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Bacteria-Mediated Effects of Antibiotics on *Daphnia* Nutrition

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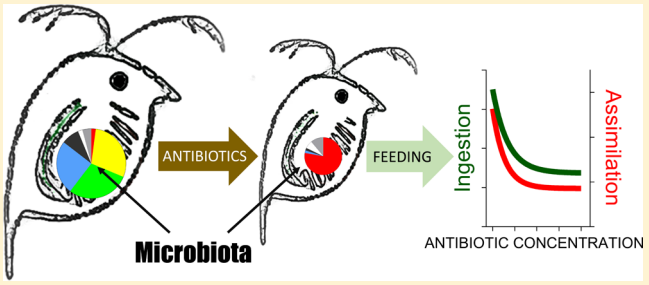
Supporting Information

ABSTRACT: In polluted environments, contaminant effects may be manifested via both direct toxicity to the host and changes in its microbiota, affecting bacteria–host interactions. In this context, particularly relevant is exposure to antibiotics released into environment. We examined effects of the antibiotic trimethoprim on microbiota of *Daphnia magna* and concomitant changes in the host feeding. In daphnids exposed to 0.25 mg L^{-1} trimethoprim for 24 h, the microbiota was strongly affected, with (1) up to 21-fold decrease in 16S rRNA gene abundance and (2) a shift from balanced communities dominated by *Curvibacter*, *Aquabacterium*, and *Limnohabitans* in controls to significantly lower diversity under dominance of *Pelomonas* in the exposed animals. Moreover, decreased feeding and digestion was observed in the animals exposed to 0.25–2 mg L^{-1} trimethoprim for 48 h and then fed ^{14}C-labeled algae. Whereas the proportion of intact algal cells in the guts increased with increased trimethoprim concentration, ingestion and incorporation rates as well as digestion and incorporation efficiencies decreased significantly. Thus, antibiotics may impact nontarget species via changes in their microbiota leading to compromised nutrition and, ultimately, growth. These bacteria-mediated effects in nontarget organisms may not be unique for antibiotics, but also relevant for environmental pollutants of various nature.



INTRODUCTION

Bacteria contribute to an overwhelming variety of functions and physiological processes of their invertebrate hosts, related to feeding,[1] reproduction,[2] development,[3] and aging.[4] The majority of these microbes are found within the gut, playing an important role in the host's digestion.[5,6] In addition, many animals, and particularly arthropods, rely on the biosynthetic capacities of their gut microbiota as a nutritional resource.[7] Environmental pressures, such as climatic factors, suboptimal diets, and contaminants may impact interactions between microbial symbionts and their hosts.[8] In polluted environments, contaminant effects may be manifested via both direct disturbance of the host physiology and disruption of bacterial communities associated with the host. Consequently, animal tolerance to environmental stressors, including contaminants, is a function of both the animal and its microbiome tolerances. In ecotoxicological context, particularly relevant are the responses of microbiome to environmental contaminants and the implications for the fitness of the host.[9]

Antibiotics are contaminants of environmental concern as they are biologically active and often have a low biodegradability.[10] Both free-living and symbiotic bacteria possess cellular targets for these compounds. Additionally, they possess a remarkable ability to adapt to their environment,[11] which includes antibiotic-resistance mechanisms; moreover, they share those adaptations within and between communities via gene transfer.[12] A broad conceptual framework has been worked out describing (1) the potential of wide use of antibiotics in animals and humans to adversely impact their health,[12] (2) environmental impacts of antibiotics on soil and sediment microbial communities,[13] and (3) spread of antibiotic resistance genes in the environment,[12] even at low antibiotic concentrations.[14] However, despite the obvious relevance, many details regarding ecology and ecotoxicology of antibiotic effects on host–symbiont relations in nontarget organisms are largely unknown.[11]

In aquatic ecology and ecotoxicology, crustaceans are routinely used as test species in field and laboratory studies. The cladoceran *Daphnia magna* is a model species used to study stress responses, physiological adaptations, and genome-environment interactions.[15] To date, *Daphnia* microbiome is comprehensively described at using 454-pyrosequencing of bacterial 16S rRNA genes[16] and cloning[17] approaches. Its host species-specificity,[16−19] independence on the sequencing platform,[16] and relative stability in animals exposed to different feeding and thermal environments[17] are remarkable. This implies that stress-induced responses in microorganisms (i.e.,

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bacteria, fungi, viruses, archaea and protozoans colonizing cells, guts, and external body surfaces[20−23]) would be detectable and informative. Yet, relatively little is known about most of the microbiota in test species, compared to more comprehensive research about their microbial parasites,[24] which found many relevant applications in ecotoxicology.[25,26]

In crustaceans, bacteria-mediated digestion contributes greatly to the breakdown and absorption of essential compounds, such as essential amino acids and vitamins.[22] Therefore, nutrition and growth penalties in animals with compromised microbiota are to be expected. Indeed, a retarded development in the antibiotic-exposed copepod *Nitocra spinipes* was linked to structural changes in its microbiota, and it was suggested that these changes resulted in the dysregulation of the host digestion.[21] We hypothesized that following exposure to an antibiotic drug, the diversity and abundance of *Daphnia*-associated microflora will decrease, with concomitant decrease in feeding activity and assimilation efficiency of the host. These hypotheses were tested by combining (1) exposure experiments, (2) assessment of dominant bacterial community members using a small subunit 16S rRNA gene (hereafter referred to as 16S) clone library approach and a qPCR of 16S to estimate total bacterial abundance, and (3) measurements of feeding, carbon incorporation, and digestion efficiency of the host (see Supporting Information, SI, Table S1, for the overview).

MATERIALS AND METHODS

Test Organism. *Daphnia magna* originated from a single clone (environmental pollution test strain *Klon 5*; the Federal Environment Agency, Berlin, Germany). The animals were cultured in 2 L M7 medium (OECD standards 202 and 211) at a density of ∼20 ind. L^{-1} and fed with a mixture of axenically grown green algae *Pseudokirchneriella subcapitata* and *Scenedesmus subspicatus* cultured at 22 °C under a light intensity of 40 μE cm^2 s^{-1}. Sterile media and glassware were used for the cultures, but no additional attempts were made to keep the *Daphnia* germ-free. In all feeding experiments, *P. subcapitata* was used as the sole food. Algal concentrations were determined using fluorescence measurements (10 AU; Turner Designs, Sunnyvale, California, USA).

Selection of the Model Substance and Its Mode of Action. Trimethoprim (TMP; diaminopyrimidine trimethoprim; 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine; CAS 738-70-5; Sigma 92131, purity, >99%) was used as a model substance. This is a bacteriostatic pharmaceutical inhibiting bacterial dihydrofolate reductase enzyme (DHFR) and thus synthesis of tetrahydrofolic acid,[27] a coenzyme in many reactions, especially in the metabolism of amino acids and nucleic acids. The selection was based on the following properties of the drug: (1) high persistence with little removal by wastewater treatments, and thus ubiquitous occurrence in ranges from very low ng L^{-1} to ∼10 μg L^{-1} in wastewater,[28] but also reaching as high as 0.3 mg L^{-1},[29] (2) slow biodegradation in natural waters,[30] (3) high correspondence between nominal and measured concentrations in the experimental incubations with similar setup (94%),[21] (4) potency to decrease microbiome diversity in the copepods,[21] and (5) low affinity for animal DHFR (∼50 000-fold less than for the corresponding bacterial enzyme), which explains low acute toxicity in *Daphnia*.[31,32] Absence of acute toxicity of TMP to *D. magna* in the concentration range used for Experiments I to III was confirmed in separate pilot tests (provided as SI). In all experiments, solvent (dimethyl sulfoxide, DMSO corresponding to a volume of 0.1% of the total incubation volume) control was used as a vehicle control (hereafter referred to as the control).

Exposure of Daphnids for Bacteria Abundance and Diversity Analyses (Experiment I). To obtain samples for analysis of bacteria abundance and community composition, neonates were assigned to two treatments, 0.25 mg L^{-1} TMP and control; five animals per replicate and three replicates per treatment. The animals were provided with food at 1.5 μg C mL^{-1} and exposed for 24 h in darkness at 20 ± 1 °C. Upon termination of the experiment, the daphnids were examined for motility and live individuals were transferred to Eppendorf tubes and stored at −80 °C for bacteria abundance and diversity analysis.

Carbon Ingestion and Incorporation Rates Measured by Radioactive Labeling (Experiment II). In this experiment, daphnids were first exposed to TMP (Part A) and then incubated with ^{14}C-labeled algae in either a pulse-feeding experiment to measure the ingestion rate (Part B) or in a longer incubation to measure carbon incorporation (Part C; SI Figure). In Part A, nonfed daphnids were incubated in M7 medium and at three TMP concentrations (0.25, 0.5, and 2 mg L^{-1}) for 48 h in 3 L beakers (one beaker per treatment, ∼30 ind. L^{-1}), gently collected on a submerged 200 μm sieve, and transferred to M7 media for 1−1.5 h before Part B commenced. The methods largely follow the standard radiotracer approach.[33]

The culture of *P. subcapitata* was labeled with 150 μCi L^{-1} $Na_2H^{14}CO_3$ (DKI: specific activity, 1.9×10^{-9} Bq $nmol^{-1}$) and incubated for 72 h to ensure uniform labeling[34] at constant illumination and 20 °C. The labeled algae were collected on a Millipore filter (0.45 μm pore size), washed three times with Milli-Q water, and diluted to the experimental concentration with M7 medium; five replicate 10 mL samples from each concentration were filtered onto Whatmann GF/F filters (0.7 mm). After the addition of a scintillation cocktail (Ultima Gold, Packard), the isotopic activity of ^{14}C was measured by liquid scintillation counting (LKB Wallac) using the channel-ratio method for calculating counting efficiency. For use in feeding trials, the filtered algae were resuspended in M7 (1.2 μgC mL^{-1}). Immediately prior to the feeding, duplicate aliquots of the radioactive food sample were filtered onto 0.45 μm membrane filters. The filters were then exposed to HC1 fumes for 15 min, immersed in 7 mL of the scintillation cocktail, and counted.

The pulse-feeding experiment (Part B) was carried out in 250 mL plastic beakers (four beakers per TMP concentration) containing a cage made from a 50 mL polyethylene centrifuge tube with a sealed nylon-mesh bottom (67 μm). The beakers were filled with suspension of unlabeled algae (1.2 μgC mL^{-1}). To measure the ^{14}C ingestion by the daphnids, 10 individuals (Instar II−III; body length 1.1−1.4 mm) per cage were incubated with unlabeled *P. subcapitata* for 1 h, then most of the medium with the unlabeled algal suspension was siphoned out and replaced with the labeled algae of the same concentration. The daphnids were allowed to feed for another 6 min; this time was assumed to be less than gut passage.[34] After this pulse-feeding, the daphnids were rinsed with Milli-Q water and transferred into vials to measure the total amount of radioactivity ingested by daphnids during the pulse feeding period.

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The ^{14}C incorporation was measured using the same setup and sampling scheme as in the pulse-feeding experiment, but with the incubation time of 3 h in the radiolabeled suspension (Part C). The beakers were incubated at 20 °C in a light cabinet and stirred frequently to prevent sedimentation of the algae. After the incubation, the daphnids were transferred to the unlabeled algal suspension for 30 min for gut depuration.

All experimental animals pooled within a replicate incubation (Parts B and C) as well as negative controls of daphnids (pooled samples from all antibiotics treatments and control) that were also sampled when Part A was terminated, were placed in vials. Each vial contained 0.5 mL of 1 N NaOH and the animals were solubilized at 60 °C overnight.[35] Subsequently, 6.5 mL of the scintillation cocktail were added and the radioactivity was counted. The radioactivity of the daphnids and the specific activity of the algal suspension were used to calculate the individual ingestion rate (mgC ind^{-1} h^{-1}; Part B), carbon incorporation rate (mgC ind^{-1} h^{-1}; Part C), and the incorporation efficiency (incorporation/ingestion). The latter was used as a proxy for assimilation efficiency that was not measured directly as respiration contribution was not assayed.

Digestive Efficiency of Daphnids (Experiment III). The proportion of dead algal cells in animal guts was assayed by a LIVE/DEAD assay employing a combination of selective staining of dead cells and chlorophyll autofluorescence.[36] The difference in the proportion of dead cells was used to infer changes in digestive efficiency in the animals exposed to TMP compared to the control animals. The daphnids from the control and TMP exposure (Experiment II, Part A) were subsequently used in a feeding experiment, where 10 adult individuals (>2.5 mm) per replicate were incubated at 1.5 μgC mL^{-1}, five replicates per treatment, for 1 h using the same type of beakers and cages as in the Experiment II (Parts B and C). Upon termination of Experiment III, the animals were sampled, rinsed, and their guts were separated from body tissues using sharp forceps. The dissected guts were placed in an Eppendorf tube (pooled for each replicate) containing 50 μL of TO-PRO-1 iodide solution (Molecular Probes Inc.; Eugene, OR), homogenized briefly with a Kontes pestle, and incubated for 20 min in darkness. Using this suspension, the LIVE/DEAD assay was conducted by counting algae (>350 cells) with an epifluorescent microscope Olympus AH2 equipped with standard long-pass blue filter (wavelength approximately 400−550 nm) at 100× magnification. The nonviable cells were identified as those with bright yellow-green nuclei, while viable cells had only red chlorophyll autofluorescence. Only cells with visibly intact membrane were considered, and staining intensity was not taken into account. The proportion of dead cells in a sample was used as a proxy for digestive efficiency of the daphnids.

DNA Extraction and PCR Amplification. Daphnids collected in Experiment I were used to evaluate TMP effects on the bacterial diversity. DNA was extracted from *Daphnia* samples using 10% Chelex.[37] Partial 16S sequences (~900 bp) were amplified from the extracted DNA using the degenerate bacterial primers: fD1 (AGAGTTTGATCMTGGCTCAG, *E. coli* positions 8 to 21)[38] and 926r (CCGTCAATTCCTTTRAGTTT; *E. coli* positions 926 to 907).[39] Each 50 μL of PCR contained 35 pmol of each primer, a ready to use PCR master mix (Fermentas, Hanover, MD), 5 μg of BSA (Fermentas), and 1 μL of template DNA. A cycling program was performed using a GeneAmp PCR System 2400 thermocycler (Applied Biosystems, Foster City, CA) as follows: 5 min at 95 °C followed by 33 cycles of 40 s at 94 °C, 1 min at 56 °C, 1 min at 72 °C, and extension at 72 °C for 7 min. PCR products were purified and concentrated by using the DNA Clean & Concentrator-5 Kit (Zymo Research, CA) prior to cloning.

Cloning and Sequencing. In total, six clone libraries were created from PCR products that were amplified from *D. magna* DNA extracts, one clone library for each replicate in the antibiotic treatment and control, 16 clones per library, 98 clones in total. Cloning with QIAGEN PCR Cloning Kit and plasmid DNA isolation with QIAGEN Miniprep Purification System were performed following the manufacturer's recommendations. For sequencing of inserted 16S gene fragments, we used the fD1 primer on a 96-capillary ABI3730XL DNA analyzer (Applied Biosystems; Uppsala Genome Center, Uppsala, Sweden). With the use of the Blastn software,[40] 16S genes were compared to GenBank sequences. To detect chimeras, the sequences were analyzed with DECIPHER 1.10 software.[41] The obtained 74 unique sequences were deposited in the GenBank (accession numbers KM603394 − KM603467; SI, Table S2).

Real-Time PCR Amplification and Quantification. Quantitative PCR (qPCR) was used to estimate 16S gene copy number that served as a proxy for total bacterial abundance. All assays were performed using StepOne Real-Time System (Applied Biosystems, Life Technologies, Foster City, CA, USA). Universal primers 534F 5′-CCAGCAGCCGCGGTAAT-3′ and 783R 5′-ACCMGGGTATCTAATCCKG-3′ were used to amplify bacterial 16S while limiting chloroplast DNA amplification;[42] the latter was of concern because algae were present in the guts. The 20 μL reactions contained 10 μL of SsoFASTTM EvaGreen SuperMix (Bio-Rad, Hercules, CA, USA), 2 μL of each primer (12.5 μM), and 2 μL of template DNA. The samples were heated for 3 min at 95 °C followed by 40 cycles of 5 s at 95 °C and 30 s at 53 °C. Negative controls were performed for all runs; all samples and controls were run in duplicate. Triplicate 10-fold dilution series of genomic *Escherichia coli* DNA served to generate a standard curve (95−102% amplification efficiency, r^2 = 0.99).

Data Analysis and Statistics. Taxonomy was assigned to each sequenced 16S gene by using the Basic Local Alignment Search Tool (BLAST). Multiple sequence alignments were performed using the MUSCLE software, version 3.5.[43] Phylogenetic relationships between 16S sequences were determined by using the maximum likelihood method[44] with the software PHYML version 2.4[45] and visualized with MEGA5. The clone libraries were combined within a treatment and classified into operational taxonomic units (OTUs) at a sequence similarity cutoff of 98%. Rarefaction curves were calculated for the overall combined data set by using the individual-based Coleman method[46] (Hughes et al., 2001) with the PAST software (Hammer et al., 2001).[47] The Good's percentage of coverage was also calculated.[48]

The Brillouin diversity index (H_B) and Brillouin relative evenness (V) were calculated using OTUs from each clone library and the PAST software. The Brillouin indices are not influenced by phylotype richness and are the most appropriate for these data as the selectivity of the PCR approach implies that the sample may not be random.[49] To compare diversity indices between antibiotic-treated and control bacterial communities, an unpaired t test was applied.

Generalized linear models (GLZ module) in STATISTICA 8.0 (StatSoft 2001, Tulsa, USA) with normal error structure and log-link function were used to evaluate whether nominal

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TMP concentration explained the variation in ^{14}C-based estimates for ingestion and incorporation rates, incorporation/ingestion ratio, and percentage of dead algal cells in the gut lumen of the test animals. The Wald statistic was used for regression coefficients, and model goodness of fit was evaluated using deviance and Pearson χ^2 statistics. Residual plots were examined to exclude remaining unattributed structure indicative of a poor model fit.

RESULTS

Bacterial Abundance. The bacterial abundance was strongly affected by TMP exposure, with 7- to 21-fold decrease in average bacterial 16S copy number in the TMP-exposed daphnids compared to the controls (Figure 1A).

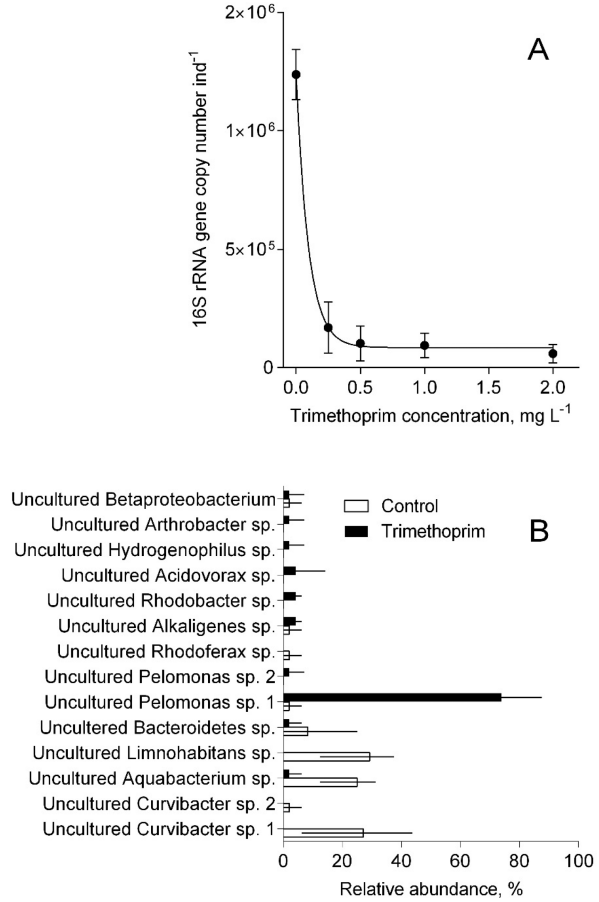

Figure 1. Quantitative (A) and qualitative (B) changes in bacterial flora associated with *Daphnia magna* exposed to trimethoprim (TMP). (A) Abundance of bacteria associated with daphnids (copy number of 16S rRNA genes ind.$^{-1}$) as a function of TMP concentration (0 to 2 mg L^{-1}); (B) Bacterial phylotypes (mean ± SD, n = 3) present in the controls and TMP-exposed (0.25 mg L^{-1}) *Daphnia magna*.

Bacterial Diversity. Although the rarefaction curves did not plateau with the current sequencing effort (SI, Figure S2), Good's coverage was 89% and 90% in TMP and control libraries, respectively (SI, Figure S3). This level of coverage indicated that the 16S gene sequences identified in these samples represent the majority of the phylotypes present.

Nearly all clones (98%) showed high levels of similarity (≥98% identity) with known sequences in the GenBank (SI, Table S2). Over 38% of the sequences represented uncultivated members of the *Pelomonas* genus, followed by genera *Curvibacter* and *Limnohabitans* (15% each), *Aquabacterium* (14%), and the Bacteroidetes Phylum (5%). Other detected genera comprised <12% of all clones sequenced. However, the proportions of these groups were profoundly different between the TMP and control libraries (Figures 1B and 2AB). A major shift from a relatively balanced community dominated by *Curvibacter* (29%), *Limnohabitans* (29%), and *Aquabacterium* (25%) in the controls to the strong dominance of the *Pelomonas* group (77%) in the exposed animals was observed (Figure 2A). Although the total number of OTUs was identical in the controls and TMP-exposed animals (nine in each group), a significantly lower diversity and microbial community evenness were observed in the exposed daphnids (Figure 3).

Feeding Activity. In the TMP-exposed daphnids, up to 68% and up to 62% decrease in ingestion rate and incorporation efficiency, respectively, were observed compared to the controls; this resulted in up to 86% decrease in the incorporation rate (Table 1A; Figure 4AB). Also, a significant decrease in the digestive efficiency was observed as indicated by the decreased proportion of dead algal cells in the guts of the exposed daphnids (Table 1B; Figure 4C).

DISCUSSION

As hypothesized, TMP exposure caused a decrease in the abundance (up to >20-fold) and a remarkable shift in the community structure of bacteria associated with *D. magna* (Figures 1, 2). These changes coincided with decline in feeding activity, digestion efficiency, and carbon uptake by the host, whereas mortality was not affected at the concentrations tested (<2 mg L^{-1}; see SI). The effects on the bacterial assemblages and feeding were observed at the lowest test concentration (0.25 mg L^{-1}), which is ∼500-fold below the reported acute toxicity values for *Daphnia* (48 h EC_{50}: 123 mg L^{-1}, 96 mg L^{-1})[31,32] and ∼20-fold below the reported serum therapeutic concentrations (1.5−9 mg L^{-1}),[50] but within the lower range of TMP minimum inhibitory concentrations (MIC) for reference bacteria strains (0.25−16 mg L^{-1}).[51] This suggests that the primary mechanism of the compromised nutrition of the TMP-exposed daphnids was related to the declined gut microflora and disrupted bacteria−bacteria and host−bacteria interactions involved in food digestion and nutrient uptake. Taken together, these results imply that antibiotics may cause profound effects on nontarget eukaryotes via changes in structure and abundance of their microbiome and possibly to compromised nutrition and, ultimately, fitness reduction of the host. This aspect of the delayed toxicity caused by antibiotic drugs must be considered in ecotoxicological evaluations, if we are to understand environmental effects of antibiotics as emerging contaminants.

The decreased feeding and energy uptake by the daphnids occurred in all TMP concentrations (Figure 4), with the 28−68% lower ingestion rate compared to the controls and a corresponding 52−86% reduction in carbon incorporation, which was the most affected end point (Table 1). The greater impact on carbon uptake reflects a net effect of the decreased food intake and reduced incorporation efficiency. Similarly, in *Daphnia* treated with a mixture of streptomycin and gentamycin, a significant inhibition of carbon uptake was observed.[52] The reduced carbon uptake would translate to a decreased net energy budget of the daphnids and thus ultimately affect their fitness. Not surprisingly, various fitness penalties have been reported in axenically cultured *Daphnia*, such as poor growth, low fecundity, and high mortality,

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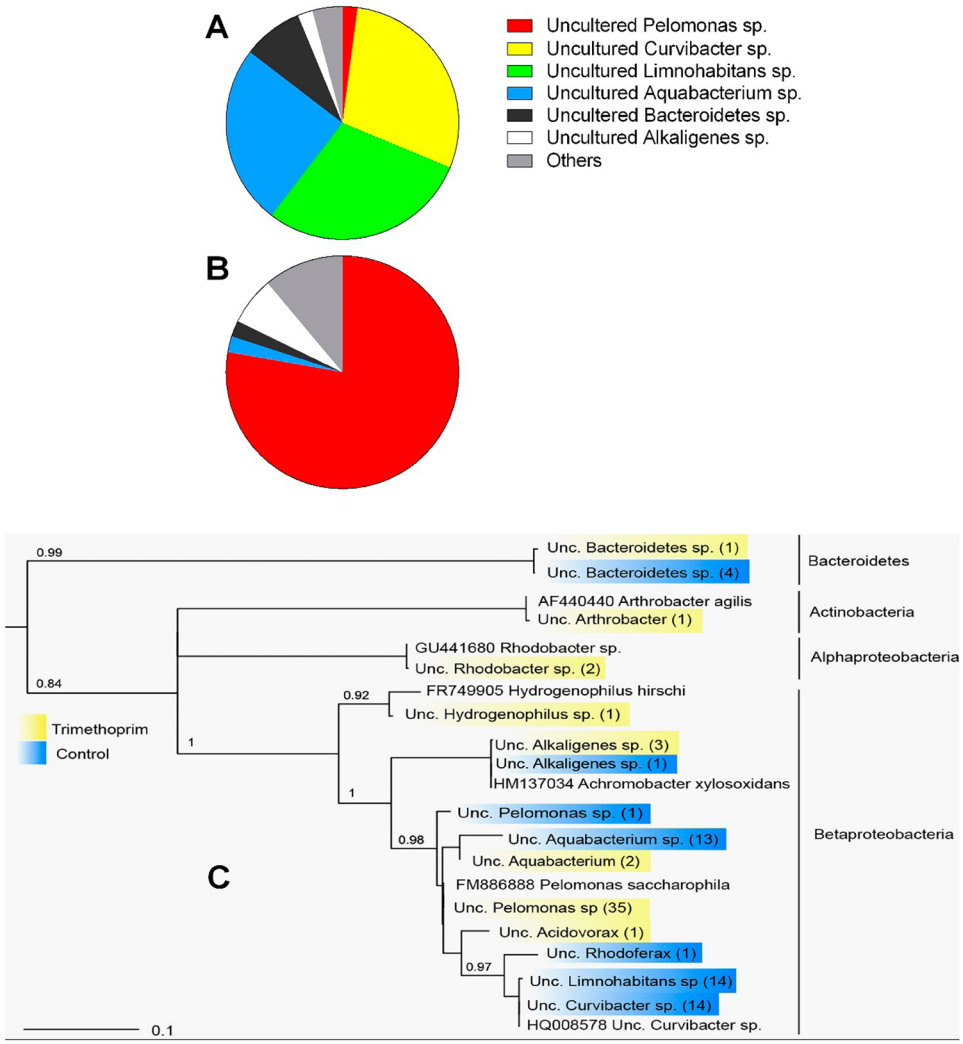

Figure 2. Phylotypes identified in clone libraries of *Daphnia magna*. Relative contribution of identified phylotypes in (A) controls and (B) TMP-exposed animals, and (C) unrooted 16S rRNA gene tree based on maximum likelihood analyses of bacteria identified in controls (blue) and TMP (yellow) libraries. Reference sequences are in black and presented with their GenBank accession numbers followed by identity descriptions. Number of 16S rRNA gene sequences identified in each clone library is indicated in brackets following the clone number. Identical sequences found within a clone library were not included. One thousand bootstrapped replicate resampled data sets were analyzed. Bootstrap values are indicated as proportion (<1) and not shown if <0.5.

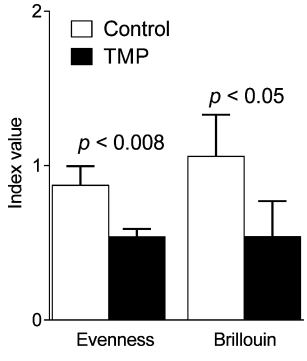

Figure 3. Diversity indices (Evenness, V, and Brillouin, H_B, indices) calculated for bacterial communities associated with *Daphnia magna* in controls and TMP (0.25 mg L^{-1}) incubations. Unpaired t test (mean ± SD, n = 3) was used for group comparisons.

Table 1. ***Daphnia magna***[a]

response variable	estimate	Wald statistic	p
(A)			
ingestion rate	−0.545	23.11	<0.0001
incorporation rate	−3.066	71.32	<0.0001
incorporation/ingestion ratio	−0.479	13.77	0.0002
(B)			
dead cells, %	−0.409	17.29	<0.0001

[a]Generalized linear models testing effects of trimethoprim exposure on (A) ingestion and incorporation rates, and incorporation/ingestion ratio (Experiment II), and (B) percentage of dead algal cells in the gut lumen (Experiment III).

compared to xenic cultures.[53,54] Similar fitness reductions have also been observed in other germ-free lines of arthropods.[2,55] It is possible that removal of antibiotics from the media will allow recolonization of daphnids with the original microbiota. Alternatively, longer exposure would allow development of a more adapted bacterial consortium, which may take over some of the ecological functions of the affected species and possess higher capacity to support nutrition. Studying such long-term effects is clearly relevant for our understanding of adaptation

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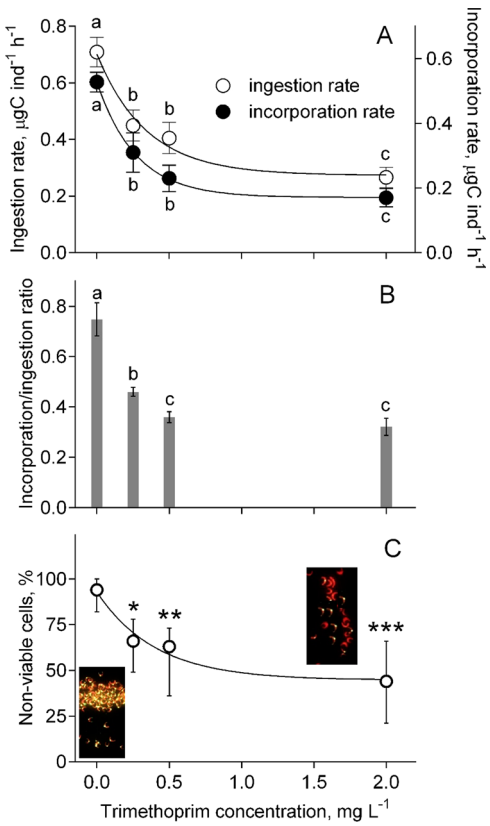

Figure 4. Feeding activity of *Daphnia magna* as a function of TMP concentration assayed in Experiment III: (A) Carbon ingestion and incorporation rates (mean ± SD, $n = 4$), (B) incorporation/ingestion ratio, and (C) digestive efficiency estimated as a proportion of dead algal cells in the gut content (median with min and max values shown as error bars; $n = 5$). Photo inserts show algal cells extracted from the daphnid guts and stained with TO-PRO-1 iodide; yellow-green staining of nuclei indicate nonviable cells, red fluorescence is due to chlorophyll, which is present in both live and dead cells. Image to the left is taken on a control sample and image to the right shows a sample from the highest TMP concentration. Means with the same letter within a variable are not significantly different; the tests are based on Box-Cox transformed values (Tukey HSD test). In percentage comparisons (panel C), asterisks indicate the significance level for the difference from the control incubations (Dunn's test: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$).

mechanisms in polluted environments and development of adequate environmental risk assessment tools.

Crustaceans harbor a microbial community consisting of bacteria, viruses, and fungi[22] inhabiting internal and external body surfaces.[56] In our experiments, all these elements (i.e., gut microflora, intracellular parasites, and epibionts) were potentially targeted by TMP. Therefore, the observed decline in the abundance of 16S genes and changes in the community could be attributed to any part of the microbiome. Many of the bacterial OTUs in our clone libraries are likely to originate from the gut as suggested by gut microflora composition in various animals and relatedness of our sequenced phylotypes with those reported from fish and invertebrates, including *Daphnia* (SI, Table S2). In particular, *Limnohabitans* have been reported to dominate gut microflora of *D. magna*[17] these bacteria were also the main contributors to the clone libraries generated from the controls (Figures 1B and 2AB). *Aquabacterium* is another genus commonly occurring in biofilms[57] but also residing in guts of various invertebrate and vertebrate species (insects,[58] birds,[59] humans[60]), as does *Curvibacter* (fish,[61] cnidarians,.[62] Similar to *Limnohabitans*, both *Aquabacterium* and *Curvibacter* dominated clone libraries in the controls but were absent in the antibiotic-treated animals (Figures 1B and 2AB). In the TMP-exposed animals, these three genera, each contributing 25−29% in the controls, were replaced by *Pelomonas* bacteria. The latter have been frequently found in lung microbiota of mammals[63] and in bird guts;[59] however, their functional role is largely unknown. It has also been reported from the gut of insects, where it occurred in low abundances and was not linked to microbially driven digestion.[64] Some *Pelomonas* possess strong antifungal properties, and it has been suggested that these bacteria may protect cnidarian *Hydra* from *Fusarium* fungus infections.[62] Therefore, one can hypothesize that the high proportional *Pelomonas* increase (from 2% in controls to 77% in TMP-exposed animals) may be associated with a concomitant increase in fungi; this, however, remains to be tested.

For a natural bacterial community, it is basically impossible to assess the effect concentrations of antibiotics, because bacteria differ in their sensitivity to specific antibiotics. Moreover, this sensitivity can change with changing ecological parameters, community composition, and evolutionary history. For example, TMP inhibits some microbial species at concentrations of 4.6 μg L^{-1},[65] while other bacteria are not adversely affected at concentrations >100 mg L^{-1}.[66] Furthermore, the response to antibiotics by natural bacterial communities, and particularly those involved in mutualistic interactions with eukaryotes, can substantially differ from the response of those in clinical environments. We observed ∼85% decline in bacteria abundance at 0.25 mg L^{-1}, giving a strong indication of EC_{50} values being much lower. However, it is difficult to assess which taxa were affected directly by the treatment and which responded indirectly to changes in other community members. Therefore, use of gnotobiotic cultures or, at least, a model species carrying a simplified bacterial community and tested under controlled conditions, would reduce the complexity of the natural environment allowing speculations at the community level.

As interest in microbiome-mediated responses emerges, the roles of microbiome in physiology and ecology of eukaryotes are being investigated. To study bacteria−host interactions, gnotobiotic cultures have been established for various model organisms,[67] including *Daphnia*.[53,54,68] Recently, it was demonstrated that in the absence of bacteria, a diet of algae alone is not sufficient for normal *Daphnia* functioning, and it was suggested that bacteria are required either as nutritional supplements or functional partners or both.[54] In germ-free Daphnia cultures, *Chlamydomonas reinhardii*, a flagellate with cellulose deficient cell walls,[69] was found nutritionally superior.[68] One can speculate that digestive capacity of the gut devoid of microbiota is lowered, especially toward refractory compounds, such as cellulose, because cellulases in crustacean gut are mostly produced by endosymbiotic bacteria.[23,70] Therefore, daphnids with compromised gut microflora would be unable to efficiently digest algal foods as it requires breakdown of the cellulose in the cell walls. Indeed, in concert with the change in bacteria abundance and consortia structure in the TMP-exposed daphnids, we observed a significant decrease in percentage of algal cells with compromised cell membrane (Figure 4C). However, despite the consensus that gut microbiota is essential for *Daphnia* nutrition and growth, the metabolic products responsible for

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nutrition disorders remain to be determined, as do the long-term means by which these functions are modified, both in laboratory and field settings.

Overall, the presence of different bacterial species does not imply a direct benefit to the host provided by specific bacteria. It is often challenging to determine whether a particular microorganism is truly autochthonous to a particular host and provides it with some benefits. While lab-reared animals will likely have different and less diverse gut microbiota compared to their wild conspecifics, they are expected to retain any symbiotic microorganisms essential for survival. Therefore, the absence of symbionts in the lab animals would suggest their absence in the field as well, but this remains to be demonstrated. Low diversity of clonal libraries observed in our *D. magna* is in agreement with earlier findings based on the cloning approach,[17] whereas, as expected, much higher diversity was found when analyzing metagenomic data on prokaryotic sequences from *Daphnia* sp.[16] In our study, the bacterial communities were undersampled, particularly in the antibiotic-treated animals, where selective removal of the dominant and TMP-sensitive taxa changed competition within microbial consortia and allowed for rare genotypes to emerge.[71] However, it is also likely that *Daphnia* clones kept in different laboratories would vary in the diversity of their microbiome, because of their past history and laboratory practices employed, with possible implications for tolerance of the host species to environmental insults.

Currently, the gut microbiota is recognized as a variable with important impacts in toxicological experiments and drug development studies,[72] and strategies for controlling this variable and rendering it informative in complex experimental settings are being developed. For well-established models, such as mice, both enough information on the microbiota and the methodologies that allow in-depth studies of the host-microbiome interactions are available.[72,73] There is also a growing recognition in ecological, evolutionary, and ecotoxicological research that microbiota contribute greatly to stress response and adaptation, thus shaping higher order interactions and systems. In the ecotoxicological context, the microbiome-mediated mode of action in eukaryotic species may not be unique for antibacterial drugs, but also relevant for other environmental pollutants of various nature exerting effects on the microbial component of the test systems.

■ ASSOCIATED CONTENT

Supporting Information

Background information on experimental design, acute toxicity tests, rarefaction and clone library coverage, as well as short description of phylotypes found in clone libraries from *Daphnia magna* exposed to trimethoprim (TMP) and in controls (C), their closest neighbors, and sequence similarity. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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