daphnia* reached maturity on Day 12, 11 and 10 for the 15, 20 and 25°C treatments, respectively, for determination of final weights (Supplementary data, Table SI).

*Daphnia* exhibited higher growth in the t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> treatments ( $\sim 0.10$  d<sup>-1</sup> at all temperatures) compared with the algae only treatments that



**Fig. 2.** Juvenile growth rates (**A**) and clutch sizes (**B**, both mean  $\pm$  SD) of *Daphnia* reared at three temperatures in food suspensions containing  $0.1 \text{ mg C L}^{-1}$  of *Scenedesmus* and either no added t-DOM (control) or  $10 \text{ mg DOC L}^{-1}$  added from leachates of beech (t-DOM<sub>beech</sub>) or hazel leaves (t-DOM<sub>hazel</sub>).

lacked t-DOM ( $\sim 0.01 \text{ d}^{-1}$ ;  $F_{2,18} = 164.3$ ,  $P < 0.001$ ), but had similar growth rates among the three temperatures based on two-way ANOVA ( $P > 0.05$ , Fig. 2A). The interaction between t-DOM treatment and temperature was not significant ( $P > 0.05$ ) and, based on Tukey's post-hoc tests, neither was the difference in growth between the two t-DOM leaf treatments ( $P > 0.05$ ). Because we stopped the algae only treatments before the t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> treatments, we confirmed with a second experiment, in which all treatments were simultaneously stopped on Day 6, that *Daphnia* provided with both algae and t-DOM grew significantly better than *Daphnia* provided with only algae (Supplementary data, Table SIII).

*Daphnia* in the algae only treatments exhibited high mortality and had not deposited eggs before the treatments were stopped. We retained several surviving daphnids from these algae only treatments (Supplementary data, Table SI) to monitor for maturity but even after 30 days these *Daphnia* had still not deposited eggs at any temperature. On the other hand, the t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> fed *Daphnia* did deposit eggs, but fecundity was low at all temperatures and variable among individuals within each replicate (fecundity range = 1–2.3 at 15°C, 0.1–0.6 at 20°C and 0.4–0.7 at 25°C; Supplementary data, Table SII and Fig. 2B). Based on two-way ANOVA performed on the available fecundity data for t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> treatments, both t-DOM treatment, temperature and their interaction had significant effects on *Daphnia* fecundity ( $F_{1,12} = 15.5$ ,  $F_{2,12} = 41.9$ ,  $F_{2,12} = 6.0$ , respectively, all  $P < 0.01$ ). Thus, fecundity was higher at 15°C than 20 and 25°C and t-DOM<sub>beech</sub> versus t-DOM<sub>hazel</sub> affected fecundity only at 20°C (Fig. 2B). Neonates released from the t-DOM subsidized daphnids that were kept at the experimental conditions were able to reach maturity under

the experimental conditions, which occurred on Day 15 (t-DOM<sub>beech</sub>) and 12 (t-DOM<sub>hazel</sub>) at 15°C, but earlier at 20°C (Day 11) and at 25°C (Day 10) in both t-DOM treatments. Together, these findings suggest that although clutch sizes were very low (mean values taken across all females in each replicate  $< 2$  eggs female<sup>-1</sup>), t-DOM supplemented *Daphnia* reached maturity faster and deposited more eggs compared with *Daphnia* receiving no t-DOM across the experimental temperatures (Fig. 2B).

### Food suspensions

Food suspensions that were made and sampled immediately contained higher DOC (ANOVA,  $F_{2,6} = 364.3$ ,  $P < 0.001$ ) and SRP (ANOVA,  $F_{2,6} = 1684$ ,  $P < 0.001$ ) in t-DOM<sub>beech</sub> > t-DOM<sub>hazel</sub> > algae only (Table I). POC concentrations were close to the target *Scenedesmus* concentration of  $0.1 \text{ mg C L}^{-1}$  in all treatment food suspensions (Table I). There were no differences among t-DOM<sub>beech</sub>, t-DOM<sub>hazel</sub> or algae only treatments for either POC or PP (ANOVA,  $P > 0.05$ , Table I) in the food suspensions. Molar particulate C:P ratios were  $232 \pm 97$  in the algae only treatment,  $359 \pm 131$  in t-DOM<sub>beech</sub> and  $262 \pm 54$  in t-DOM<sub>hazel</sub>.

As expected, incubating food suspensions (without *Daphnia*) at the experimental temperatures for 24 h resulted in higher bacterial cells numbers in the t-DOM leaf treatments compared with the algae only treatment (Fig. 3). Differences in bacterial counts among treatments (algae only < t-DOM<sub>beech</sub> = t-DOM<sub>hazel</sub>) and temperatures (15 < 20 < 25°C) were significant (two-way ANOVA, Table II). However, based on a significant t-DOM\*temperature interaction (Table II), algae only, t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> treatments had similar bacterial cell counts at 15°C (Fig. 3).

**Table I:** Dissolved organic carbon (DOC, mg L<sup>-1</sup>), soluble reactive phosphorus (SRP, μg L<sup>-1</sup>), particulate organic carbon (POC, mg L<sup>-1</sup>) and particulate phosphorus (PP, μg L<sup>-1</sup>) measured from food suspensions that were created exactly as per the experimental conditions, but without *Daphnia*, and sampled immediately

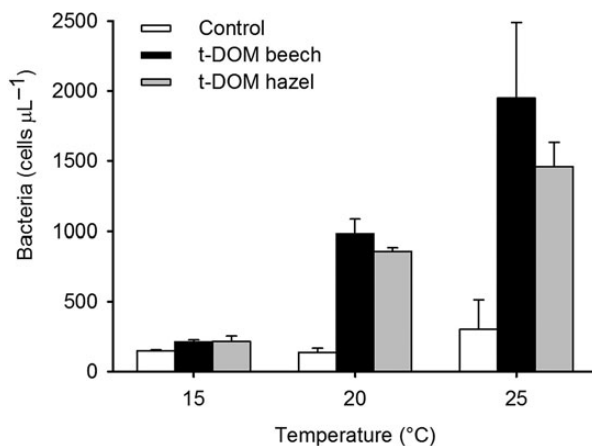
Treatment	No t-DOM	t-DOM <sub>beech</sub>	t-DOM <sub>hazel</sub>
Dissolved			
DOC	1.14 ± 0.13 <sup>a</sup>	10.01 ± 0.62 <sup>b</sup>	8.53 ± 0.39 <sup>c</sup>
SRP	4.01 ± 0.30 <sup>a</sup>	145.55 ± 0.97 <sup>b</sup>	44.04 ± 1.33 <sup>c</sup>
Particulate			
POC	0.16 ± 0.03 <sup>a</sup>	0.17 ± 0.03 <sup>a</sup>	0.17 ± 0.02 <sup>a</sup>
PP	1.88 ± 0.45 <sup>a</sup>	1.36 ± 0.45 <sup>a</sup>	1.68 ± 0.25 <sup>a</sup>

Each value is the mean ± SD of three replicates. Different superscript letters denote significantly different values (based on ANOVA and Tukey's post-hoc tests) among the three treatments.

**Table III:** Fatty acids of the different food types provided to *Daphnia* in the experiment

Fatty acid	Algae	t-DOM <sub>beech</sub>	t-DOM <sub>hazel</sub>
ΣTotal	8.39 ± 1.57	0.25 ± 0.03	0.35 ± 0.03
ΣSAFA	2.92 ± 0.53	0.21 ± 0.02	0.31 ± 0.03
ΣMUFA	0.84 ± 0.18	np	0.01 ± 0
ΣPUFA	4.63 ± 0.88	0.04 ± 0.01	0.03 ± 0
18:2n-6	1.21 ± 0.24	np	np
18:3n-6	0.08 ± 0.02	np	np
18:3n-3	3.01 ± 0.57	np	np
18:4n-3	0.31 ± 0.06	0.01 ± 0.01	np
20:2n-6	np	0.02 ± 0.01	np
20:4n-6	np	np	0.02 ± 0.01
20:5n-3	np	Np	np
22:6n-3	np	Np	np

Food types consisted of algae (*Scenedesmus obliquus*) and t-DOM leaf leachates from two different trees (beech and hazel). Sum (Σ) of SAFA, MUFA and PUFA and individual PUFA are reported as μg FAME • mg dry weight sample<sup>-1</sup>. Values are mean ± SD of three replicates for each food type. "np" means not present.



**Fig. 3.** Bacterial counts (cells μL<sup>-1</sup>, mean ± SD) from food suspensions that excluded *Daphnia* and were incubated in darkness for 24 h at the experimental temperatures. "Control" treatments had no added t-DOM. Both t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> treatments had 10 mg DOC L<sup>-1</sup> added from leaf leachates. All treatments had 0.1 mg C L<sup>-1</sup> of *Scenedesmus obliquus*.

**Table II:** Results of two-way ANOVA and Tukey's post-hoc tests to determine the influence of t-DOM (no t-DOM, t-DOM<sub>beech</sub>, t-DOM<sub>hazel</sub>), temperature (15, 20, 25°C) and the t-DOM\*temperature interaction on bacterial counts (cells μL<sup>-1</sup>) from food suspensions that excluded *Daphnia* and were incubated for 24 h in darkness

Response variable	Effect	F	df	P
Bacteria	t-DOM	83.35	2,18	<0.001
	Temp	81.38	2,18	<0.001
	t-DOM*Temp	11.69	4,18	<0.001

The *Scenedesmus* culture fed to *Daphnia* during the experiment had 34× and 24× more total fatty acids than the t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> leachates, respectively (Table III). *Scenedesmus* fatty acids were dominated by C18 polyunsaturated fatty acids (PUFA, almost entirely 18:2n-6 and 18:3n-3, Table III), whereas t-DOM leachates were dominated by saturated fatty acids (SAFA) with almost no monounsaturated fatty acids (MUFA) or PUFA (Table III).

## DISCUSSION

Our findings provide experimental evidence that t-DOM (directly) or t-DOM-driven bacterial production (indirectly) supports *Daphnia* growth and reproduction when algal food is strongly limiting and without additional intermediate trophic links (e.g. heterotrophic protists). Bacteria efficiently sequester DOC (Hessen, 1992; Faithfull *et al.*, 2011; Lennon *et al.*, 2013) and phosphorus (Hessen and Andersen, 1990; Forsström *et al.*, 2013) and rapidly (within 24 h) exploited the higher DOC and SRP in both t-DOM leachates based on higher bacterial cell counts in the t-DOM compared with algae only treatments (at 20 and 25 but not 15°C). *Daphnia* consumption of t-DOM-driven bacteria may therefore explain their higher growth and reproduction in the t-DOM compared with the algae only treatments at 20 and 25°C. Consistent with this suggestion, the growth rates observed here for t-DOM supplemented *Daphnia* are consistent with previously reported values for *Daphnia* provided with bacteria and some algae (~0.1 d<sup>-1</sup> when fed 80:20 bacteria:algae) (Wenzel *et al.*, 2012) and with empirical findings of increasing zooplankton allochthony with

increasing availability of t-DOM-supported bacterial production (Berggren *et al.*, 2014; Tanentzap *et al.*, 2014).

Bacterial cell numbers in our experimental food suspensions were, however, low compared with natural systems. For example, Arvola *et al.* (Arvola *et al.*, 1992) reported bacterial cell numbers ranging from 2200 to 7300 cells  $\mu\text{L}^{-1}$  in the epilimnion and 17 000–41 000 cells  $\mu\text{L}^{-1}$  in the hypolimnion of a small humic lake, compared with  $<2500$  cells  $\mu\text{L}^{-1}$  in our experimental food suspensions following 24 h incubation (Fig. 3). Further, higher bacterial availability on its own cannot explain the higher growth and reproduction of *Daphnia* in the t-DOM treatments at 15°C in our experiment because bacterial cell numbers were very low ( $<250$  cells  $\mu\text{L}^{-1}$ ) and similar among t-DOM and algae only treatments (Fig. 3). This could suggest some benefit of the added t-DOM itself. The leaf leachates contained P and a small amount of energy-yielding SAFA. These and other compounds may have been obtained directly from the water by *Daphnia*, which are capable of directly taking up DOC based on radiolabel tracer studies (Speas and Duffy, 1998), possibly via consumption of DOC-uptaking epibiont bacteria on their filter combs (Eckert and Pernthaler, 2014). *Daphnia* could also possibly consume precipitated t-DOM as in black fly larvae (Ciborowski *et al.*, 1997) or marine crustaceans (Camilleri and Ribi, 1986) or t-DOM that has adsorbed onto particles as in roundworms (Höss *et al.*, 2001). Both algae (added experimentally) and bacterial cells (present naturally) were available for t-DOM adsorption and could have acted as vehicles for increased *Daphnia* acquisition of C and P in the t-DOM compared with algae only treatments. We also cannot rule out the potential presence of fungi in our experimental treatments for *Daphnia* consumption. It is not possible to further separate the importance of direct versus indirect t-DOM uptake pathways in our experiment because attempts to exclude bacteria (in subsequent experiments, data not shown) using antibiotics failed, as *Daphnia* died within 6 days. Running the experiment under sterile conditions was also not feasible. Additional work is therefore needed to separate the importance of direct and indirect (e.g. via bacteria) t-DOM pathways to zooplankton. Exploring how our findings compare to those for other herbivorous zooplankton with different feeding strategies is also warranted.

Despite the significant growth rate increase in all the t-DOM treatments compared with the algae only treatments, *Daphnia* could not exploit their potential for higher growth at higher temperatures as hypothesized (Giebelhausen and Lampert, 2001; Masclaux *et al.*, 2012). Under the experimental conditions, temperature was therefore not limiting growth rate. Further, despite different SRP concentrations, the t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> food suspensions had similar bacterial cell counts following 24 h incubation, suggesting

that these two leachates were qualitatively similar resources for bacterial growth. Similar bacterial counts between the two leachates may explain why neither *Daphnia* growth nor fecundity consistently differed between the t-DOM<sub>beech</sub> and t-DOM<sub>hazel</sub> treatments across the experimental temperatures. *Daphnia* growth at all temperatures and in both leachates may therefore have been limited by dietary nutrients not available (or available in insufficient quantities) in the algae, t-DOM and bacteria. Freshwater bacteria are generally considered as an important source of P, but lack other essential nutrients such as sterols (Martin-Creuzburg *et al.*, 2011) and some PUFA (Taipale *et al.*, 2012) that are involved in high and low temperature adaptation, respectively. It is therefore possible that cholesterol and PUFA were increasingly limiting *Daphnia* growth at the lower and higher experimental temperatures, respectively, based on existing knowledge of *Daphnia* nutrition (Sperfeld and Wacker, 2011; Masclaux *et al.*, 2012). This may have prevented *Daphnia* from achieving higher growth rates at higher temperatures even though bacterial cell numbers increased with increasing temperature. Maturity and fecundity, on the other hand, did show a slight effect with temperature. *Daphnia* at 20°C unexpectedly took 2 days longer (20 days) to reach maturity than at 15°C (18 days) in both t-DOM treatments for reasons that are not known. But as expected, *Daphnia* reached maturity fastest at 25°C. The ability of *Daphnia* to deposit larger, although still very small, clutches at the lowest temperature (15°C) could indicate that lower metabolic demands allowed more energy to be allocated to reproduction, or that lower temperatures decreased the food quantity or quality thresholds for reproduction.

Our study is the first to show that t-DOM, directly or indirectly (via bacteria), supports zooplankton growth and reproduction in the absence of sufficient high quality algae or protist food sources. Parts of the experimental conditions were realistic because algal concentrations below the limiting levels of 0.3–0.5 mg C L<sup>-1</sup> for *Daphnia* (Giebelhausen and Lampert, 2001) are possible in both clear-water and also humic lakes (Karlsson *et al.*, 2003), and summer temperatures reach  $>20^\circ\text{C}$  in many freshwater bodies. Inputs of fresh, little-degraded t-DOM, such as the leaf leachates prepared for our experiment, are also possible and known to stimulate bacterial production during precipitation and runoff events in natural water bodies (Bergström and Jansson, 2000). Increased relative bacterial to phytoplankton production has also been linked to increased reliance on bacteria by zooplankton in humic and clear-water lakes (Karlsson *et al.*, 2003) and increased zooplankton biomass in freshwater deltas (Tanentzap *et al.*, 2014). Our experimental findings should promote future investigations into the potential importance of t-DOM for other herbivorous zooplankton during certain times of the year or in specific habitats where t-DOM inputs may be large or the

availability of high quality food sources may be limiting. Our findings also stress, however, that the growth and fecundity of *Daphnia* at three temperatures, and when provided with two different t-DOM sources, remained very low compared with previously reported values for *Daphnia* feeding on sufficient algal food (Wenzel *et al.*, 2012). t-DOM or t-DOM-supported bacteria may therefore be unable to continually sustain *Daphnia* populations across the range of water temperatures tested here.

## SUPPLEMENTARY DATA

Supplementary data can be found online at <http://plankt.oxfordjournals.org>

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