The ISME Journal (2014) 8, 1808–1819

www.nature.com/ismej

ORIGINAL ARTICLE

Bacterial epibionts of *Daphnia*: a potential route for the transfer of dissolved organic carbon in freshwater food webs

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The identification of interacting species and elucidation of their mode of interaction may be crucial to understand ecosystem-level processes. We analysed the activity and identity of bacterial epibionts in cultures of *Daphnia galeata* and of natural daphnid populations. Epibiotic bacteria incorporated considerable amounts of dissolved organic carbon (DOC), as estimated via uptake of tritiated leucine: three times more tracer was consumed by microbes on a single *Daphnia* than in 1 ml of lake water. However, there was virtually no incorporation if daphnids were anaesthetised, suggesting that their filtration activity was essential for this process. Microbial DOC uptake could predominantly be assigned to microbes that were located on the filter combs of daphnids, where the passage of water would ensure a continuously high DOC supply. Most of these bacteria were *Betaproteobacteria* from the genus *Limnohabitans*. Specifically, we identified a monophyletic cluster harbouring *Limnohabitans planktonicus* that encompassed sequence types from *D. galeata* cultures, from the gut of *Daphnia magna* and from daphnids of Lake Zurich. Our results suggest that the epibiotic growth of bacteria related to *Limnohabitans* on *Daphnia* spp. may be a widespread and rather common phenomenon. Moreover, most of the observed DOC flux to *Daphnia* in fact does not seem to be associated with the crustacean biomass itself but with its epibiotic microflora. The unexplored physical association of daphnids with heterotrophic bacteria may have considerable implications for our understanding of carbon transfer in freshwater food webs, that is, a trophic 'shortcut' between microbial DOC uptake and predation by fish.
The ISME Journal (2014) **8,** 1808–1819; doi:10.1038/ismej.2014.39; published online 3 April 2014
Subject Category: Microbe-microbe and microbe-host interactions
Keywords: *Daphnia*; epibionts; symbiosis; fresh water; food webs; dissolved organic carbon

Introduction

In order to accurately estimate element fluxes or predict ecosystem responses, it is essential to understand food web architecture (Bascompte, 2010). Unexplored trophic links may considerably alter food web structure, and can, once investigated, substantially change our understanding of ecosystem carbon metabolism (Corno *et al.*, 2012; Kuwae *et al.*, 2012). Therefore, interacting species and their mode of interaction need to be identified for more accurate assumptions about ecosystem functionality and stability (Thébault and Fontaine, 2010; Mougi and Kondoh, 2012).

The lack of information about the physical interactions between heterotrophic bacteria and zooplankton, such as the crustacean genus *Daphnia*, may neglect an important aspect of freshwater food webs (Tang *et al.*, 2010). Indeed, little is known about the microbial communities associated with healthy *Daphnia*, as opposed to the plethora of research about the respective roles of either bacterio- or zooplankton within aquatic food webs, or about microbial parasites of *Daphnia* (see, for example, Ebert, 2008; Newton *et al.*, 2011; Miner *et al.*, 2012). Recent studies, however, point to other system-relevant associations between bacteria and *Daphnia*, for example, the transfer of microbes from lower to higher water layers via attachment–detachment processes (Grossart *et al.*, 2010). The gut microflora of *Daphnia magna* was reported to be dominated by members of the genus *Limnohabitans* (Freese and Schink, 2011), that is, by common inhabitants of the pelagic zone of freshwater epilimnia that typically co-occur with phytoplankton (Šimek *et al.*, 2005, 2011). High bacterial diversity, including phylotypes related to *Limnohabitans*, was also found when analysing prokaryotic sequences from metagenomic data of *Daphnia* spp. (Qi *et al.*, 2009).

Whereas algae are generally regarded as the main food for daphnids, heterotrophic bacteria are considered less important (see, for example

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Received 26 September 2013; revised 6 February 2014; accepted 12 February 2014; published online 3 April 2014

Peterson *et al.*, 1978; Nagata and Okamoto, 1988; Martin-Creuzburg *et al.*, 2011). However, some bacteria, notably filamentous morphotypes, are also consumed by *Daphnia* sp. that, in turn, may directly affect bacterial community structure at least during particular seasons (Langenheder and Jürgens, 2001; Pernthaler *et al.*, 2004; Taipale *et al.*, 2008). In addition, it is conceivable that the associations of zooplankton with heterotrophic bacteria may also have substantial implications for biogeochemical processes such as the transfer of carbon through the food web: although daphnids feed on organic carbon from the particulate fraction (Cole *et al.*, 2006), the attached bacteria would likely consume dissolved organic carbon (DOC). Apart from a single more recent report (Speas and Duffy, 1998), uptake of DOC by *Daphnia* (or by their epibionts) has been addressed by studies dating from the beginning of the last century (see, for example, Kerb, 1911; Krogh, 1930). This largely unexplored trophic link might, however, be of great relevance for lake carbon cycling; for example, in the context of the much debated question of to what extent internal primary production or terrestrial carbon sources support freshwater ecosystems (see, for example, Grey *et al.*, 2001; Pace *et al.*, 2004; Brett *et al.*, 2009). In such studies, the biomass of homogenised daphnids is proportionally assigned to allochthonous or autochthonous sources—by analysis of the isotopic ratios of carbon atoms—in order to model the fluxes of organic carbon through the food web. The zooplankton epibionts are considered as part of the *Daphnia* biomass; however, their specific metabolic abilities (that is, consumption of DOC) might considerably affect the interpretation of such assessments. Moreover, epibionts will be consumed by fish together with their host and might, thus, form a shortcut through the food web due to the transfers of organic matter, directly deriving from DOC, to fish.

We studied the uptake of dissolved leucine by *Daphnia galeata* or their epibionts to gain first insight into the importance of this trophic link, and we compared it with their ingestion of leucine-incorporating planktonic microbes. Furthermore, we localised and identified a prominent genus of microbial epibionts responsible for this uptake on cultured *D. galeata* as well as on daphnids from mixed natural populations in a lake (Lake Zurich, Switzerland). Using leucine and *N*-acetyl-D-glucosamine (NAG) as model substrates, we then assessed the metabolic activity of *Daphnia* epibionts in Lake Zurich.

Materials and methods

Sampling and sample preparation

Lake Zurich was sampled weekly at ~1000 h between 26 April and 24 May 2012 (coordinates 47°31' N, 8°58' E). Chlorophyll *a* and temperature were measured using a multiple-wavelength probe (TS-16–12 fluoroprobe, bbe Moldaenke GmbH, Kronshagen, Germany) and a multi-parameter probe (6600 multi-parameter, water quality monitoring, YSI Incorp., Yellow Springs, OH, USA), respectively. The sample taken on 26 April was from the depth of maximum Chlorophyll *a* (8 m), whereas later samples were collected from 5 m depth because of the onset of the clear water phase (Figure 5). Zooplankton was collected using a Ruttner sampler, concentrated with a 40 μm net from a volume of 5 l and directly fixed with formaldehyde (final concentration, 4%) for determination of abundances. Live daphnids were collected using the same device and transported to the laboratory in a clean jar. A third set of daphnids were immediately anaesthetised with carbon dioxide-enriched water for later experiments. The daphnids were kept in a laboratory incubator at *in situ* temperature for a maximum of 3 h before the experiments.

Next, 50 ml of lake water was fixed with formaldehyde (final concentration, 1%) for the analysis of total bacterial abundances and the proportions bacteria affiliated with the *Beta-Proteobacteria* genus *Limnohabitans*. Subsamples of 4 ml were filtered onto white polycarbonate membrane filters (type GTTP, 45 mm diameter, 0.2 mm pore size; Millipore, Billerica, MA, USA) for the counting of *Limnohabitans*-related bacteria. The remaining sample was stored at 4 °C for flow cytometric determination of total cell numbers.

Tracer experiments

In experiment I (Figure 1), *D. galeata* females were kept in sterile (0.2 μm prefiltered and autoclaved) lake water (sLW) and fed with *Scenedesmus subspicatus* approximately every second day. Adult individuals were washed three times with sLW, and 6 sets of 4 individuals were transferred to 50 ml Erlenmeyer flasks containing 10 ml of sLW. Twelve additional individuals were anaesthetised in commercial carbon dioxide-enriched water, washed twice in sLW and 3 sets of 4 individuals were placed in 50 ml Erlenmeyer flasks containing 10 ml of sLW. Daphnids were acclimatised for 1 h in the dark at 20 °C before tracer addition.

Nine sets of 30 μm prefiltered lake water (10 ml each) were incubated for 1 h with 10 nM of tritium-labelled leucine (specific activity: 120 Ci mmol^{-1}) or NAG (specific activity: 60 Ci mmol^{-1}, American Radiolabeled Chemicals, Inc., St Louis, MO, USA) to label the microbial community. Leucine is a widely used marker for biomass production in aquatic microbial ecology (Kirchman *et al.*, 1985). It is incorporated into protein, and there is a large set of data from freshwater systems for comparison (Kubitschek, 1968; Jørgensen, 1992; del Giorgio and Cole, 1998). NAG is the subunit of chitin, the main polymer of the daphnia carapace. Thus, bacteria living on a chitinous surface might arguably have the ability to incorporate NAG (Köllner *et al.*, 2012;

Beier and Bertilsson, 2013). The labelled communities were then distributed to triplicate Erlenmeyer flasks containing four live daphnids (treatment Rw) or four anaesthetised daphnids (treatment A), respectively. The third set of labelled microbial communities was filtered onto nitrocellulose membrane filters (type GSWP, 45 mm diameter, 0.22 µm pore size; Millipore) to determine microbial incorporation of the tracer. The filtrate was also collected and added to triplicate Erlenmeyer flasks each containing four daphnids (treatment F).

Live and anaesthetised daphnids were incubated for 1 h at 20 °C on a laboratory rocker (10 r.p.m. over a tilt angle of ±11°). Thereafter, all daphnids were individually picked and anaesthetised in carbon dioxide-rich water. Three individuals from each Erlenmeyer were then transferred to separate scintillation vials with 500 µl Soluene350 (Perkin Elmer Inc., San Jose, CA, USA) to solubilise the tissue and incubated at 50 °C for ~6 h. For one experiment, six *D. galeata* individuals were dissected after labelling and colons and the outer carapaces were separated for uptake measurements. When the daphnids were dissolved, 0.5 ml of scintillation cocktail (Rotiszint eco plus, Carl Roth GmbH, Karlsruhe, Germany) was added and radioactivity was measured in a scintillation counter ($n = 9$ for each treatment and date). The average uptake values of three daphnids from the same Erlenmeyer flask were treated as a single replicate in order to avoid pseudoreplication. One daphnid from each treatment and date was fixed with EtOH, placed on a cover slip and dissected under a binocular microscope for later fluorescence *in situ*

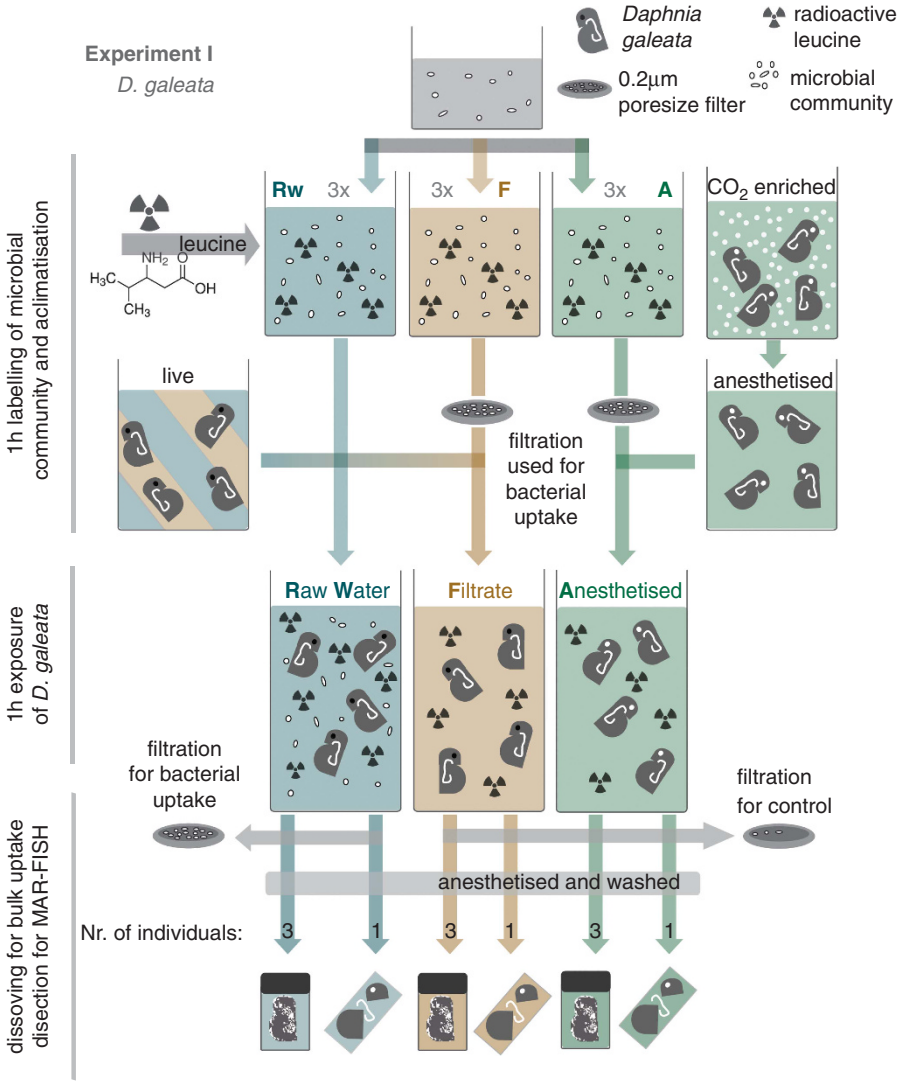

Figure 1 Schematic depiction of the setup of experiment I. For the raw water (Rw) and filtrate (F) treatments, active *D. galeata* individuals were incubated for 1 h together with the lake water microbial assemblages (Rw) or in lake water filtrates (F). Both Rw and F treatments had been preincubated with tritiated leucine for 1 h before the addition of daphnids. In the anaesthetised treatment (A), daphnids were placed in CO_2-enriched mineral water before incubation in the lake water filtrates (negative control). Subsequently, daphnids were either dissected for microautoradiography (MAR-FISH, one animal), or total leucine uptake per individual was determined by scintillation counting (three animals). All treatments were done in triplicates.

hybridisation (FISH) and microautoradiography–FISH analysis (see below).

Experiment II (Supplementary Figure S1) was essentially performed as described for experiment I, except that daphnids were collected from Lake Zurich, and the microbial communities in the A and F treatments were removed already before addition of the radioactive tracer to assess the total potential uptake of *Daphnia* sp. epibionts. Separate triplicate sets of water samples (10 ml) were used to determine tracer uptake by the pelagic bacterial assemblages. Finally, all incubations for experiment II were performed at *in situ* (lake water) temperatures.

Staining and microscopic analysis

Dissected daphnids on the cover slips were overlaid with a drop of 0.1% low melting point agarose and dried at 45 °C. The cover slip was incubated in 90% EtOH for 45 min to fix the bacterial community. FISH and microautoradiography–FISH with probe R-BT065 (Šimek *et al.*, 2001) (targeting bacteria affiliated with *Limnohabitans*) were essentially conducted as described before (Pernthaler *et al.*, 2002; Alonso and Pernthaler, 2005), although on the cover slips, and predigestion was reduced to a lysozyme treatment of 20 min only. Microscopic imaging of the filter apparatus was done on an inverse confocal laser scanning microscope (CLSM Leica SP2, Leica Microsystems, Wetzlar, Germany) at the Centre for Microscopy and Image Analysis of the University of Zurich. Images were further processed with the software package Imaris x64 version 7.5.2 (Bitplane AG, Zurich, Switzerland) and arranged using Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA).

Bacteria were counted on the filter combs of dissected and stained Lake Zurich daphnids from 26 April. Cells on a single filter comb and the corresponding appendages of seven daphnids were quantified and the result was multiplied by four (to account for the number of combs per individual).

Statistical analysis

Differences between the Rw and A treatments compared with the F treatment were determined by one-way analysis of variance after natural logarithm transformation of the data to ensure normal distribution, with comparison of the means by Holm–Sidak *post hoc* test. The analyses were carried out using SigmaPlot 11 (Systat Software, Chicago, IL, USA).

Phylogenetic analysis

Four 16S rRNA gene clone libraries were constructed to analyse the phylogenetic composition of *Limnohabitans* bacteria on daphnids from Lake Zurich on cultured *D. galeata* and in the surrounding media (Lake Zurich water from 5 m depth and *D. galeata* cultivation medium). For this purpose, daphnids and water samples from Lake Zurich were obtained on 31 March. All daphnids were washed 3 times in sterile, UV-treated deionised water. DNA was isolated from ~13–16 daphnids or 250 ml of filtered water using the PowerSoil DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The 16S rRNA gene sequences affiliated with *Lhb* were amplified using the R-Bt065 oligonucleotide (Šimek *et al.*, 2001) as a forward primer (position: 065) and GM4r (position: 1492), a general bacterial reverse primer (Muyzer *et al.*, 1995), resulting in a fragment of 1442 bp. PCR conditions were tested with *Limnohabitans planktonicus* strain II-D5 (Kasalicky *et al.*, 2010), and were optimised in a reaction volume of 25 µl GoTaq Green Master Mix (Promega Co., Madison, WI, USA) as follows: 94 °C for 300 s, 94 °C for 60 s, 57 °C for 30s, 72 °C for 120 s (steps 2–4 repeated 30 times) and 72 °C for 600 s. Three purified PCR products of each sample were pooled and cloned into competent *Escherichia coli* cells, according to the manufacturer's protocol (pGEM-T Easy Vectors; Promega Co.). Of each transformation, 120 clones were picked and screened for inserts and positive clones were sequenced using the primers listed above plus primer GM1f (position: 518, Muyzer *et al.*, 1993). The sequences were assembled using DNA Baser Sequence Assembler (Heracle BioSoft S.R.L., Pitesti, Romania), aligned with the SINA web aligner (Pruesse *et al.*, 2007) and merged into the SILVA SSU reference database 110 using the software package ARB (Ludwig *et al.*, 2004). Uchime (Edgar *et al.*, 2011) was used to exclude chimeric sequences. Bootstrapped Maximum Likelihood trees (1000 repetitions; Stamatakis *et al.*, 2008) were calculated that comprised the sequences from this study, *Limnohabitans* sp. isolates as published in Kasalický *et al.* (2013) and *Limnohabitans* sequences obtained from *D. magna* gut analysis (Freese and Schink, 2011). The same set of sequences was clustered into operational taxonomic units (OTUs; 99% identity) using Mothur (Schloss *et al.*, 2009), and the OTU clustering pattern was compared with the results of the phylogenetic analysis. All sequences from this study are deposited in the EMBL database with accession numbers HF96498 – HF968621.

Results and discussion

Uptake of dissolved leucine by epibionts of D. galeata

We labelled a natural microbial community with tritiated leucine, and let *D. galeata* feed on it for 1 h (experimental setup: Figure 1). The amount of radiolabel in *D. galeata* because of their incorporation of both dissolved leucine and microbes (raw water treatment (Rw), Figure 1) was compared with the uptake of dissolved leucine only in a treatment where daphnids were placed into the sample after removing the labelled microbial cells by filtration

(treatment Filtrate (F), Figure 1). The amount incorporated by additionally feeding on microorganisms did not significantly exceed the uptake of substrate from the dissolved fraction only (Figure 2), indicating that leucine-incorporating heterotrophic microbes were of minor importance as a food source for daphnids (Peterson *et al.*, 1978; Nagata and Okamoto, 1988; Martin-Creuzburg *et al.*, 2011). Substantial amounts of radioactivity were detected in daphnids maintained on the filtrate, suggesting uptake of dissolved substrate by the animals (Figure 2): free-living bacteria in 1 ml of water incorporated 0.1 ± 0.04 pmol h^{-1} leucine, whereas three times higher uptake rates (0.3 ± 0.04 pmol h^{-1}) were observed per individual daphnid (Figure 2). The level of incorporation of dissolved leucine in this study far exceeds the previously reported uptake of custom labelled algal exudates by daphnids (Speas and Duffy, 1998). This might be ascribed to methodological issues, for example, a low labelling efficiency of the exudates.

To further explore the notion that leucine was readily taken up by epibiotic bacteria, labelled *D. galeata* individuals were dissected and the separated pieces were overlaid with a photographic emulsion to microscopically localise the deposition of radioactivity on the animal and in bacterial cells by microautoradiography and catalysed reporter deposition FISH (Alonso and Pernthaler, 2005). Strongest labelling was detected around the trunk limbs; particularly on the setae and appendages of trunk limbs 3 and 4 (TL3 and 4, Figure 3). Most uptake on these body surfaces could be assigned to single bacterial cells (Figure 3). The setae and setula of trunk limbs 3 and 4 serve as food-capturing filter sieves (see, for example, Fryer, 1991), and daphnids incessantly filter water through these structures. Besides protecting from protistan grazers and abiotic stressors (Tang *et al.*, 2010, 2011), a steady supply of organic carbon and nutrients may render the filter apparatus an ideal habitat for epibiotic bacteria. This interpretation is further supported by our finding that almost no label was incorporated when daphnids were anaesthetised before being placed in the filtrate (treatment anaesthetised (A), Figure 1). Thus, active filtration by the animals seemed to be a prerequisite for substrate uptake by the epibionts. Furthermore, the proportions of incorporated radioactivity in the external body parts of dissected daphnids were between 4 and >80 times higher (mean, 29.5) then in the colons (data not depicted). Epibiotic bacteria have also been described from the feeding appendages of marine copepods, as well as on setae of other crustaceans such as the deep-sea Yeti crab, *Kiwa hirsuta* (Carman and Dobbs, 1997; Goffredi *et al.*, 2008). It should be noted that ~20% of the filter combs in 20 analysed daphnids were nearly uncolonised by bacteria. This might be because of moulting processes that have been shown to reduce the parasite loads on daphnids (Duneau and Ebert, 2012), and thus likely also affect the densities of other epibionts and the substrate uptake.

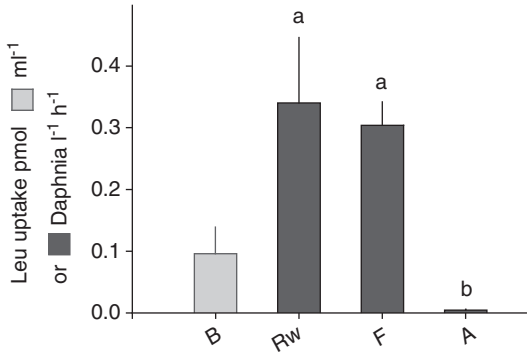

Figure 2 Leucine uptake by the heterotrophic bacterial community (B) after 1 h of labelling by active *D. galeata* after 1 h of incubation in the labelled raw water (Rw), and by active (F) and anaesthetised (A) *D. galeata* maintained for 1 h in tracer containing 0.2 μm prefiltered lake water. Error bars represent the standard errors of triplicate water samples (B) or the standard errors of measurements from three replicates (Rw, F and A). Different lowercase letters above the bars of Rw, F and A indicate significant differences between treatments (analysis of variance (ANOVA), $P<0.5$).

Active epibionts affiliated with Limnohabitans

Previous studies centred on the identity of *Daphnia*-associated microbes hint at the importance of *Betaproteobacteria*, in particular of bacteria related to the genus *Limnohabitans* (Peter and Sommaruga, 2008; Qi *et al.*, 2009; Freese and Schink, 2011). Therefore, we performed FISH on dissected individuals of *D. galeata* using an oligonucleotide probe for a phylogenetic cluster (*Lhb*) that includes the type strains *L. planktonicus* and *L. parvus* (Šimek *et al.*, 2001). A large proportion of the epibionts on the *D. galeata* filter apparatus were affiliated with *Lhb* (Figure 3), whereas <0.5% of cells in the surrounding cultivation water were hybridised with this probe. Most of the epibiotic *Lhb* bacteria showed visible incorporation of leucine (Figure 3), as detected by microautoradiography–FISH (27). Planktonic *Limnohabitans* spp. are known to readily incorporate this substrate (Horňák *et al.*, 2006; Salcher *et al.*, 2013). Cultures of *L. planktonicus* have, moreover, been shown to profit from the presence of algae that has been ascribed to their utilisation of algal exudates (Šimek *et al.*, 2011). In addition to the advantageous supply of such fresh DOC to *Limnohabitans* spp. on *Daphnia* filter combs by the filtration activity itself, it is also conceivable that these bacteria might further profit from the products of 'sloppy feeding', that is, organic compounds released by the physical breaking of algal cells (see, for example, Riemann *et al.*, 1986; Carman, 1994).

FISH on segments of various dissected *daphnids* from Lake Zurich confirmed the presence of epibiotic *Lhb* bacteria on natural zooplankton populations

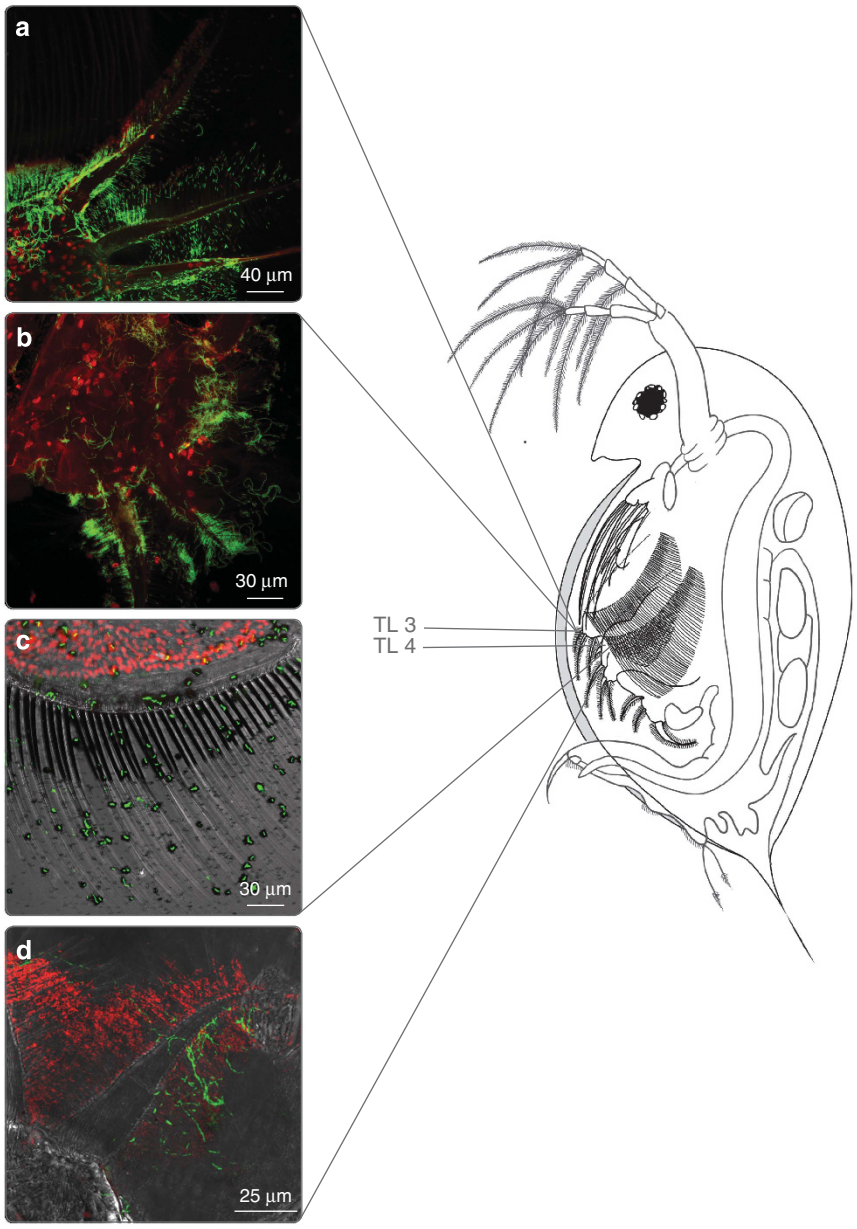

Figure 3 Confocal photomicrographs of *Daphnia* spp. epibionts (left), and localisation of the depicted structures in a schematic drawing of a daphnid (right). Green cells are hybridised with probe R-Bt065, targeting *Lhb* bacteria. Depicted in red are other DNA-containing objects, that is, bacterial cells that are not *Lhb* and nuclei of *Daphnia*. (**a**, **b**) *D. galeata* feeding appendages with hybridised *Lhb cells*. (**c**) *D. galeata* feeding combs with hybridised *Lhb* cells surrounded by black halos from microautoradiography staining that indicates the uptake of tritiated leucine by these bacteria. (**d**) Feeding appendage of daphnid from Lake Zurich with hybridised *Lhb* and numerous other bacteria. TL3 and TL4 indicate trunk limbs 3 and 4.

(Figure 3). Although only up to 3% of the heterotrophic bacteria in lake water were affiliated with *Lhb* (Figure 5), filter combs of *daphnids* were typically covered by bacteria from this genus that were, moreover, visibly incorporating leucine. In addition, there were clear morphological differences between planktonic *Lhb* and those associated with daphnids, for example, only the latter formed filamentous morphotypes (Figure 3). Similar to observations in cultures of *D. galeata,* some filter combs of daphnids in Lake Zurich were also virtually free of bacteria (in 3 out of 17 analysed individuals). In addition, other unidentified bacteria were occasionally found to dominate on the filter seata (Figure 3).

Indications for a core group of epibiotic Limnohabitans sp.
To investigate the phylogenetic relationship of *Lhb* bacteria on daphnids from various sources, we constructed 16S ribosomal DNA clone libraries using the sequence of the *Lhb* probe as a forward primer together with a general bacterial reverse primer. By this we identified bacteria associated

with *D. galeata* and Lake Zurich daphnids as well as from the respective surrounding water.

No sequence obtained from the cultivation water of *D. galeata* was from the genus *Limnohabitans*. In contrast, *Lhb* sequences were obtained from water samples of Lake Zurich. These sequences were, however, considerably different from the ones retrieved from daphnids (Figure 4). These findings indicate a degree of specificity of the associations between *Lhb* bacteria and their host (Wahl *et al.*, 2012).

Highest similarity was detected between *Lhb* sequences retrieved from daphnids from Lake Zurich and from the cultured *D. galeata* (Figure 4). The sequences clustered in two shared OTUs (99% identity level), as well as forming one specific OTU per source population. One of the shared clusters also included sequences retrieved from the digestive tract of *D. magna* (Freese and Schink, 2011) and the type strain *L. planktonicus* (Figure 4; Hahn *et al.*, 2010). It thus seems that there are core phylotypes closely affiliated with *L. planktonicus* that are commonly associated with different species of daphnids from various habitats, as well as a more variable set of other microbiota (Grossart *et al.*, 2009). *L. planktonicus* was originally isolated from pelagic samples of a freshwater reservoir (Kasalicky *et al.*, 2010). Subsequent analysis, however, revealed, that this species is not common in the pelagic zone of lacustrine waters (Jezbera *et al.*, 2013). It is thus possible that these bacteria in fact predominately inhabit an epibiotic niche. Epibiosis may however not be the exclusive place of occurrence for a particular genotype: the human pathogen *Vibrio cholera* is present in high abundances in the mouth area of marine copepods, but these bacteria are also found free-living in costal marine waters, although at low densities (Huq *et al.*, 1983;

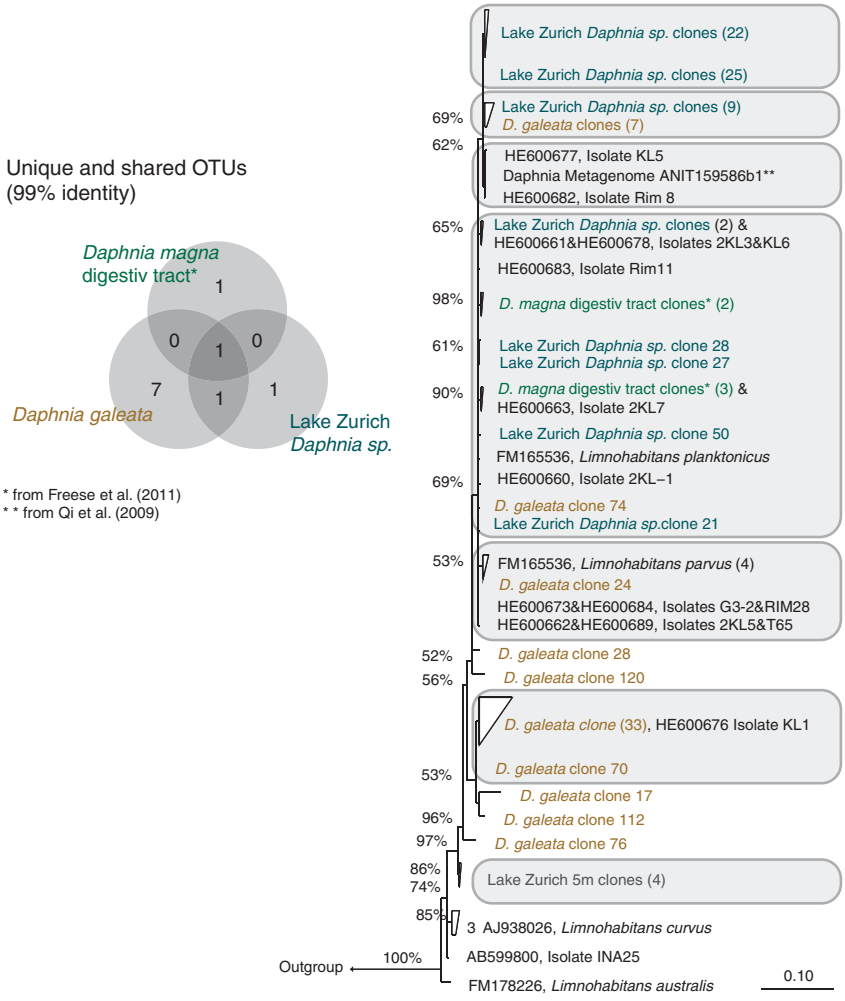

Figure 4 The left panel indicates shared and unique OTUs (99% identity cutoff) of 16S rRNA gene sequences of *Lhb* bacteria from cultured *Daphnia galeata*, from Lake Zurich daphnids, and from cultured *D. magna* (Freese *et al.*, 2009). The right panel indicates phylogenetic analysis (Maximum Likelihood method) of *Limnohabitans* spp. including sequences from cultured strains (Kasalický *et al.*, 2013) and *Daphnia* metagenome (Qi *et al.*, 2009). The individual OTUs are depicted as grey boxes, the broken line links sets of sequences from a single OTU. Values in brackets refer to the numbers of sequences in 'collapsed' clusters depicted as wedges. Only bootstrap values of >50% (1000 replications) are reported. Scale bar, 10% estimated sequence divergence.

Heidelberg *et al.*, 2002; Cottingham *et al.*, 2003). Thus, *V. cholera* may be part of the 'rare biosphere' within pelagic communities while being abundant on zooplankton. A similar occurrence pattern might be hypothesised for bacteria related to *L. planktonicus*.

Interestingly, closely related *Lhb* sequences were found on the filter combs of *D. galeata* and in the *D. magna* digestive tract. A possible explanation lies in the feeding physiology of *Daphnia*, that is, part of the *Lhb* population on the filter apparatus might be ingested and transported into the digestive system. Freshwater bacteria have been observed to pass the gut of daphnids alive (King *et al.*, 1991). Whether or not *Lhb* bacteria play a role in the digestion processes within the colon of the animals remains to be explored, as well as the mode of interaction between the Daphnids and *Lhb* epibionts on the filter combs. It should be emphasised that dead *D. galeata* were never found to be inhabited by *Lhb* bacteria ($n = 6$ inspected individuals), as has already been observed for *D. magna* (Freese and Schink, 2011). This may indicate that the interaction between *Lhb* bacteria and the animals is not of a pathogenic nature. Moreover, *Lhb* bacteria might actively leave the surface of *Daphnia* when the animals moult or die, as has been described for protistan epibionts (Willey and Threlkeld, 1995; Bickel *et al.*, 2012).

Activity of Daphnia epibionts in Lake Zurich: implication for food webs

In order to assess the *in situ* relevance of DOC uptake by daphnids (or their epibionts, respectively), we sampled Lake Zurich throughout April and May, starting at the onset of the clear water phase after the spring phytoplankton bloom. *Daphnia* reached abundances of up to 13 adult and 28 juvenile individuals l^{-1} (Figure 5), and were dominated by members of the *D. longispina* species complex (*D. galeata*, *cucullata, longispina* and hybrids; data: water supply Zurich). The uptake of dissolved leucine by daphnids and by bacterioplankton was determined on four dates (Figures 5 and 6). These experiments were in principle designed as described for the *D. galeata* cultures, with minor modifications (see Supplementary Figure S1). On three of the four dates, we additionally tested for uptake of NAG (Figure 6), a substrate that is not incorporated by *Lhb* (Eckert *et al.*, 2012). High uptake rates of both dissolved leucine and NAG were detected, although with large variations between dates (Figure 6). Interestingly, the proportional amount of NAG taken up by the daphnids via consumption of labelled bacteria (normalised to the labelled bacterioplankton community, that is, (Rw–F)/B) was consistently higher by approximately fivefold than that of leucine, indicating that *Daphnia* preferentially fed on NAG- rather than leucine-incorporating bacteria. This might be explained by the high NAG uptake rates of large filamentous

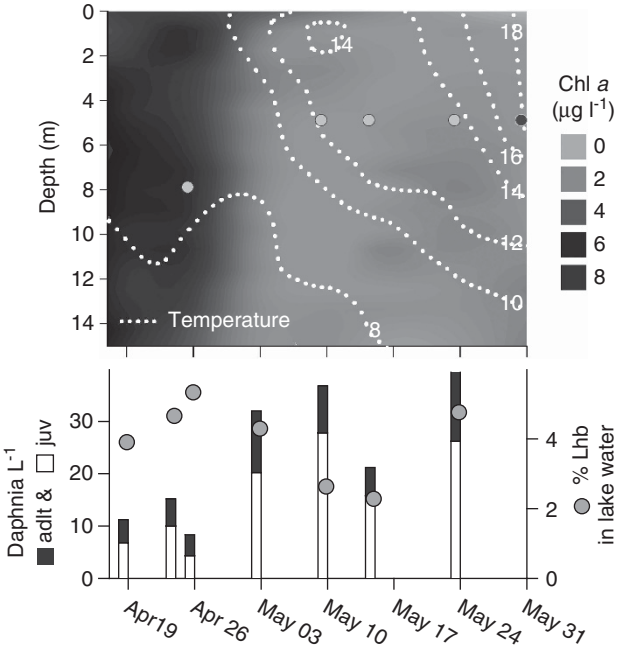

Figure 5 Development of chlorophyll *a*, temperature and of the populations of *Daphnia* sp. and pelagic *Lhb* bacteria in Lake Zurich from 17 April to 31 May 2013. Upper panel indicates Chlorophyll *a* concentrations and temperature between 0 and 15 m depth. Grey circles indicate the dates and depth of samplings for the incubations with radiolabelled tracers, and for DNA extraction to identify *Lhb* epibionts (last time point). Lower panel indicates abundances of juvenile (juv) and adult (adlt) daphnids and proportions of pelagic *Lhb* bacteria of all bacterioplankton cells.

bacteria that are more likely to be grazed by daphnids than, for example, small rod-shaped cells specialised for leucine incorporation (Langenheder and Jürgens, 2001; Pernthaler *et al.*, 2004; Kragelund *et al.*, 2008; Eckert *et al.*, 2013). Alternatively, there might be NAG uptake by some phytoplankton species that are consumed by daphnids (Nedoma *et al.*, 1994). Thus, the transfer mode of DOC to daphnids may in fact differ for individual organic compounds: Although the direct incorporation of heterotrophic bacteria seemed unimportant for leucine uptake of zooplankton, their foraging on NAG-labelled microbes was of greater relevance.

The observed uptake of both NAG and leucine by daphnids incubated in bacteria-free filtrates is evidence for a high, temporarily variable DOC incorporation *in situ*, likely mediated via the attached bacterial flora. This is suggested by the high proportion of tracer incorporation on the external body surfaces as compared with the digestive tract (data not depicted). Each daphnid approximately hosted 10^5 bacteria on their filter combs, as estimated from counts on individuals from 26 April. Assuming that 50–60% of the tracer is incorporated on these surfaces (as concluded from our laboratory experiment, data not depicted), the per-cell activity of bacteria on the filter combs is more than two orders of magnitude higher than of

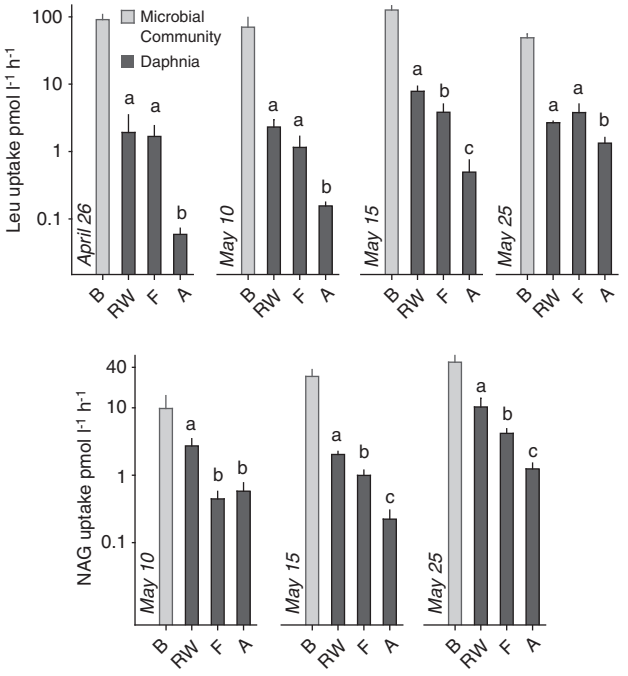

Figure 6 Uptake of tritiated leucine (upper panels) and NAG (lower panels) by the heterotrophic bacterial community (B) after 1 h of incubation, by lake daphnids after 1 h of incubation in this labelled raw water (Rw), and by active (F) and anaesthetised lake daphnids (A) maintained for 1 h in tracer containing 0.2 μm prefiltered lake water. The experimental dates are indicated in each panel. Error bars represent the standard errors of triplicate water samples (B) or the standard errors of measurements from three replicates (Rw, F and A). Different lowercase letters on the bars of Rw, F and A indicate significant differences between the treatments ($P<0.5$).

free-living lake bacteria. This is in agreement with findings from marine systems that zooplankton-associated bacteria are metabolically more active than free-living bacteria (Møller *et al.*, 2007). Moreover, attached bacteria move through the water column with the host (Grossart *et al.*, 2010). Daphnids tend to search for patches with higher food concentration (Larsson and Kleiven, 1996; Dodson *et al.*, 1997) and might thereby give an additional advantage to attached bacteria, that is, by transporting them to hot spots of organic carbon and inorganic nutrients (Grossart *et al.*, 2010).

If extrapolated to the total daphnid population in lake water, the incorporation amounted to up to 8% of the leucine and nearly 9% of the NAG uptake by the bacterioplankton. Although this proportion may seem small at a first glance, such direct transfer of low-molecular-weight DOC to zooplankton may nevertheless be of considerable importance. The positive effect of *Daphnia* on the size of fish populations in lakes is mainly attributed to the fact that *Daphnia* feed on primary producers and are in turn consumed by fish that is, the cascade algae–daphnia–fish is considered to be highly efficient because of its shortness (Stockner and Porter, 1988; Stockner and Shortreed, 1989). Similarly, the prokaryotic epibionts on daphnids may form a more direct link between microbial DOC uptake and fish predation. Such a shortcut would circumvent the passage of substrates through intermediate levels of the microbial food web, thereby avoiding the significant respiration losses associated with these trophic transitions (Lindeman, 1942; Pomeroy and Wiebe, 1988; Stockner and Shortreed, 1989). This may be illustrated by a highly simplified lake food web consisting of planktivorous fish, *Daphnia*, phytoplankton, protists, bacteria and DOC. In this food web, fish feed on *Daphnia* that feed on protists and phytoplankton. The latter provides DOC for bacteria that are in turn incorporated by protists. However, the pathway of the transfer of organic carbon changes when epibiosis is included: if fish feed on *Daphnia*, they also ingest the attached bacteria. Thus, fish concomitantly consume organisms that are considered to belong to different trophic levels, and all of these organisms will contribute to their biomass. Considering the '*Rule of 10*' concept (stating that only 10% of energy is transferred from a given trophic level to the next one; Lindeman, 1942), at least 0.1% of the total microbial DOC (leucine) uptake would be transferred to fish via the epibiotic bacteria. In comparison, <0.01% of DOC would reach fish through the 'classical' microbial loop because of the losses that occur across two or more intermediate trophic levels (that is, protists, daphnids). Thus, 10 times more carbon would be transferred to fish from epibiotic bacteria than from free-living bacteria. This suggests that zooplankton epibionts such as *Limnohabitans* sp. might play a disproportionally important role for the transfer of DOC from both autochthonous and terrestrial sources to the top trophic levels in lacustrine ecosystems.

Acknowledgements

We are particularly indebted to Piet Spaaks, Marina Manca and Esther Keller for providing us with *Daphnia galeata* and the introduction to *Daphnia* handling, and to Julia Huber for Daphnia artwork. Michaela Salcher is acknowledged for providing DNA from Lake Zurich, Jörg Villiger for bioinformatic assistance, Eugen Loher for help with sampling and Thomas Posch for comments on data analysis and the manuscript. We also thank Karel Šimek and Voijta Kasalický for interesting discussions about *Limnohabitans*. The assistance and support of the Centre for Microscopy and Image Analysis at the University of Zurich is acknowledged. This study was funded by the Swiss National Science Foundation (Project 31EE30-132771) as part of the European Collaborative Research Program (EUROCORES) Ecological and Evolutionary Functional Genomics (EuroEEFG) of the European Science Foundation.

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