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Zooplankton grazing reduces the persistence of an anthropogenic pollution marker in lake water

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Abstract

Wastewater treatment plant effluents release microbiological pollutants, including the *intI*1 gene (integrases of class 1 integron), which has been proposed as a target for monitoring anthropogenic pollution in surface waters. This gene correlates with antibiotic resistance genes, making it an important proxy for genetic contamination in aquatic environments. It is currently unclear whether *intI*1 found in lake water is mainly present due to continuous seeding or if autochthonous bacteria harbor this gene. To better understand the fate and dynamics of class 1 integrons in aquatic systems, we resorted to classical limnological monitoring of *intI*1 (qPCR) over multiple years in three different size fractions: free-living bacteria, particle-attached bacteria, and zooplankton-attached bacteria. We also conducted experiments to elucidate the impact of grazers on the abundance of *intI*1. The monitoring of different size fractions of the Lake Maggiore microbial community showed a particle-bound lifestyle for *intI*1-hosting bacteria. Most of these bacteria originated from both a wastewater effluent that discharges into Lake Maggiore and the lake water itself (amplicon sequencing). We hypothesize that these bacteria grow on particles in open waters, making them particularly vulnerable to grazing by large filter feeders such as *Daphnia*. Therefore, the presence of *Daphnia* reduced the abundance of *intI*1 in lake water, whereas this was not true for other grazers such as *Rotaria macrura* or *Poterioochromonas* sp. Our study shows that the food web structure and temporal changes in the lake influence the abundance of *intI*1 and consequently the assessment of anthropogenic pollution.

Monitoring is required to evaluate and manage the impact of anthropogenic pollution in aquatic environments, and thanks to low costs and easy reproducibility, DNA-based methods are considered the future for the field (Zheng et al. 2020). One of the most important polluters of surface waters in industrialized countries is the effluent of wastewater treatment plants (WWTP), which can release fecal bacteria (Marano 2020), and is a source of other microbiological pollutants, including antibiotic resistance genes (ARGs) (Munk et al. 2022). Beta-lactam, tetracycline, sulfamidic, and quinolone resistance genes have been suggested as potential monitoring targets for this kind of pollution (Abramova et al. 2023). Among these, the frequency of the *intI*1 gene exhibits a strong correlation not only with anthropogenic pollution in general but also with antibiotic resistance specifically, making it one of the most promising targets for DNA-based environmental monitoring (Gillings et al. 2015; Zheng et al. 2020; Abramova et al. 2023). Integrons are genetic elements that capture and express genes, and the *intI* genes encode for integron integrases, proteins that catalyze the recombination of exogenous gene cassettes with a specific recombination site (Gillings 2014). Therefore, integrons are important in the acquisition and dissemination of ARGs. Thanks to its strong correlation with anthropogenic pollution, quantifying *intI*1 in microbial community DNA extracts from water samples is a potent and emerging method to evaluate the level of genetic contamination in aquatic environments (Zheng et al. 2020).

Generally, it seems that the quantity of *intI*1 can be linked to the proportion of WWTP effluents entering freshwaters,

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suggesting a continuous external introduction, rather than a spread by growth of bacteria hosting class 1 integrons (Corno et al. 2023; Haenelt et al. 2023). In terms of water quality, *intI*1 abundances seem to readily increase in experimental communities with the addition of even very low quantities of wastewaters treated at the highest standards (Subirats et al. 2019) and the stronger the contamination the more abundant the class 1 integrons seem to be in natural microbial communities (Di Cesare et al. 2022*b*). In fact, so far only two studies have observed an increase of the abundance of the gene without apparent chemical water pollution or direct seeding, suggesting growth of *intI*1 containing bacteria within the natural water body (Lee et al. 2021; Haenelt et al. 2023).

Which ecological factors influence the abundance of *intI*1 is largely unknown. The integrons themselves are not mobile genetic elements, and *intI*1 is usually encoded in the chromosome (Gillings 2014). Thus, its fate in freshwater systems, for long-term persistence, is determined by the ecological success of the bacterial carrier. Abiotic factors such as low nutrient availability and biotic factors such as competition with the autochthonous bacterial community and predation are important variables that reduce the ecological success of many allochthonous bacteria entering the lake from WWTP outflows (Korajkic et al. 2019). This is mainly attributed to the fact that such genotypes are adapted to fast growth in high nutrient conditions, which makes them, on the one hand, poor competitors in oligotrophic systems and, on the other hand, a preferential prey for heterotrophic nanoflagellates. Given that *intI*1 can reside in various bacterial hosts across different taxa, it remains to be tested which factors could influence the overall abundance of this gene (Lee et al. 2021).

To illustrate the potential complexity of the interactions and how they might influence *intI*1 dynamics one could make the following considerations: in a study in Lake Maggiore, we found that the gene was enriched in the sediment compared to the water column, suggesting that there was a general trend of the gene sinking down with particulate organic matter from surface waters and accumulating in depth, with close to no resuspension from the sediment (Di Cesare et al. 2020). This would imply that the bacteria hosting *intI*1 are less commonly free-living but might rather resort to a particle-associated lifestyle (Di Cesare et al. 2020). This aggregation or attachment might protect *intI*1-hosting bacteria from small flagellated grazers (Corno and Jürgens 2008) but might make them more vulnerable to filter feeders, such as *Daphnia* or certain rotifers since they preferentially feed on larger particles (Langenheder and Jürgens 2001).

In order to better understand the fate of class 1 integrons in aquatic systems we resorted to classical limnological monitoring of the *intI*1 gene over multiple years in three different size fractions: (1) bacterial community mostly free-living, (2) particle-attached bacteria, and (3) zooplankton-attached bacteria. Then, we conducted experiments with natural bacterial communities with model organisms from multiple trophic levels, such as the crustacean *Daphnia obtusa*, the flagellate *Poterioochromonas* sp, and the rotifer *Rotaria macrura*. Since *Daphnia* seemed to impact the abundances of *intI*1, experiments to elucidate the role of *Daphnia* grazing on its abundance were further conducted. Thereby we tried to disentangle the effect of *Daphnia* on microbes deriving from a WWTP effluent and its allochthonous *intI*1-hosting bacteria and on *intI*1-hosting bacteria that are naturally found within the lake ecosystem.

Materials and methods

Sampling and filtration

The water samples were taken in Lake Maggiore, a large subalpine lake, monthly from January 2019 to December 2021 at two sampling sites: Ghiffa (WGS84 coordinates: 45°56′N, 8°38′E) and Pallanza (45°55′N, 8°32′E), the former a pelagic site and the 2nd a semi-littoral site, with a higher concentration of particulate material and a greater influence from WWTP effluents (Bertoni and Callieri 1982). The water samples from the lake were collected in a single operation by a sampler that collects a 5-liter integrated sample from the thermocline depth up to the surface (Bertoni et al. 2010). Water samples were stored at 4°C in the dark until processing within 24 h. The natural bacterial community was: (1) prefiltered over 10 μm and filtered on 0.2-μm polycarbonate filters (47 mm diameter) to obtain the fraction of bacteria not attached to animals (bacterial community of mostly free-living cells) and (2) filtered on GF/C filters (porosity around 1.2 μm, 47 mm diameter) to get the particle-associated fraction. The zooplankton-attached fraction (3) of the bacterial community was obtained in the following way: zooplankton were washed twice with sterile distilled water on a 50-μL mesh net to separate the animals (on the net) from the associated bacteria (in the filtrate). The animals were then stored in a TRIS/EDTA/NaCl (each 0.1 M) buffer and frozen at −20°C until analyses. In particular, the zooplankton organisms were collected monthly from January 2019 to December 2021 in Lake Maggiore only in the pelagic zone of the lake (Ghiffa station), using a nylon net of 50-μm mesh, 0–50 m depth vertical hauls, in order to reach 1 g d.w. for each sample.

Differences in both the occurrence and abundance of *intI*1 across the three filtration fractions, sampling year, and sampling locations were tested using linear models in R version 4.2.2 (R Core Team 2022) in R-Studio. Model fit was evaluated using the *performance* package, which showed that no transformation of the data was needed (Lüdecke et al. 2021). Interactions between the tested variables were removed from the models when they were not significant. For significant categorical predictors with more than two levels, post hoc tests were performed by estimated marginal means (EMMs) using the *emmeans* package (Lenth 2023).

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Food web experiment and analysis

The experiment was performed to test the effect of predators of bacteria in the food web of Lake Maggiore, by adding different predators to a natural bacterial community containing *intI*1 (Fig. 1). The predators were chosen from the filter feeder crustacean *D. obtusa* (with densities of one adult animal in 100 mL), the filter feeder bdelloid rotifer *R. macrura* (one animal in 10 mL), and the mixotrophic nanoflagellate *Poterioochromonas* sp. (10^3 cells mL^{-1}). These organisms were chosen to study the impact of grazing (predation) on bacteria, as previously investigated by Ricci (1984), de Bernardi et al. (1985), and Corno and Jürgens (2006). All possible combinations of one, two, and three predator types were added in flasks with 100 mL of Lake Maggiore water prefiltered over 5 μm with a microbial abundance of 10^6 cells mL^{-1}. Surface water samples from Lake Maggiore were taken manually from the shore. Each combination was conducted in triplicate. The treatments were incubated for 72 h, then the whole community was filtered on 0.2-μm polycarbonate filters (25 mm diameter). Samples were stored in TRIS-EDTA buffer and frozen at −20°C until further molecular analyses (quantification of the *intI*1 and 16S rRNA genes by quantitative real-time PCR [qPCR]). Flagellate and bacterial abundances at the beginning of the experiment were determined by the flow cytometer Accuri C6 (Becton Dickinson), equipped with a 20-mW 488-nm Solid-State Blue Laser and a 14.7-mW 640-nm Diode Red Laser.

A linear model was conducted to test the effect on *intI*1 of the presence of the different predators in the food web and their interaction. Model quality was evaluated using the *performance* package, which showed that the log-transformation of the abundance data generated a better model quality. Plots were made using the packages *ggplot2* and *cowplot* with a logarithmic scale for the gene abundance. Interactions were omitted from the final model if not significant.

Daphnia gradient experiment and analysis

Two experiments were performed, using different water matrices: the 1st one with lake water and the 2nd one with lake water and WWTP effluent water. This was done in order to see whether *Daphnia* had a distinct effect on bacteria deriving from WWTP effluent or natural *intI*1-hosting bacteria (Fig. 1).

i. Lake water: A sample of Lake Maggiore surface water was taken manually from the shore. Samples of *D. obtusa* derived from a long-term culture, kept in a small garden pond at the CNR-IRSA in Verbania. Animals were collected from the garden and cultured under laboratory conditions up to the 2nd generation to avoid maternal effects (Ebert 1993; LaMontagne and McCauley 2001; Lampert 2001); these laboratory-adapted animals were used in the experiments. *D. obtusa* was added to the flask, with 100 mL of lake water and a quantity of animals in a gradient with the following numbers of animals per vessel: 0, 3, 6, 9, 12, and 15. Each treatment was conducted in triplicate. The treatments were incubated for 72 h at 22°C in the dark, which should be long enough to see a clear impact on the

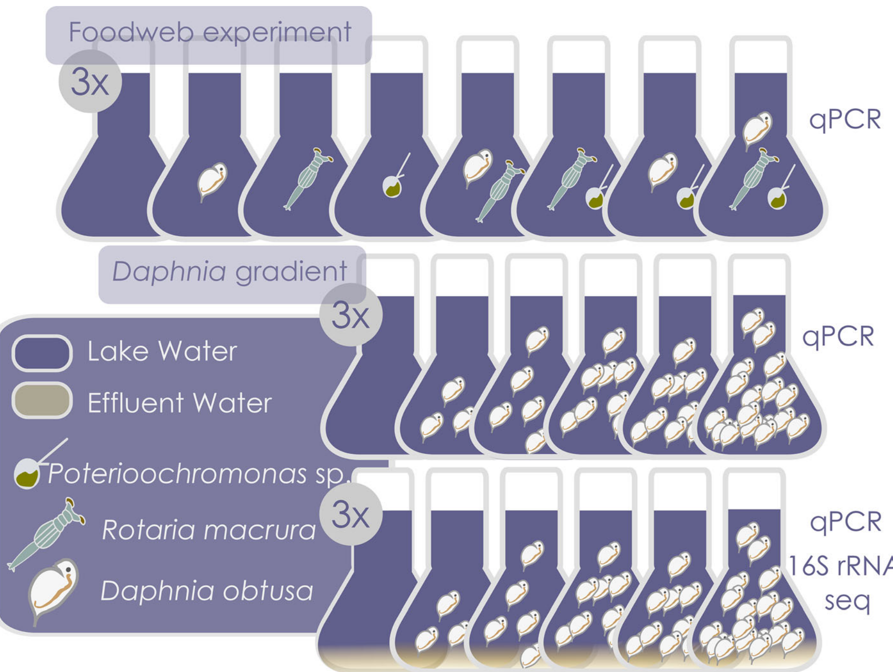

Fig. 1. Schematic description of the experimental set up of the three experiments (food web, *Daphnia* in lake water, *Daphnia* in lake water with WWTP effluent addition) conducted in the study and the analysis conducted on the extracted DNA.

community (Degans et al. 2002). The bacterial community at the end of the experiment was prefiltered over 25 μm to remove the animals, filtered on 0.2-μm polycarbonate filters (25 mm diameter) and kept frozen at −20°C for quantification of the *intI*1 and 16S rRNA genes by qPCR.

ii. Lake water with 10% WWTP effluent water: Experiment II was essentially performed as described for experiment I, except that we used a mix of 90% lake water with 10% WWTP effluent water. The effluent water samples were collected at the large municipal WWTP of Verbania (45°56′N, 8°33′E; 51,000 Population Equivalent [PE] in Eastern Piedmont, Northern Italy). This WWTP receives domestic sewage and an amount of pretreated hospital sewages. These samples were used for both qPCR (to quantify the *intI*1 and 16S rRNA genes) and microbial community analysis (by 16S rRNA gene amplicon sequencing). Original lake water and WWTP effluent water was also filtered and extracted for t0 analysis.

For both experiments the relationship between the abundance of *intI*1 and number of animals was tested in a linear model. Model quality was checked with the performance package. Plots were made using the packages *ggplot2* and *cowplot* with a logarithmic scale for the gene abundance.

DNA extraction

The filters were processed to extract DNA by using a commercial kit (DNeasy UltraClean Microbial Kit, QIAGEN) according to the manufacturer's instructions with the modifications mentioned by Di Cesare et al. (2015). The zooplankton organisms were preserved in a TRIS/EDTA/NaCl (each 0.1 M) buffer, aliquots (0.5 mL) of these bulk samples were placed in the PowerBead tubes of the PowerSoil DNA extraction kit (QIAGEN) and supplemented with 1% SDS and 250 μg mL^{-1} of Proteinase K at −20°C until DNA extraction. The samples were homogenized using the Precellys instrument in two cycles, each at 6000 rpm for 1 min. Subsequently, they were incubated at 52°C for 2 h. Then, each incubated sample was processed for the DNA extraction following the manufacturer's instruction of the PowerSoil DNA extraction kit (QIAGEN). DNA extraction negative controls were included in the extraction protocol and we verified that no nucleic acids were extracted from there.

qPCR analysis

qPCR was conducted for all DNA samples (Fig. 1). The DNA extracts were tested by qPCR for the abundance of the class 1 integron integrase (*intI*1) and the 16S rRNA genes. The *intI*1 gene abundance was normalized to the abundance of the 16S rRNA gene. All qPCRs were performed using the RT-thermocycler CFX Connect (Bio-Rad). The primer pairs used to quantify the 16S rRNA gene were Bact1369 forward primer 5′-CGGTGAATACGTTCYCGG-3′ and Prok1492 reverse primer 5′-GGHTACCTTGTTACGACTT-3′ and to quantify the *intI*1 gene were intI1LC5 5′-GATCGGTCGAATGCGTGT-3′ and intI1LC1 5′-GCCTTGATGTTACCCGAGAG-3′ (Barraud et al. 2010; Di Cesare et al. 2015). These primers are commonly used and suggested for the assessment of *intI*1 in environmental samples (Okonkwo 2023). The protocols and programs used to quantify the 16S rRNA and *intI*1 genes were previously described by Di Cesare et al. (2015, 2016). The standard curve and inhibition tests were performed as previously described (Di Cesare et al. 2013), and no inhibition was determined. Negative controls (min 4) were included in all runs. The efficiency of reactions, R^2, and the limits of quantification were determined as previously described (Bustin et al. 2009). The mean value ± standard deviation of the efficiency of reactions and R^2 were 97.17 ± 6.73 and 0.99 ± 0.01, respectively. The limits of quantification (LOQ) are shown in Supporting Information Table S2. The correct size of all qPCR products was evaluated by electrophoresis (30 min at 80 V, 1% agarose gel).

16S rRNA gene sequencing and analysis

Amplicon sequencing of the 16S rRNA gene was conducted for the samples mixing lake and wastewater with a *Daphnia* gradient and for the t0 lake and wastewater used in the experiment (Fig. 1). We sequenced the V3/V4 of the 16S rRNA gene. Using the universal bacterial primers S-D-Bact-0341-b-S-17 (forward primer, 5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (reverse primer, 5′-GACTACHVGGGTATCT AATCC-3′) (Herlemann et al. 2011), sequencing was conducted on an Illumina MiSeq platform by Macrogen. The raw amplicon reads of the bacterial community are available in the NCBI database and can be accessed under project ID PRJNA962832. Data processing was performed in R version 4.2.2. Raw reads were processed using the DADA2 package version 1.26 (Callahan et al. 2016). Additional packages that were used to analyze the data and data handling include phyloseq package version 1.42.0 (McMurdie and Holmes 2013), Biostrings package version 2.66.0 (Pagès et al. 2023) and *ggplot2*. The DADA2 pipeline provided in the online tutorial (https://benjjneb.github.io/dada2/tutorial.html) was used with only minor adjustments. Briefly, fastq files were quality checked, filtered (maxEE 2 and 2), and trimmed (left trim 50 bp for *R*1 and *R*2 and right trim 25 bp for *R*2). The obtained sequences were merged into a unique sequence and, through error correction, amplicon sequence variants (ASVs) were determined. They were then checked for chimeric sequences and used for the taxonomic assignment through the SILVA database version 138.1 (Quast et al. 2013). ASVs that were not identified at least at the level of phylum or that were identified as either Archaea, chloroplast, or mitochondria were removed from the dataset. The original dataset was composed of 4498 ASVs and 4310 ASVs were retained after processing. Read numbers were not normalized since the numbers per samples were similar (range 80,736–110,287). Beta-diversity was evaluated using both abundance and occurrence-based Bray–Curtis dissimilarity with the *vegan* package and a *nonmetric multidimensional*

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Table 1. Output from the linear models testing the effect of the categorical predictors fraction, sampling location (place) and year on the occurrence, presence/absence (a), and the abundance (b) of *int*I1.

(a) Presence/absence	**df**	***F***	***p***
Fraction	2	333.8	< 0.0001***
Place	1	0.0	0.8293
Year	2	0.4	0.6821
(b) Abundance	**df**	***F***	***p***
Fraction	2	62.0	0.0026**
Place	1	0.2	0.6623
Year	2	29.6	0.0545

df, degrees of freedom.
** $p < 0.01$ *** $p \langle 0.001$

Table 2. Output from the linear model testing the effect of the various trophic levels on the abundance of *intI1* in the microcosms. Flagellate = *Poterioochromonas* sp., *Daphnia* = *Daphnia obtusa*, Rotifer = *Rotaria macrura*.

	Estimate	**s.e.**	***t***	***p***
(Intercept)	0.18	0.04	4549.0	0.0002***
Flagellate	−0.03	0.04	−0.7	0.5209
Daphnia	−0.11	0.04	−2673.0	0.0146*
Rotifer	−0.04	0.04	−0.9	0.3871

s.e., standard error.
* $p < 0.05$; *** $p < 0.001$.

scaling (nmds) was calculated and plotted with the *phyloseq* package. In order to evaluate the impact on *Daphnia* grazing on the removal of WWTP-derived microbiota compared to the lake microbiota we calculated the Bray–Curtis dissimilarity of each microcosm community to the original lake water and WWTP effluent samples, respectively, and plotted the data per number of *Daphnia*.

Read numbers of genera were correlated to the abundance of *D. obtusa* in all mesocosm samples using Spearman correlation and genera that correlated negatively with *Daphnia* abundance ($r > 0.8$) were used for further analysis. We then categorized bacterial genera in "freshwater derived" or "WWTP derived" or common to both types of water "neutral" using AncomBC analysis (Lin et al. 2022). Thus, we plotted all genera that were significantly negatively correlated to the abundance of *D. obtusa* and checked whether they were more commonly related to WWTP effluent or lake water.

Results

*intI*1 dynamics in Lake Maggiore

The *intI*1 gene was detected in 99% of the samples of the bacterial community (water fraction filtered at 0.2 μm), a significantly higher occurrence compared to the other two fractions where it was detected in 61% (particle-associated bacteria, > 1.2 μm) and 32% (zooplankton-associated bacteria, > 50 μm). No differences were found for the gene occurrence or quantity for the two different sampling stations (Table 1). *intI*1 was quantifiable only in 8 samples out of 36 from the zooplankton-associated fraction, but, when quantifiable, its normalized abundances were notably high (Fig. 2). Despite

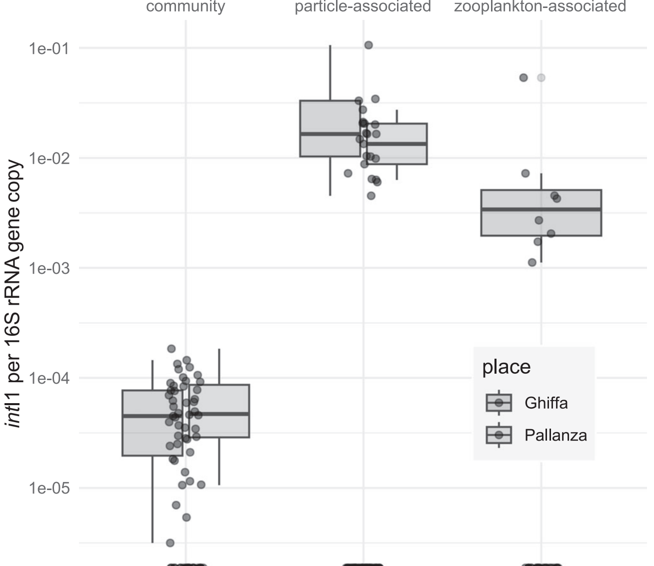

Fig. 2. Boxplots of normalized abundance of the *intI*1 gene in three different water fractions of samples with quantifiable *intI*1 abundances of bacterial community and particle-associated at both stations, Ghiffa and Pallanza, and in the zooplankton-associated fraction, Ghiffa station only.

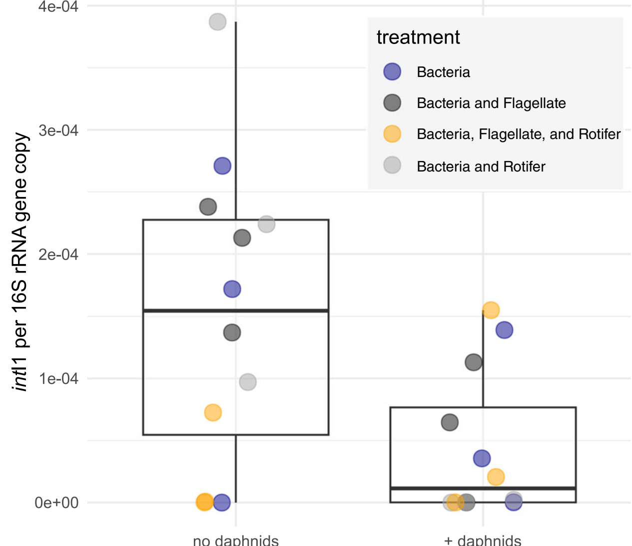

Fig. 3. Normalized abundance of the *intI*1 gene in experimental microcosms with and without the addition of *Daphnia obtusa*. Colors indicate the presence of flagellates and rotifers alone and together in the microcosms.

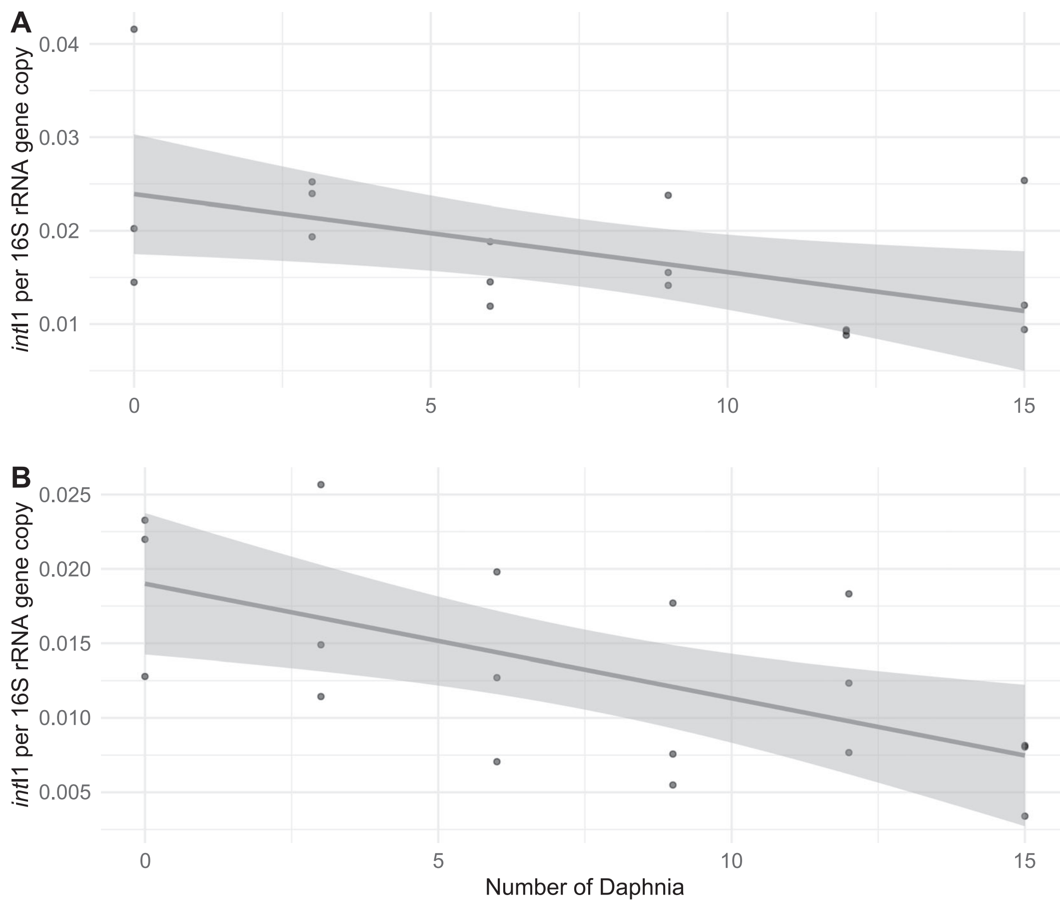

Fig. 4. Normalized *intI*1 abundances in microcosms per number of *Daphnia* added to 100 mL for: (**a**) only lake water and (**b**) lake water with 10% WWTP effluent. Gray line and shaded area are the regression line and the confidence interval of the linear model.

the lower number of quantifiable samples in the bacterial community fraction of 2019, the abundances of the *intI*1 gene did not show significant changes over the years (Supporting Information Fig. S1). The gene abundance was only slightly higher in the first months of the year in the bacterial community fraction, and this was even more pronounced in the first 7 months of the year for both the particle-associated and zooplankton-associated fractions (Supporting Information Fig. S1). The post hoc test conducted between the three tested fractions shows no significant difference (Supporting

Table 3. Output from the linear models testing the effect of the number of *Daphnia* on the abundance of the *intI1* gene in the experiment (a) with 100% lake water (LW) and (b) 10% WWTP effluent water (WW) and 90% LW.

(a) 100% LW	Estimate	s.e.	*t*	*p*
(Intercept)	0.0239	0.0030	7.9	< 0.0001***
# *Daphnia*	−0.0008	0.0003	−2.5	0.0233*
(b) 10% WW and 90% LW	**Estimate**	**s.e.**	***t***	***p***
(Intercept)	0.019	0.002	8.5	< 0.0001***
# *Daphnia*	−0.001	0.000	−3.1	0.0067**

s.e., standard error.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 4. Output from the linear models testing the effect of the number of *Daphnia* on distance of the microbial community to the original community of lake water (LW) and wastewater treatment plant effluent water (WW) 100% lake water (a) or 10% WWTP effluent water and 90% LW (b) for abundance-based Bray–Curtis dissimilarity.

(a) LW	Estimate	s.e.	*t*	*p*
(Intercept)	0.494	0.011	44.8	< 0.0001***
# *Daphnia*	0.008	0.001	6.5	< 0.0001***
(b) WW	**Estimate**	**s.e.**	***t***	***p***
(Intercept)	0.833	0.014	59.8	< 0.0001***
# *Daphnia*	0.004	0.002	2.6	0.0124*

s.e., standard error.
* $p < 0.05$; *** $p < 0.001$.

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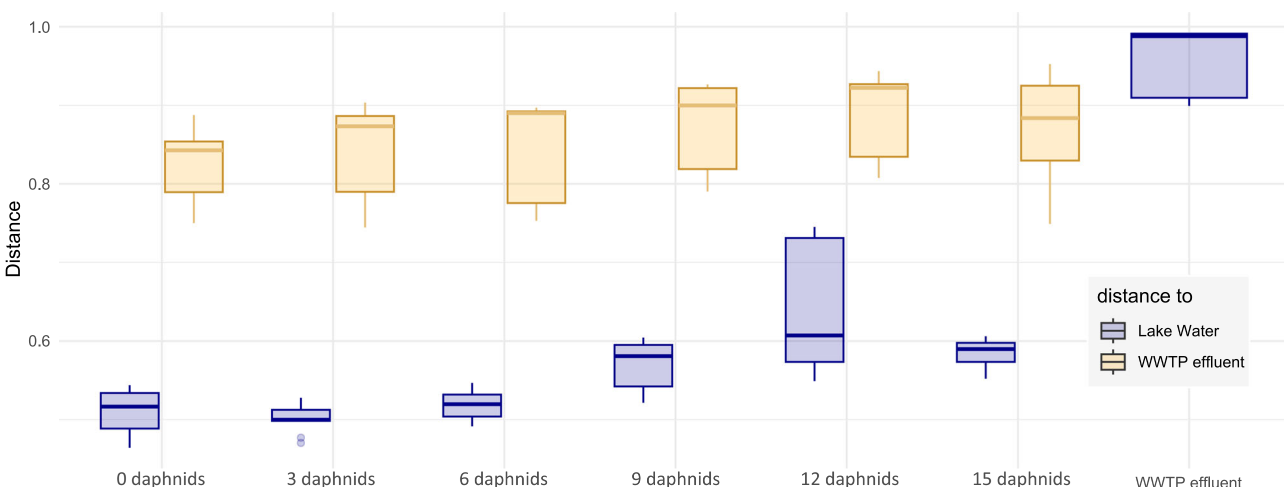

Fig. 5. Beta-diversity of the microbial community of each microcosm (denoted by the number of *Daphnia* in 100 mL of microcosm). Dissimilarity to either the original lake water or original WWTP effluent water community calculated with abundance-based Bray–Curtis dissimilarity.

Information Table S1). Despite being detected in fewer samples, normalized copy numbers of the *intI*1 gene per 16S rRNA gene were significantly higher in the samples from the particle-associated bacteria compared to those from the bacterial community (Supporting Information Table S1B). The mean abundance of *intI*1 was two to three orders of magnitudes higher in particle-attached bacteria compared to the bacterial community fraction (mostly free-living cells not associated to particles larger than 10 μm).

Food web impact on *intI*1 abundance and impact of *Daphnia* grazing on microbial community

By adding filter feeders, the crustacean *D. obtusa* and/or the bdelloid rotifer *R. macrura*, and/or *Poterioochromonas* sp. in all possible combinations, we constructed artificial food webs in microcosm experiments and tested their impact on the abundance of *intI*1. Statistical analysis showed that only the addition of *Daphnia* had a significant negative effect on the abundance of *intI*1 in the system (Table 2; Fig. 3).

In order to quantify the impact of *Daphnia* on *intI*1 abundance, we conducted two experiments with a gradient of *Daphnia*, one with only lake water and one with the addition of 10% WWTP effluent. In all the samples, *intI*1 was positive and quantifiable. In the microcosms with 100% lake water, the overall abundance of *intI*1 per sample ranged from 8.8×10^{-3} to 4.2×10^{-2} *intI*1 per copy of 16SrDNA (Fig. 4). In the microcosms with lake water with 10% WWTP effluent, its concentration ranged from 3.4×10^{-3} to 2.6×10^{-2} gene copies per 16SrRNA gene copy. The abundance of the *intI*1 gene decreased significantly in the water when the number of *Daphnia* increased in both treatments (Fig. 4; Table 3).

The differences in microbial community composition (beta-diversity) were measured as Bray–Curtis dissimilarity in the WWTP effluent, in the lake water before the experiment and in the water with increasing numbers of *Daphnia* (Supporting Information Fig. S2A). Samples from WWTP effluent and in the lake water formed separated clusters. Even the samples with 10% of WWTP effluent without *Daphnia* formed a separated cluster (Supporting Information Fig. S2A). In the samples with *Daphnia* (D), the variability of the composition of the microbial community between replicates increased.

The distance to both Lake Water and WWTP community increased significantly with the increase of *Daphnia*, for both abundance-based and presence absence-based Bray–Curtis dissimilarity (Table 4; Supporting Information Table S3; Fig. 5; Supporting Information Fig. S2B). The complete output of the AncomBC analysis is presented in Supporting Information File S1. Of a total of 25 genera that were negatively correlated with *Daphnia* abundance, 9 were characteristic of lake water, 7 were neutral, and 9 originated from WWTP (Fig. 6).

Discussion

The analysis of different size fractions of the Lake Maggiore microbial community strongly suggests a particle-bound lifestyle for *intI*1-hosting bacteria. Such a result provides support to the formerly suggested attachment of *intI*1-hosting bacteria to sinking particles, such as small organic particles or lake snow (Grossart and Simon 1993). Generally, *intI*1 was temporarily enriched, and no difference was seen between the two sampling stations, which might suggest specific growth of certain *intI*1-hosting bacteria within the system, as compared to continuous seeding in from WWTP effluents.

In the fraction above 50 μm, which we defined as zooplankton-associated bacteria, in addition to bacteria potentially associated to large biofilms on very large particles, we could only observe sporadic spikes of the *intI*1 gene. This strongly suggests a sudden intense enrichment of the

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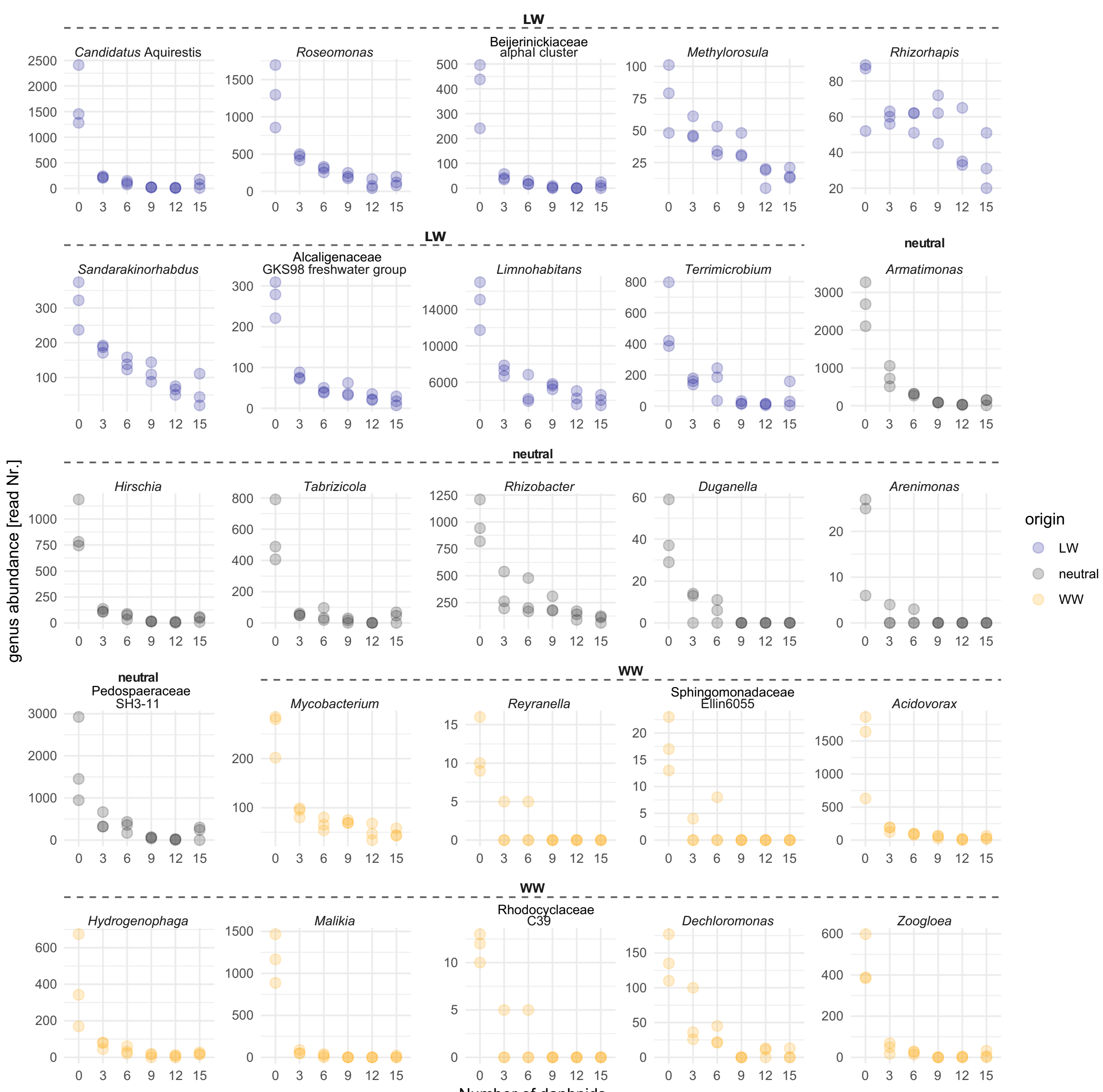

Fig. 6. Number of reads for genera that are significantly negatively correlated with the number of *Daphnia* in the treatments. The colors of the points denote whether a specific genus was significantly overrepresented in wastewater effluent water (yellow), lake water (blue), or neither (gray) according to AncomBC analysis.

zooplankton microbiota with specific *intI*1-hosting bacteria. Zooplankton microbiota composition is considered to be very flexible (Eckert et al. 2021), and such short-term enrichments of zooplankton microbiota were observed for, for example, *Escherichia coli*, and even natural antibiotic resistance gene containing bacteria (*tet*A) and *intI*1 (Eckert et al. 2016; Di Cesare et al. 2018, 2022*a*). However, this does not seem to be the case in our system since the addition of *Daphnia* generally decreased *intI*1-hosting bacteria even in the food web experiment where DNA extractions were done from the whole community and thus included also the bacteria associated with the animal itself.

Despite all three tested grazers feed on bacteria (Ricci 1984; Corno 2006), only *Daphnia* had a clear and linear impact on *intI*1. However, the size fraction of bacterial removal might be very different due to different feeding mechanisms:

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nanoflagellates target medium-sized unicellular bacteria as preferential prey (Corno and Jürgens 2008). The size range of particles that are preferentially ingested by *R. macrura* is yet to be studied, but other bdelloid rotifers with similar feeding mechanisms and head size (i.e., *Philodina* sp.) were also shown to preferentially ingest smaller-sized particles in the size range of single-cell bacteria (Lapinski and Tunnacliffe 2003). On the contrary, *Daphnia* feeding predominantly removes larger bacteria such as filaments and/or aggregates (Langenheder and Jürgens 2001). Evidence of this is also given in the here presented 16S rRNA sequencing data where the obligate filamentous Candidatus genus *Aquirestis* was strongly removed in the presence of *Daphnia* (Hahn and Schauer 2007). Moreover, our experiment does not suggest that *Daphnia* filter feeding reduced *intI*1-hosting bacteria through a cascading food web effects (Zöllner et al. 2003) but through direct removal of the bacteria themselves, which again hints to a predominant particle-attached or aggregated lifestyle.

Distance to the original community, for both lake water and WWTP effluent, increased with increasing number of *Daphnia* abundances. In terms of occurrence-based beta-diversity metrics, *Daphnia* seemed to have a stronger effect on the lake water bacteria, whereas in terms of abundance-based metrics *Daphnia* seemed to impact WWTP-derived bacteria in a stronger manner. Notwithstanding these differences, it seems, however, that the effect was similar on both microbial communities. A similar pattern was seen when bacterial genera that were significantly negatively correlated to the abundance of *Daphnia* were classified as either WWTP or LW derived. Also, here important genera were categorized as deriving from either of the sources or even as neutral. The stronger effect of removal of *intI*1 might not be related to a bacterial genus in particular but simply to a preferential growth on particles of WWTP-derived bacteria compared to typical free-living pelagic bacteria from the lake.

Many of the genera that were found to be reduced by *Daphnia* are known to grow in aggregates or on surfaces, such as *Sandarakinorhabdus* aggregated with Cyanobacteria (Cai et al. 2018), *Armatimonas* growing on aquatic plants (Tamaki et al. 2011), *Hirschia*, growing on many types of surfaces (Chepkwony et al. 2019). *Reyranella* can develop biofilm and has been suggested as a potential utilizer of plastic as a carbon source (Gorokhova et al. 2021). *Acidovorax* is commonly found in wastewater (Palanisamy et al. 2022), and it is also known to be present in aquatic environments (Chen et al. 2020). *Malikia* is an important member of biofilms developed in WWTPs (Ziegler et al. 2016; Naz 2018). *Dechloromonas* is particularly abundant in biological phosphorus-removal systems of WWTPs (Petriglieri et al. 2021), where is prone to develop biofilm on metal surfaces (Li et al. 2020) and polymers (Shen et al. 2016). *Zoogloe* encompasses denitrifying bacteria (Zhang et al. 2023) and has been found as an important component of the algal–bacteria granular sludge (Liu et al. 2023). It can exist in aggregate form (Chakraborty et al. 2011) contributing to the development of biofilm on various polymers (Shen et al. 2013; Walczak et al. 2015; Fang et al. 2020). *Mycobacterium* is one of the bacterial genera hosting the highest number of potential pathogenic species (Bartlett et al. 2022), prone to form aggregates (Borrego et al. 2000) and develop biofilm, eventually causing infectious diseases (Esteban and García-Coca 2018). All in all, unsurprisingly, many of the removed bacteria can either be found in aggregates or even filamentous forms and are therefore preferentially ingested by *Daphnia*.

In conclusion, this study shows that most of the *intI*1-hosting bacteria, deriving from both wastewater and lake water, persist on particulate substrates in lacustrine environments, making them particularly vulnerable to grazing by large filter feeders such as *Daphnia* sp. In terms of dynamics, it seems that some *intI*1-hosting bacteria manage to attach to particles and even grow on them, subsequently sinking to the sediment where the gene accumulates (Di Cesare et al. 2020). The fact that the tested food web structures influenced the abundance of *intI*1 differently, demonstrates that, depending on the community composition of the lake in different seasons, the persistence of *intI*1 might vary. This, in turn, hampers the assessment of anthropogenic pollution using this gene as a proxy in natural waterbodies.

Data availability statement

The raw amplicon reads of the bacterial community are available in the NCBI database and can be accessed under project ID PRJNA962832. All other data and R scripts can be found here https://github.com/EsterME/Daphnia_intI1.

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Conflict of Interest

None declared.

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