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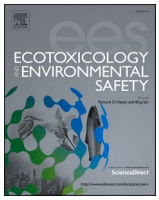

Evolution of pesticide tolerance and associated changes in the microbiome in the water flea *Daphnia magna*

Lizanne Janssens [a], Marlies Van de Maele [a], Vienna Delnat [a], Charlotte Theys [a], Shinjini Mukherjee [b], Luc De Meester [c,d,e,f], Robby Stoks [a,*]

[a] *Evolutionary Stress Ecology and Ecotoxicology, University of Leuven, Debériotstraat 32, 3000 Leuven, Belgium*
[b] *Laboratory of Reproductive Genomics, University of Leuven, ON I Herestraat 49, 3000 Leuven, Belgium*
[c] *Freshwater Ecology, Evolution and Biodiversity Conservation, University of Leuven, Debériotstraat 32, 3000 Leuven, Belgium*
[d] *Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany*
[e] *Institute of Biology, Freie Universität Berlin, Berlin, Germany*
[f] *Berlin-Brandenburg Institute of Advanced Biodiversity Research (BBIB), Berlin, Germany*

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ABSTRACT

Exposure to pesticides can have detrimental effects on aquatic communities of non-target species. Populations can evolve tolerance to pesticides which may rescue them from extinction. However, the evolution of tolerance does not always occur and insights in the underlying mechanisms are scarce. One understudied mechanism to obtain pesticide tolerance in hosts are shifts toward pesticide-degrading bacteria in their microbiome. We carried out experimental evolution trials where replicated experimental populations of the water flea *Daphnia magna* were exposed to the pesticide chlorpyrifos or a solvent control, after which we performed acute toxicity assays to evaluate the evolution of chlorpyrifos tolerance. Additionally, we quantified changes in the microbiota community composition of whole body and gut samples to assess which sample type best reflected the pesticide tolerance of the *Daphnia* host. As expected, chlorpyrifos-selected clones became more tolerant to chlorpyrifos as shown by the higher $EC_{50\ 48\ h}$ (36% higher) compared with the control clones. This was associated with shifts in the microbiome composition whereby the abundance of known organophosphate-degrading bacterial genera increased on average ~4 times in the chlorpyrifos-selected clones. Moreover, the abundances of several genera, including the organophosphate-degrading bacteria *Pseudomonas*, *Flavobacterium* and *Bacillus*, were positively correlated with the $EC_{50\ 48\ h}$ of the host populations. These shifts in bacterial genera were similar in magnitude in whole body and gut samples, yet the total abundance of organophosphate-degrading bacteria was ~6 times higher in the whole body samples, suggesting that the gut is not the only body part where pesticide degradation by the microbiome occurs. Our results indicate that the microbiome is an important mediator of the development of tolerance to pesticides in *Daphnia*.

1. Introduction

Exposure to pesticides can have detrimental effects on aquatic communities of non-target species, even at legally accepted concentrations (Stehle and Schulz, 2015). Populations can, however, evolve tolerance to pesticides which may rescue them from extinction. Evolution of pesticide tolerance in non-target species has been shown, for example, against carbaryl in waterfleas (Jansen et al., 2011b) and against chlorpyrifos in amphibians (Cothran et al., 2013). Despite the importance of "evolutionary rescue" (Bell, 2017), it does not always occur and there is in general mixed evidence for genetic adaptation to environmental pollution in natural populations (Loria et al., 2019). Powerful ways to evaluate whether populations can evolve tolerance to stressors are experimental evolution trials, as these allow unambiguously linking of a manipulated stressor to an evolutionary response in replicated settings (Kawecki et al., 2012). To advance our understanding on when to expect evolution of tolerance to pollutants, combining experimental evolution with a study of potential underlying mechanisms may be especially insightful.

Several mechanisms can underlie the evolution of pesticide tolerance in a species. Most attention went to two mechanisms directly encoded in the genes of the species under study. A first mechanism is the buildup of

* Corresponding author.
E-mail address: robby.stoks@kuleuven.be (R. Stoks).

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target site tolerance caused by a mutation of the target site of the pesticide (e.g. acetylcholinesterase for organophosphates), resulting in no or a lower inhibition of the target enzyme by the pesticide (Stenersen, 2004). A second mechanism is the buildup of metabolic tolerance caused by overexpression of detoxification genes (Stenersen, 2004). More recently, changes in the microbiome have been revealed as a very different mechanism to obtain pesticide tolerance in the host (Kikuchi et al., 2012). Yet, the latter mechanism is never considered in the context of experimental evolution of tolerance to pesticides.

Evidence is accumulating that the microbiome can play an important role in determining the phenotype of its host and its tolerance to stressors (Lynch and Hsiao, 2019), including xenobiotics (Kikuchi et al., 2012; Senderovich and Halpern, 2013; Ceja-Navarro et al., 2015). Exposure to xenobiotics such as pesticides may cause a shift in the bacterial community composition of the host whereby bacterial species able to degrade the xenobiotic increase in abundance, while sensitive bacterial species decrease in abundance and possibly even disappear (Russell et al., 2011). Degradation of xenobiotics by bacteria can occur via catabolism whereby the xenobiotic compound is used as source for essential elements such as for example carbon, nitrogen and phosphorus, and via co-metabolism whereby the toxic compound is biotransformed into less toxic metabolites but not used as energy source (Singh and Walker, 2006). Several bacteria are able to degrade pesticides (summarized in the reviews by Russell et al., 2011 and Kumar et al., 2018). For example, some key genera often associated with the degradation of pesticides are *Pseudomonas* and *Flavobacterium* (organophosphates), *Rhizobium* (carbamates), and *Bacillus* (pyrethroids). Shifts in the microbiome toward more pesticide-degrading bacteria may eventually provide pesticide tolerance of the host. For example, Chen et al. (2020) showed that inoculation of silk worms with *Stenotrophomonas* significantly increased their resistance to chlorpyrifos.

The water flea *Daphnia* plays an important role in aquatic food webs as food for fish and as grazer of phytoplankton, and is an important model organism in ecotoxicology (Miner et al., 2012). An increasing number of studies documented the importance of the microbiome for *Daphnia* fitness, and *Daphnia* is becoming a model organism for host-microbiome-environment interactions (Akbar et al., 2022). Daphnids are colonized with bacteria throughout their entire body cavity and gut (Qi et al., 2009; Eckert and Pernthaler, 2014). These microbiota help the daphnids in food uptake and digestion (Brune and Dietrich, 2015; Chevalier et al., 2015), acquisition of essential nutrients (Nikoh et al., 2011), resistance against infections (Kamada et al., 2013) and the breakdown of harmful compounds such as toxins of cyanobacteria (Macke et al., 2017). As a result, the survival and growth of *Daphnia* is affected by its microbiome community composition (Sison-Mangus et al., 2015; Callens et al., 2018; Motiei et al., 2020). Recent evidence is showing that the microbiome can play an important role in the tolerance of *Daphnia* to pollutants. For example, mercury-tolerant microbiota can ameliorate survival and fecundity of *Daphnia* exposed to mercury (Fong et al., 2019). More information on several issues is needed to further develop *Daphnia* as model organism for microbiome-based ecotoxicological research. One such issue is whether the gut microbiome rather than the whole body microbiome plays a role in toxicant tolerance. The gut microbiome is known to differ in composition from the whole body microbiome (Callens et al., 2016). Yet, it remains to be studied whether pesticide-degrading bacteria are more associated with the gut or not, hence which type of samples can best be studied to unravel the role of the microbiome in shaping pesticide tolerance. Another issue is whether the evolution of pesticide tolerance of *Daphnia* is associated with changes in the microbiome toward pesticide-degrading bacteria.

The aim of current study was to study experimental evolution of pesticide tolerance in *Daphnia* and to determine whether this was associated with predictable changes in its microbiome. To that end, we carried out an experimental evolution trial where replicated experimental populations of *Daphnia* were exposed to the pesticide chlorpyrifos or a solvent control, after which we performed acute toxicity assays to assess the evolution of chlorpyrifos tolerance. This organophosphate pesticide, that functions through the inhibition of acetylcholinesterase, is among the most hazardous chemicals to aquatic organisms (Johnson et al., 2017). While recently banned in several countries, it is still widely used (Rahman et al., 2021). Additionally, we collected samples at the end of the experimental evolution trial to quantify changes in the microbiota community composition of the *Daphnia*. We compared microbiome samples of the whole body and the gut to assess which sample type best reflected the pesticide tolerance of the *Daphnia* host. We expected the chlorpyrifos-selected *Daphnia* populations to have evolved a higher tolerance to chlorpyrifos and the bacterial communities of these host populations to have (partially) shifted towards chlorpyrifos-degrading bacteria.

2. Methods

The overall research strategy was to perform an experimental evolution trial in aquaria and to subsequently test in separate assays for micro-evolution of chlorpyrifos tolerance. Additionally, at the end of the experimental evolution trial, we compared the microbiota community composition of the *Daphnia* between the two selection regimes.

2.1. Source population and pre-experimental rearing

D. magna collected in Langerodevijver (50°49′41″ N, 4°38′21″ E) in Flanders were used for the selection experiment. This shallow lake is situated in a natural area without agriculture, and is surrounded by dykes making it unlikely that the *Daphnia* clones have already developed tolerance to pesticides (Coors et al., 2009). Specifically for chlorpyrifos in Flanders, it was shown that *D. magna* populations show a higher tolerance when the percentage of conventional agriculture in the landscape surrounding the pond increases (Almeida et al., 2021). Note that any (partial) tolerance in the study population (while unlikely) would make any findings of (further) evolution of tolerance conservative and would introduce no bias toward "false positive" effects.

To prepare the experiment, we reared 180 clonal lineages (unique genotypes confirmed by microsatellite analyses, following Jansen et al., 2011a) under standard conditions of light (14:10 L:D), temperature (20 °C) and food (0.2×10^8 cells of the algae *Acutodesmus obliquus*, 5 days per week) for at least two generations. The offspring (second to fifth brood) of these mothers was used to inoculate the selection aquaria.

2.2. Experimental evolution trial

For the experimental evolution trials we applied two treatments for two weeks: exposure to two pulses of 0.35 μg/L chlorpyrifos (dissolved in 0.35 μL/L ethanol) or two pulses of 0.35 μL/L ethanol (solvent control). To start the experiment we randomly composed three start populations, each consisting of a unique set of 60 clones from Langerodevijver. From each start population, we obtained six identical sets of the 60 clones through parthenogenetic reproduction; of which three sets were used for the pesticide-exposed selection treatment and three sets for the control treatment. This is a powerful approach as it allows genetically identical start populations to be used in both selection treatments (see Van Doorslaer et al., 2009). This resulted in a total of 18 selection units (aquaria).

We used 30 L aquaria filled with a mixture of 6 L filtered (mesh size 125 μm) pond water originating from five natural ponds in Flanders where *D. magna* occurs (to obtain a diverse community of free living bacteria) and 9 L of dechlorinated tap water. One week before the inoculation of the *D. magna* juveniles, we added 15 mL of 1×10^8 cells/mL of the alga *Acutodesmus obliquus* as food source. During the experiment, we weekly added 5 mL of 1×10^8 cells/mL of algae to keep the food level constant. In each aquarium we inoculated three individuals (maximum 5 days old; as in Jansen et al., 2011b) from each of the 60 clones of a given start population (180 individuals per aquarium in

total). Two weeks after inoculating the aquaria with the daphnids, the chlorpyrifos exposure treatment started by giving the first pulse; one week later a second chlorpyrifos pulse was given.

The chosen chlorpyrifos concentration was based on a pilot study where we observed approximately 50% mortality across two weeks in adult daphnids when being exposed to two pulses of 0.35 μg/L chlorpyrifos (one pulse per week). The actual chlorpyrifos concentration present in each aquarium exposed to chlorpyrifos was measured using Ultra-performance liquid chromatography MS/MS with Triple Quadrupole Mass Spectrometry at different time points. The initial concentration directly after the first pulse was 0.30 ± 0.03 μg/L (mean ± SE, n = 9 aquaria), one week later before giving the second pulse it was 0.11 ± 0.01 μg/L, immediately after the second pulse 0.38 ± 0.03 μg//L and one week later at the end of the selection experiment 0.14 ± 0.01 μg/L. One week after the second chlorpyrifos pulse, *D. magna* individuals were collected to start clonal lineages for the acute toxicity assays and to determine the microbiota community composition.

2.3. Acute toxicity assays

Per aquarium we collected five adult daphnids to start clonal lineages, hence fifteen isolates per start population-by-selection treatment combination (total of 90 isolates). In one of the chlorpyrifos-exposed aquaria, there were no survivors at the end of the experiment. We therefore chose to collect more daphnids from the other replicate aquaria of the same start population of this treatment to still obtain fifteen isolates after selection for this start population. We tested all 90 isolates with microsatellite markers to identify unique clones (as in Jansen et al., 2011a). This resulted in 64 unique clonal lineages, from which 34 clones originated from the control aquaria (twelve from the first, eleven from the second and eleven from the third start population) and 30 clones from the chlorpyrifos-selected aquaria (eight from the first, eleven from the second and eleven from the third start population). To minimize maternal effects, the 64 isolates were kept as individual cultures for at least two generations before their offspring was used in the acute toxicity assays.

We carried out acute toxicity assays to determine the tolerance to chlorpyrifos of all 64 clonal lineages based on OECD (2004) guideline 202 for an acute immobilization test in *Daphnia*. We exposed second to fifth clutch neonates (< 24 h old) to one of five chlorpyrifos concentrations (ranging from 0.2 to 3.0 μg/L, spacing factor of 2.0 between the concentrations) or a water control. Before starting the assays, we tested the immobility of fifteen clones, randomly distributed among the start populations and selection treatments, in a solvent control with 3.0 μL/L ethanol, which corresponds with the maximum ethanol concentration the daphnids were exposed to in the highest chlorpyrifos test concentration. As there was no difference in immobility between the water control and the ethanol control, we decided not to use a solvent control in the actual assays. Note that the lowest NOEC of ethanol reported for aquatic invertebrates is > 10,000 times higher than the highest ethanol concentration used in current experiment (UNEP, 2004). Per clonal lineage, eight replicates were tested at each concentration (resulting in 48 jars per clonal lineage). Given the size of the experiment (3072 jars in total), tests were set up over a period of two months. The order in which the clones were tested was randomized across aquaria and selection treatments. During the toxicity assays, five neonates were placed together in a 100 mL glass jar filled with 30 mL of the medium. Animals were kept at standard conditions (20 °C and 14:10 light:dark cycle) and not fed during the toxicity test. Note that this does not preclude a role for the gut microbiome in mediating pesticide tolerance as *Daphnia* keep on filtering water, hence taking up water and pesticides in the gut, in the absence of food (M. Van de Maele, pers. obs.; Ebert, 2005). After 48 h, all individuals were scored for immobility and the percentage of mortality was calculated by dividing the number of dead individuals by the total number of individuals in a jar, multiplied by 100.

2.4. Determination of the microbiota community composition

At the end of the experimental evolution trial, twenty mature *Daphnia* per aquarium were collected for the determination of the microbiota community composition (protocol based on Houwenhuyse et al., 2021). One set of 10 *Daphnia* was pooled and as whole bodies stored at − 80 °C to determine the whole body bacterial composition. In the second paired set of 10 *Daphnia,* the guts were immediately extracted under a stereomicroscope using sterilized dissecting needles, pooled and stored at − 80 °C. Given the extinction of one of the chlorpyrifos-exposed aquaria, we obtained nine paired sets of pooled *Daphnia* from the control aquaria and eight paired sets of pooled *Daphnia* from the chlorpyrifos-exposed aquaria. Hence, we obtained for the nine control and eight chlorpyrifos-exposed aquaria, each time one whole body microbiome sample and one gut microbiome sample.

In a first step, microbial DNA was extracted using the NucleoSpin Tissue Kit (Machery Nagel) following the protocol provided by the manufacturer. To characterize the microbiome composition, we amplified the V3-V4 hypervariable region (460 bp) of the bacterial 16 S rRNA using the 16S-IllumTS-F and 16S-IllumTS-R primers (Klindworth et al., 2013). We conducted the PCRs using a Biometra TOne Thermocycler (Westburg) and quantified the DNA using the Quant-iTTM Picogreen kit (Thermo Fisher). The purified amplicons were merged and paired-end sequenced (2 × 250) using a MiSeq Reagent Kit v2 (600 cycle) on an Illumina MiSeq platform (KU Leuven Genomics Core, Leuven, Belgium).

After Illumina sequencing, the QIIME2 pipeline v2020.6 (Bolyen et al., 2019) was used to process the raw sequence data. First, the paired-end demultiplexed sequence reads were imported and their ASCII Phred33 quality control scores were checked. A total of 1215,043 reads, with an average of 35,737 reads per sample (min. = 129 and max. = 112,957 reads) was obtained. Using the denoise function of DADA2 within QIIME2, both forward and reverse reads were trimmed for 14 bp and truncated at 240 bp. Furthermore, the paired-end sequences were filtered, denoised and dereplicated, chimeras were filtered and an amplicon sequence variants (ASVs) table was created using this denoise function. After quality control, on average ~83% of the data per sample remained. A total of 1455 ASVs across all samples was obtained with an average length of 426.46 bp (s.e.= 17.94). Taxonomy was assigned to the ASVs using the Silva 138 SSU Ref NR 99 database (Quast et al., 2013) and a naive Bayesian classifier. This classifier contained the extracted sequences of the V3-V4 region using the function qiime feature-classifier extract-reads with parameters forward primer (16S-IllumTS: CCTACGGGNGGCWGCAG), reverse primer (16S-IllumTS: GACTACHVGGGTATCTAATCC) and truncation length (460 bp). Finally, a phylogenetic tree was generated using the function qiime phylogeny align-to-tree-mafft-fasttree.

The R package Decontam (version: 1.12.0; Davis et al., 2018) was used to look for contaminating DNA features but none were present. Cleaning and filtering of the dataset was performed in R (see details in Appendix A). Samples were rarefied to an equal sampling depth of the sample with the fewest reads (i.e. 13,797) using the "rarefy_even_depth" function of the phyloseq package (version: 1.36.0; see details in Appendix B).

2.5. Statistical analyses

Mortality in the acute toxicity assays was analyzed in Statistica using a general linear model (GLM) with selection treatment of the experimental evolution trial and pesticide test concentration of the acute toxicity assay, and their interaction as independent variables. Start population and clone nested in the interaction between start population and selection treatment were included as random factors (as in Van Doorslaer et al., 2009). The significant interaction between selection treatment and pesticide concentration was further explored using linear contrasts, whereby we compared the mortality in a given pesticide concentration with the water control. We corrected for multiple testing

using the false discovery rate (FDR) procedure. Additionally, we performed a Mann-Whitney U test to test for an effect of the selection treatment on the $EC_{50\ 48\ h}$ values. Therefore, we ran per aquarium a generalized linear model with a binomial error distribution and logit link function to determine the dose-response relationship. In 14 aquaria, overdispersion was observed and therefore we used a quasibinomial error distribution to account for this. The $EC_{50\ 48\ h}$ value was estimated as $-\beta_0/\beta_1$ with β_0 the intercept and β_1 the slope of the fitted dose-response curve (Coors et al., 2009).

The microbiome of the *Daphnia* collected in the aquaria at the end of the selection experiment was analysed at the ASV level. To investigate differences in α-diversity, the Shannon index and Faith's Phylogenetic Diversity were calculated with the "estimate_richness" function of the phyloseq package (version: 1.36.0; McMurdie and Holmes, 2013) in R (version: 4.1.0). Afterwards, a general linear mixed model (GLMER) was performed with selection treatment, sample type (whole body sample vs gut sample) and their interaction as independent variables using the packages lme4 (version: 1.1–27.1; Bates et al., 2015), car (version: 3.0–11; Fox and Weisberg, 2011) and afex (version: 1.0–1; Singmann et al., 2016). Aquarium nested in selection treatment was included as a random factor. For the β-diversity, a permutation MANOVA was performed with selection treatment, sample type and their interaction as independent variables using Bray-Curtis and Weighted UniFrac distance metrics. PERMANOVAs were run with 10,000 permutations with a set seed of 10,000 (allowing reproducibility) using the "adonis2″ function of the vegan package in R (version: 2.5–7; Oksanen et al., 2020). β-diversity metrics were plotted with non-metric multidimensional scaling (NMDS) using the "ordinate" function of the phyloseq package (Bray--Curtis: k = 3 with stress = 0.13) and the ggplot2 package (version: 3.3.5; Wickham, 2016) in R.

To identify bacterial genera that differed between the control and chlorpyrifos-selected samples, ASVs were grouped, whereby we only included those taxa that represented at least 0.1% of the reads (Houwenhuyse et al., 2021). We observed a significant interaction between the selection treatment and the sample type for the β-diversity in the PERMANOVA (see results), indicating different responses in microbial community composition to the selection treatment in gut and whole body samples. Therefore, we performed Mann-Whitney U tests to compare the abundance between the control and chlorpyrifos-selected samples with selection treatment as grouping factor (following Chen et al., 2020), separately for the whole body samples and the gut samples. Additionally, we performed Mann-Whitney U tests to compare the abundance between the whole body and gut samples with sample type as grouping factor, separately for the control and chlorpyrifos-selected samples. All Mann-Whitney U tests were performed in Statistica with FDR correction for multiple testing. Given that only 12.6% of the ASVs could be identified at the species level and xenobiotic degrading capacities are often shared between species of the same genus and often only known at the genus level (see for example review by Kumar et al., 2018), we will present the results at the genus level.

To investigate relationships between the abundance of certain bacteria and the level of chlorpyrifos tolerance, we performed in Statistica Spearman correlation analyses at the aquarium level between the relative abundance of a given genus (both at the whole body and at the gut level) and the $EC_{50\ 48\ h}$. We only included genera that represented at least 0.1% of the reads and used an FDR correction for multiple testing.

3. Results

3.1. Mortality during the acute toxicity assays

There was a significant Selection treatment x Pesticide concentration interaction ($F_{5,\ 2951} = 26.49$; $p < 0.001$), indicating that the selection treatment affected the tolerance to chlorpyrifos. Clones originating from control aquaria showed mortality at the three highest test concentrations (0.8 μg/L: 9%, 1.5 μg/L: 38%; 3.0 μg/L 73%; contrasts with water control: all $p < 0.001$). Clones originating from chlorpyrifos-exposed selected aquaria, on the other hand, were only affected at the two highest concentrations (1.5 μg/L: 16%, 3.0 μg/L: 53%; contrasts with water control: both $p < 0.001$) and the mortality was lower in comparison with the sensitive clones (contrast analyses at both concentrations: $p < 0.001$) (Fig. 1A). The $EC_{50\ 48\ h}$ was 36% higher in chlorpyrifos-selected clones than in control clones ($Z = -2.54$; $df = 17$; $p = 0.011$) (Fig. 1B).

3.2. Patterns in the Daphnia microbiome

The bacterial α-diversity of the *Daphnia* microbiome was higher in the whole body than in the gut samples, but only significantly so for the Shannon index (main effect Sample type: $\chi_1 = 135.59$; $p < 0.001$) (Fig. 2A-B). It was also higher in the chlorpyrifos-selected samples, both for the Shannon index ($\chi_1 = 5.01$; $p = 0.025$) and the Faith's phylogenetic diversity ($\chi_1 = 6.32$; $p = 0.012$). Sample type and selection treatment did not interact with each other for the α-diversity (both $p > 0.54$) (Fig. 2A-B).

The bacterial communities differed between gut and body samples both based on the Bray-Curtis ($F_{1,\ 30} = 35.09$; $p < 0.001$) and weighted UniFrac distance metrics ($F_{1,\ 30} = 65.46$; $p < 0.001$) (Fig. 2C-D). Both metrics also showed differences in bacterial communities between control and chlorpyrifos-selected samples (Bray-Curtis: $F_{1,\ 30} = 3.91$; $p = 0.022$; weighted UniFrac distance metrics: $F_{1,\ 30} = 5.59$; $p = 0.015$). Moreover, for both indices there was a trend for a Sample type x

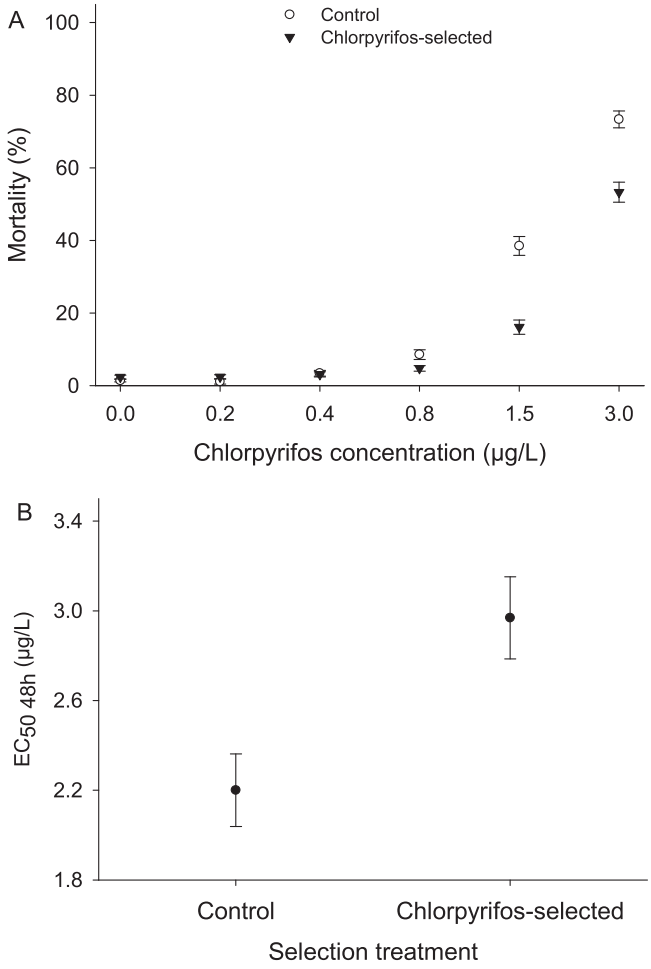

Fig. 1. (A) *D. magna* mortality after 48 h as a function of the selection treatment and chlorpyrifos test concentration in the acute toxicity assay, and (B) $EC_{50\ 48\ h}$ as a function of the selection treatment. Given are means ± 1 SE.

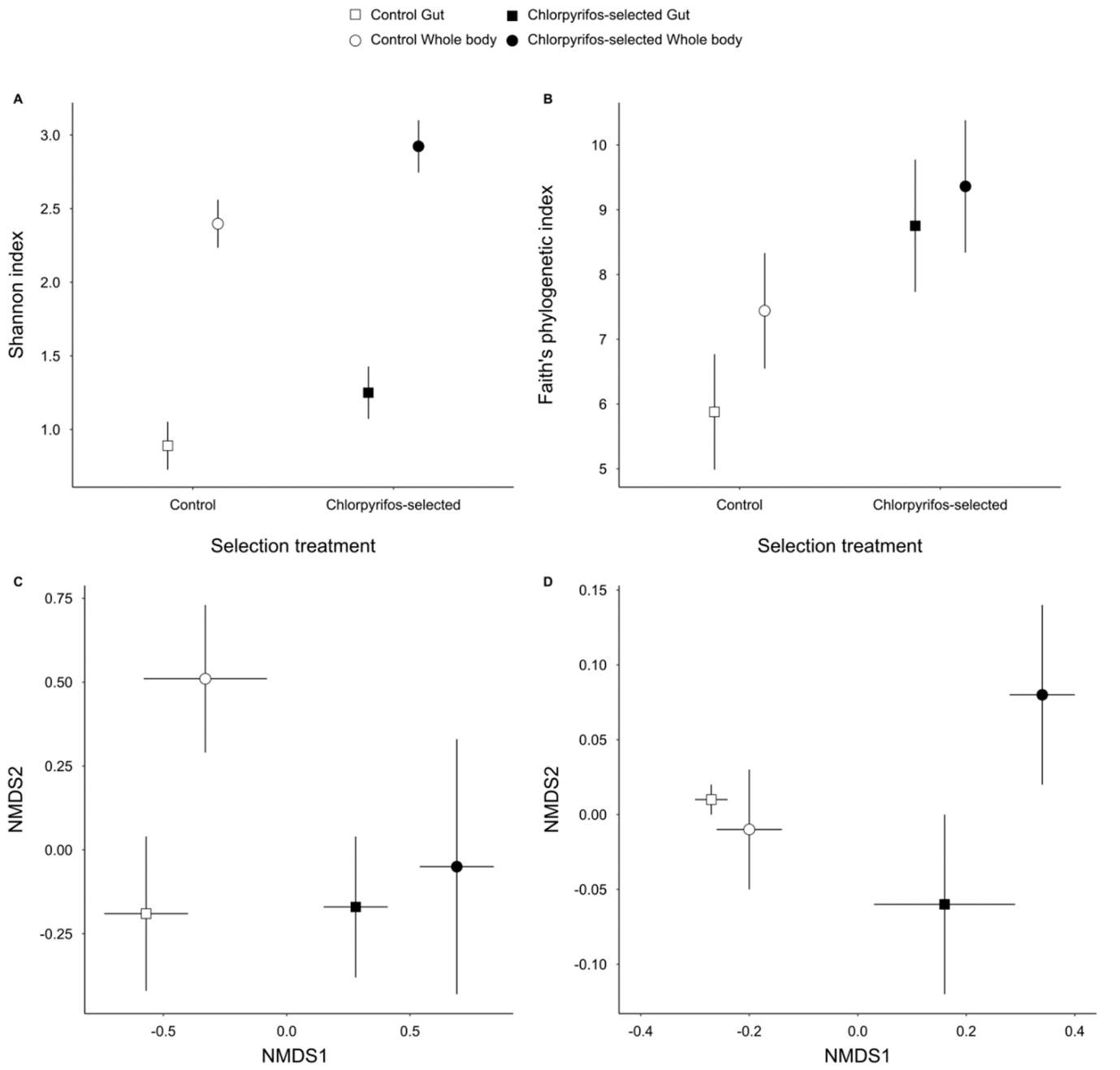

Fig. 2. Patterns in the diversity of the bacterial communities of *D. magna* gut and whole body samples as a function of the chlorpyrifos selection treatment. (A) Shannon and (B) Faith's Phylogenetic indices of alpha diversity (means ± 1 SE). (C-D) NMDS plots visualizing the patterns in the diversity (means with 95% CI) based on (C) the Bray-Curtis dissimilarity and (D) weighted UniFrac distance metrics.

Selection treatment interaction (Bray-Curtis: $F_{1,\ 30} = 2.59$; $p = 0.063$; weighted UniFrac distance metrics: $F_{1,\ 30} = 3.13$; $p = 0.063$) suggesting a different response to the pesticide between the gut and whole body bacterial communities (Fig. 2C-D). A detailed comparison of the most common genera in the gut and body samples in the *Daphnia* originating from the control aquaria and from the chlorpyrifos-selected aquaria is given in Appendix C.

When comparing the microbiome samples of *Daphnia* originating from control aquaria and chlorpyrifos-selected aquaria, ten genera showed a different abundance, of which four were shared between gut and whole body samples (Fig. 3). Both in the gut and in the whole body samples, there was in the chlorpyrifos-selected *Daphnia* a higher abundance of *Bacillus* (gut: × 79.7, $p = 0.040$; whole body: × 43.1, $p = 0.017$), *Aquabacterium* (gut: × 6.7, $p = 0.0096$; whole body: × 2.9, $p = 0.022$), and *Flavobacterium* (gut: × 2.6, $p = 0.033$; whole body: × 2.7, $p = 0.020$), while *Pseudomonas* was even only detected in chlorpyrifos-selected samples (gut: $p = 0.0041$; whole body: $p = 0.0052$). Additionally, in the chlorpyrifos-selected gut samples, a higher abundance of *Polynucleobacter* (× 38.0, $p = 0.0072$) and *GKS98_freshwater_group* (× 6.9, $p = 0.042$) was detected compared to the control samples. Similarly, in the chlorpyrifos-selected whole body samples, a higher abundance of *Pedobacter* (× 7.0, $p = 0.014$), *Luteolibacter* (× 5.0, $p = 0.023$), *Limnohabitans* (× 1.7, $p = 0.037$) and *Cavicella* (× 2.6, $p = 0.044$) was detected.

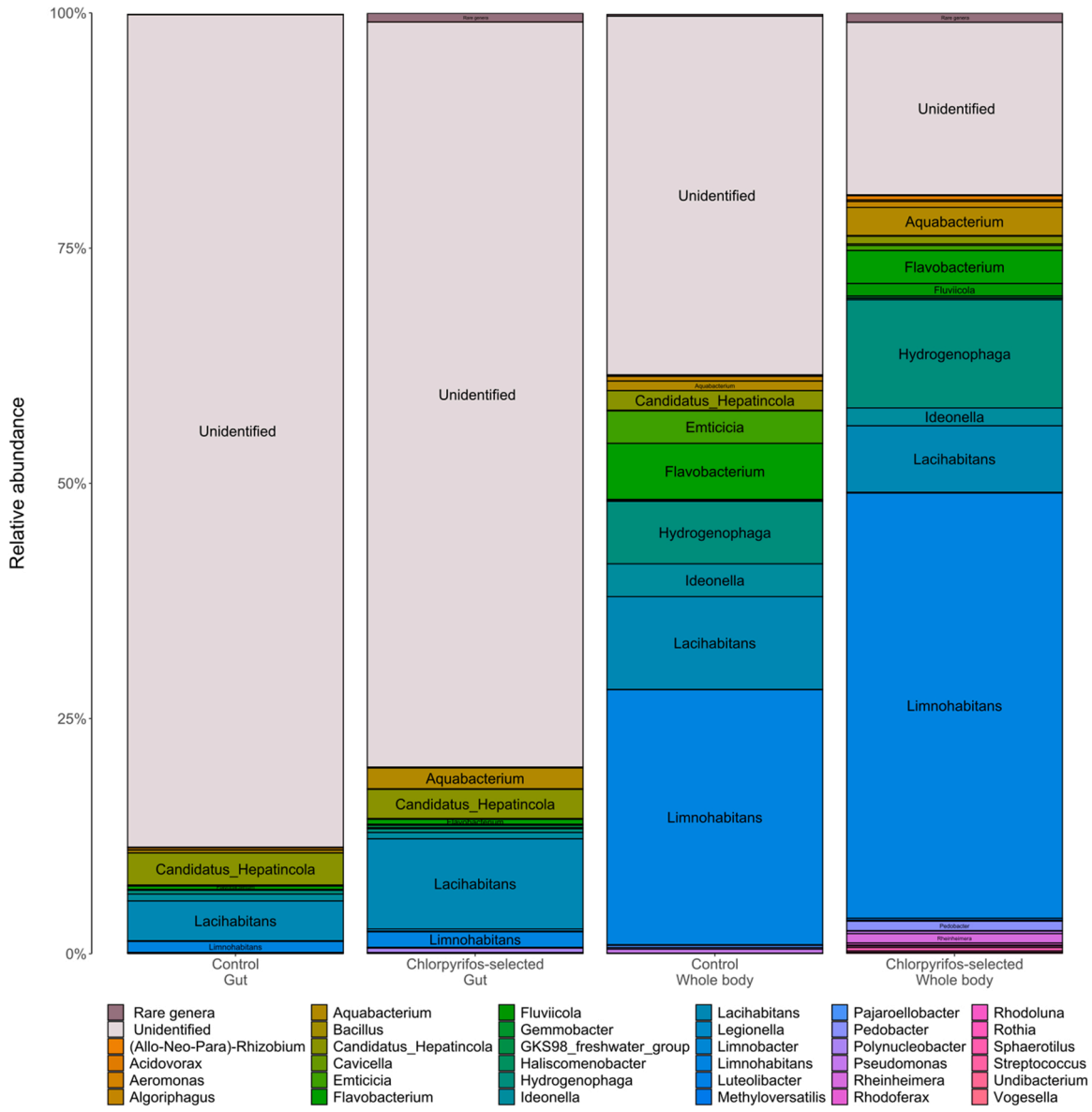

Fig. 3. Relative abundance of the bacterial genera in *D. magna* gut and whole body samples as a function of the chlorpyrifos selection treatment.

3.3. Correlations between abundance of bacterial genera and Daphnia pesticide tolerance

When looking at the gut microbiome, the abundance of five bacterial genera was positively correlated with the $EC_{50\ 48\ h}$ of the *Daphnia*: *Pseudomonas* ($r = 0.66$; $p = 0.015$), *GKS98_freshwater group* ($r = 0.66$; $N = 17$ aquaria; $p = 0.030$), *Polynucleobacter* ($r = 0.53$; $p = 0.041$), *Aquabacterium* ($r = 0.54$; $p = 0.045$) and *Flavobacterium* ($r = 0.56$; $p = 0.048$). For the whole body microbiome, three bacterial genera were positively correlated with the $EC_{50\ 48\ h}$: *Flavobacterium* ($r = 0.80$; $p = 0.0018$), *Pseudomonas* ($r = 0.75$; $p = 0.0031$) and *Bacillus* ($r = 0.54$; $p = 0.048$). In addition, there was also a negative correlation between the $EC_{50\ 48\ h}$ and the abundance in the whole body samples of *Ideonella* ($r = -0.59$; $p = 0.045$).

4. Discussion

4.1. Experimental evolution of Daphnia chlorpyrifos tolerance

As expected, *D. magna* clones collected from aquaria exposed to chlorpyrifos during the experimental evolution trials were more tolerant to chlorpyrifos in terms of survival. This higher chlorpyrifos tolerance was demonstrated both by a higher "lowest observed effect concentration" (LOEC) (1.5 μg/L vs 0.8 μg/L) and a higher $EC_{50\ 48\ h}$ (3.0 μg/L vs 2.2 μg/L) in the chlorpyrifos-selected clones in comparison with the control clones. This matches other experimental evolution trials showing an increased pesticide tolerance to chlorpyrifos (Ejaz et al., 2017) and other pesticides, for example carbaryl (Jansen et al., 2011b). These example studies used a longer exposure duration (Ejaz et al., 2017: nine generations; Jansen et al., 2011b: 54 days) and/or higher exposure concentration (Jansen et al., 2011b: 4 times the $EC_{50\ 48\ h}$) in comparison to the current study where the daphnids were only exposed for two pulses spread over two weeks to the $EC_{50\ 48\ h}$ of the adults (0.35 μg/L), which is a realistic exposure scenario in water bodies close to agricultural land (Stehle and Schulz, 2015). Current design therefore illustrates that under such realistic conditions, rapid evolution of pesticide tolerance may occur.

Two non-exclusive mechanisms may have contributed to the rapid evolution of tolerance. First, clonal sorting may have occurred whereby clones were sorted that were part of the standing genetic variation of the

natural population. Standing genetic variation was recently shown to cause the rapid evolution of tolerance to pesticides in the Colorado potato beetle (Pelissie et al., 2022). Several pathways that were present in these 'sorted' clones can explain the increased chlorpyrifos tolerance. One possibility is that these clones showed target site tolerance (Stenersen, 2004) caused by a mutation of acetylcholinesterase, resulting in no or a lower inhibition of this enzyme by chlorpyrifos as shown in mosquitoes tolerant to chlorpyrifos (Duron et al., 2006). Also, metabolic tolerance may have played a role (Stenersen, 2004) caused by over-expression of detoxification genes, such as carboxylesterases, phosphotriesterases and other esterases, as shown in organophosphate-resistant mosquitoes (Labbé et al., 2009). Note that new mutations that conveyed tolerance are unlikely to have occurred during the short experimental evolution trials. Second, our data suggest that also the microbiota may have contributed to the tolerance of *Daphnia* against this pesticide (see below).

4.2. Impact of chlorpyrifos on the microbiome communities

The microbiome was more diverse in the chlorpyrifos-selected samples compared to the control samples as indicated by the patterns in the α-diversity. A similar observation was done in wasps after exposure to the herbicide atrazine (Wang et al., 2020). A possible explanation is that the chlorpyrifos molecules served as an extra carbon-rich food source, thereby attracting additional bacteria and resulting in a higher diversity (see also Akbar et al., 2014). However, also the opposite has been observed (e.g. in response to exposure to the organophosphate fenitrothion in mosquitoes (Dada et al., 2018)) whereby the microbiome community became dominated by the few bacterial taxa able to use this compound as food source, thereby apparently outcompeting the other taxa.

As indicated by the analyses of β-diversity, the microbial community between the two selection regimes differed and this was the case in both sample types. This matches our expectations as xenobiotics are expected to lead to changes in the microbiota composition with a shift towards xenobiotic-tolerant bacteria, as shown for example for metals (Lapanje et al., 2010; Senderovich and Halpern, 2013; Fong et al., 2019), antibiotics (Gorokhova et al., 2015; Akbar et al., 2020) and pesticides (reviewed in Chiu et al., 2020) including organophosphates (Chen et al., 2020). These bacteria are thought to possess enzymes that are able to transform or degrade the xenobiotic, thereby providing access to additional nutrients to the bacteria, which as a result can increase in abundance (Russell et al., 2011). Note that many ASVs could not be identified at the genus level, yet this does not affect the patterns in α- and β-diversity as our analyses use ASV-specific labels.

Based on an extensive literature search for bacteria known to degrade organophosphates, 43 genera containing organophosphate-degrading species and/or strains were identified (Appendix D). Twenty out of these genera were present in the *Daphnia* of current study. Note that given more research on xenobiotic degrading enzymes and genes in bacteria is needed, as well as profound testing of more bacteria for their degradation capacities (Kumar et al., 2018), it is possible that more genera than those identified in current study are able to degrade organophosphates. We compared the abundance of these 20 genera between samples from control and chlorpyrifos-selected aquaria (summarized in Table 1). The bacterial degradation of chlorpyrifos (and other pesticides) can occur through two pathways. A first possibility is catabolism whereby the bacteria utilize the chlorpyrifos molecule as source of carbon and phosphorus, as is for example the case for *Enterobacter* (Singh et al., 2003a). A second possibility is co-metabolism whereby the chlorpyrifos molecule is biotransformed, but not used as energy source or as a constitutive element by the bacteria. For example, *Flavobacterium* (Mallick et al., 1999), *Pseudomonas* (Serdar et al., 1982) and *Micrococcus* (Guha et al., 1997) are bacteria that can transform chlorpyrifos by hydrolysis to produce dethylthiophosphoric acid (DETP) and 3,5,6-trichloro-2-pyridinol (TCP). The bacterium-induced hydrolysis of the organophosphorus compounds occurs via bacterial enzymes including organophosphorus hydrolase and phosphotriesterase (both present in *Pseudomonas*, Singh and Walker, 2006), organophosphorus acid anhydrolase (present in *Alteromonas*, Singh and Walker, 2006) and phosphonatase (present in *Bacillus*, Singh and Walker, 2006). Little is known about the further degradation of DETP and TCP, but research has shown that DETP can be used as carbon and phosphorous source by *Enterobacter* (Singh et al., 2003a) and as sulfur source by *Pseudomonas* (Cook et al., 1980), and that TCP can be mineralized by *Pseudomonas* by reductive dechlorination (Feng et al., 1998).

The abundance of the 20 known organophosphate-degrading bacterial genera detected in current study was approximately four times higher in the chlorpyrifos-selected samples compared to the control samples, both in the gut and in the whole body samples (Table 1). Several of the organophosphate-degrading bacteria were only detected in the chlorpyrifos-selected samples: *Acinetobacter* (whole body), *Azospirillum* (gut), *Bacillus* (whole body), *Caulobacter* (gut), *Chryreobacterium*, *Lactobacillus*, *Mycobacterium* (gut), *Pseudomonas*,

Table 1
Overview of the 20 known organophosphate-degrading bacteria found in the microbiome of *D. magna* in current study.

Genus	Relative abundance control gut (%)	Relative abundance chlorpyrifos-selected gut (%)	Relative abundance control body (%)	Relative abundance chlorpyrifos-selected body (%)	Reference for organophosphate degrading capacity
Acinetobacter	0.0048	0.026	0	0.062	Chanika et al. (2011)
Azospirillum	0	0.0031	0.0024	0.0010	Foster et al. (2004)
Bacillus	0.00081	0.064	0.016	0.69	Li et al. (2008)
Brevundimonas	0.00081	0.020	0.0081	0.062	Li et al. (2008)
Caulobacter	0	0.022	0.0032	0.14	Singh et al. (2003b)
Chryseobacterium	0	0.0041	0	0.0062	Ramya et al. (2016)
Corynebacterium	0.0032	0	0.033	0.052	Kim et al. (2009)
Enterobacter	0	0	0.00081	0.0021	Awad et al. (2011)
Exiguobacterium	0.00081	0.0052	0.0032	0.0010	Lopez et al. (2005)
Flavobacterium	0.30	0.78	1.73	4.71	Brown (1980)
Lactobacillus	0	0.0072	0	0.010	Zhang et al. (2014)
Mcyobacterium	0	0.0020	0	0	Seo et al. (2007)
Paracoccus	0.0024	0.010	0.0024	0.0010	Xu et al. (2008)
Pseudomonas	0	0.096	0	0.80	Dumas et al. (1989)
Rhodococcus	0	0.0040	0	0	Phugare et al. (2012)
Rhizobium	0.0064	0.016	0.028	0.76	Kim et al. (2009)
Sphingomonas	0.0097	0.033	0.014	0.0041	Li et al. (2008)
Staphylococcus	0.0024	0.0052	0.0089	0.16	Baishya and Sharma (2014)
Stenotrophomonas	0.0024	0	0.00081	0	Li et al. (2008)
Vibrio	0	0.0021	0.0032	0	Agarry et al. (2013)
TOTAL %	**0.34**	**1.13**	**1.85**	**7.46**	

Rhodococcus (gut) and *Vibrio* (gut). Others, such as *Bacillus, Brevudimonas, Flavobacterium*, *Rhizobium* and *Staphylococcus*, showed a much higher abundance (up to more than ten times higher) in chlorpyrifos-selected samples. Moreover, for *Pseudomonas* and *Flavobacterium,* we could demonstrate a positive correlation between the abundance of these bacteria and the $EC_{50\ 48\ h}$, indicating they indeed contribute to chlorpyrifos tolerance in *D. magna*. We cannot exclude the possibility that some bacterial species of these known 'organophosphate-degrading bacterial genera' do not have this function. These bacterial species would then, however, not contribute to the here reported patterns, making these patterns conservative. In addition, we note that many ASVs could not be identified at the genus level, therefore we may have missed organophosphate-degrading bacterial genera.

Also other studies demonstrated an increase in pesticide-degrading bacteria in the microbiome after exposure to pesticides. Wang et al. (2020) showed that exposure to the herbicide atrazine resulted in an increased abundance of the atrazine-degrading bacteria *Pseudomonas* and *Serratia* in wasps. Almeida et al. (2017) showed in chlorpyrifos-resistant fruit flies a higher abundance of *Enterococcus, Delftia, Leclercia* and *Microbacterium*, genera which were not only able to grow on chlorpyrifos-containing medium, but were also able to biodegrade the toxicant. Similarly, Dada et al. (2018) showed in mosquitoes resistant to another organophosphate (fenitrothion) a higher abundance of 21 bacterial genera including four genera which also showed a higher abundance in the chlorpyrifos-selected samples in current study *(Bacillus, Pseudomonas*, *Staphylococcus* and *Vibrio*). The study by Chen et al. (2020) went one step further by performing mono-inoculation studies in silkworms and demonstrated that inoculation with *Stenotrophomonas* decreased the chlorpyrifos-induced mortality of the host.

4.3. Comparison between gut and whole body microbiomes

As expected, the microbial diversity was higher in the whole body compared to the gut samples and this regardless of the pesticide exposure. There was also a clear difference in community composition between the whole body and the gut microbiome samples. Also, when only looking at the organophosphate-degrading bacteria, there was a clear distinction between the gut and body samples with in the absence of the pesticide the gut being inhabited by 11 organophosphate-degrading bacteria (corresponding with 0.34% of the total bacterial community) in comparison with 14 genera in the whole body (corresponding with 1.13% of the total bacterial community). In the absence of the pesticide, *Limnohabitans* made up approximately 27% of the bacterial community in the whole body samples, while this genus represented only 1% of the bacterial community in the gut. Callens et al. (2016) also showed a much higher abundance of *Limnohabitans* in the microbiome of whole body samples compared to the gut samples of *D. magna*. In addition, Callens et al. (2016) demonstrated a higher abundance of *Chlorochromatium*, *Hydrogenophaga* and *Flavobacterium* in the whole body samples compared to the gut samples. The two latter genera were also more abundant in the whole body samples in current study, suggesting these genera mainly occur outside the gut. Eckert and Pernthaler (2014) demonstrated high abundances of bacteria including *Limnohabitans* to be responsible for the uptake of dissolved organic carbon on the filter combs of daphnids, illustrating that bacteria can be functionally associated with several body parts of *Daphnia*.

Despite the differences between the sample types in microbial composition in the *Daphnia* not exposed to the pesticide, both sample types showed an increase in the abundance of organophosphate-degrading bacteria when exposed to the pesticide. Indeed, both in the whole body and in the gut samples the abundance of *Bacillus* (on average 60 times higher) and *Flavobacterium* (on average 2.5 times higher) strongly increased and *Pseudomonas* even only was present in the chlorpyrifos-selected samples. Moreover, when comparing the presence of organophosphate-degrading bacteria in general (Table 1), both sample types showed a similar response, with an approximately four times higher abundance of these bacteria in the chlorpyrifos-selected samples. Most of these organophosphate-degrading bacteria were shared between the gut and whole body (e.g. *Bacillus, Flavobacterium, Pseudomonas, Rhizobium*), while some were unique for the gut (*Rhodococcus, Mycobacterium*) or whole body (*Enterobacter*) (Table 1). Overall, these congruent patterns in both sample types can be easily explained because the guts were also included in the whole body samples. Despite the similar response to the pesticide between gut and body samples, the total abundance of organophosphate-degrading bacteria was approximately six times higher in the whole body samples, suggesting that the gut is not the only body part where pesticide degradation by the microbiome occurs. Other body parts where bacteria degrade pesticides could be the filter combs where the water with dissolved pesticide molecules passages to enter the body (as shown for example for the uptake of dissolved organic carbon (Eckert and Pernthaler, 2014)) and the egg sack where these bacteria could play a role in preventing pesticide residues being carried over to the eggs. Given that in both sample types a similar increase in organophosphate-degrading bacteria in the chlorpyrifos-selected samples was observed, both are relevant to be used in microbiome studies linking pesticide tolerance to bacterial communities. The use of whole body samples might, however, be more powerful given (i) the higher number of ASVs that could be identified at the genus level (on average 84% in the whole body versus 28% in the gut) and (ii) the higher relative abundance of pesticide-degrading bacteria in the whole body microbiome compared to the gut microbiome (approximately six times higher).

4.4. Conclusions and future perspectives

Using experimental evolution where we exposed *D. magna* to two realistic chlorpyrifos pulses over two weeks, we obtained daphnid populations tolerant to the pesticide. This was associated with shifts in the microbiome composition with an increase of chlorpyrifos-degrading bacteria such as *Pseudomonas*, *Flavobacterium* and *Bacillus.* Our results thereby suggest a role for the microbiome in the evolution of pesticide tolerance of the *Daphnia* as further supported by the correlations between the abundance of certain bacterial genera and the $EC_{50\ 48\ h}$. Yet, follow-up experiments are needed to demonstrate the causal role of these bacteria in shaping the evolution of *Daphnia* tolerance. An interesting approach would be to carry out a reciprocal microbiome transplant experiment whereby control and pesticide-selected *Daphnia* clones are inoculated with the microbiome of control or pesticide-selected clones and the effects on chlorpyrifos tolerance evaluated (cfr Macke et al., 2017 for tolerance to toxic cyanobacteria). Additional interesting avenues for future research are to monitor the microbiome in organophosphate-tolerant *Daphnia* if they are contained in pesticide-free water for long time, and to directly compare the microbiomes of *Daphnia* collected at sites with contrasting organophosphate contamination. Our study adds to the emerging insight that besides target site tolerance and metabolic tolerance, that are directly encoded in the host's genome, also the microbiome may be an important driver of tolerance to pollutants.

CRediT authorship contribution statement

Lizanne Janssens: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft. **Marlies Van de Maele:** Methodology, Investigation, Writing – review & editing. **Vienna Delnat:** Formal analysis, Software, Visualization, Writing – review & editing. **Charlotte Theys:** Formal analysis, Software, Visualization, Writing – review & editing. **Shinjini Mukherjee:** Methodology, Writing – review & editing. **Luc De Meester:** Funding acquisition, Methodology, Writing – review & editing. **Robby Stoks:** Funding acquisition, Resources, Conceptualization, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data accessibility

Data used for the statistics will be made available in Dryad Digital Repository after acceptance of the manuscript. R and command line code that are used in this study are available in GitHub at https://github.com/viennadelnat/microbiome-pesticide-selection.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113697.

References

Agarry, S.E., Olu-Arotiowa, O.A., Aremu, M.O., Jimoda, L.A., 2013. Biodegradation of dichlorovos (organophosphate pesticide) in soil by bacterial isolates. J. Nat. Sci. Res. 3, 12–16.
Akbar, S., Gu, L., Sun, Y., Zhou, Q., Zhang, L., Lyu, L., Huang, Y., Yang, Z., 2020. Changes in the life history traits of *Daphnia magna* are associated with the gut microbiota composition shaped by diet and antibiotics. Sci. Total Environ. 705, 135827.
Akbar, S., Gu, L., Sun, Y., Zhang, L., Lyu, K., Huang, Y., Yang, Z., 2022. Understanding host-microbiome-environment interactions: Insights from *Daphnia* as a model organism. Sci. Total Environ. 808, 152093.
Akbar, S., Sultan, S., Kertesz, M., 2014. Bacterial community analysis in chlorpyrifos enrichment cultures via DGGE and use of bacterial consortium for CP biodegradation. World J. Microbiol. Biotechnol. 30, 2755–2766.
Almeida, L.Gd, Moraes, L.A.Bd, Trigo, J.R., Omoto, C., Cônsoli, F.L., 2017. The gut microbiota of insecticide-resistant insects houses insecticide-degrading bacteria: a potential source for biotechnological exploitation. Plos One 12, e0174754.
Almeida, R.A., Lemmens, P., De Meester, L., Brans, K.I., 2021. Differential local genetic adaptation to pesticide use in organic and conventional agriculture in an aquatic non-target species. Proc. R. Soc. B 288, 20211903.
Awad, N.S., Sabit, H.H., Abo-Aba, S.E.M., Bayoumi, R.A., 2011. Isolation, characterization and fingerprinting of some chlorpyrifos-degrading bacterial strains isolated from Egyptian pesticides-polluted soils. Afr. J. Microbiol. Res. 5, 2855–2862.
Baishya, K., Sharma, H.P., 2014. Isolation and characterization of organophosphorus pesticide degrading bacterial isolates. Arch. Appl. Sci. Res. 6, 144–149.
Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67, 1–48.
Bell, G., 2017. Evolutionary rescue. Annu. Rev. Ecol., Evol. Syst. 48, 605–627.
Bolyen, E., Rideout, J.R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME2. Nat. Biotechnol. 37, 852–857.
Brown, K.A., 1980. Phosphotriesterases of *Flavobacterium* sp. Soil Biol. Chem. 12, 105–112.
Brune, A., Dietrich, C., 2015. The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. Annu. Rev. Microbiol. 69, 145–166.
Callens, M., Hajime, W., Yasuhiko, K., Miura, J., Decaestecker, E., 2018. Microbiota inoculum composition affects holobiont assembly and host growth in Daphnia. Microbiome 6, 56.
Callens, M., Macke, E., Muylaert, K., Bossier, P., Lievens, B., Waud, M., Decaestecker, E., 2016. Food availability affects the strength of mutualistic host-microbiota interactions in *Daphnia magna*. ISME J. 10, 911–920.
Chanika, E., Georgiadou, D., Soueref, E., Karas, P., Karanasios, E., Tsiropoulos, N.G., Tzortzakakis, E.A., Karpouzas, D.G., 2011. Isolation of soil bacteria able to hydrolyze both organophosphate and carbamate pesticides. Bioresour. Technol. 102, 3184–3192.
Chen, B., Zhang, N., Xie, S., Zhang, X., He, J., Muhammad, A., Sun, C., Lu, X., Shao, Y., 2020. Gut bacteria of the silkworm *Bombyx mori* facilitate host resistance against the toxic effects of organophosphate insecticides. Environ. Int. 143, 105886.
Chevalier, C., Stojanovic, O., Trajkovski, M., 2015. Gut microbiota orchestrates energy homeostasis during cold. Cell 163, 1360–1374.
Chiu, K., Warner, G., Nowak, R.A., Flaws, J.A., Mei, W., 2020. The impact of environmental chemicals on the gut microbiome. Toxicol. Sci. 176, 253–284.
Ceja-Navarro, J.A., Vega, F.E., Karaoz, U., Hao, Z., Jenkins, S., Lim, H.C., Kosina, P., Infante, F., Northern, T.R., Brodie, E.L., 2015. Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. Nat. Commun. 14, 7618.
Cook, A.M., Alexander, M., Daughton, C.G., 1980. Desulfuration of dialkyl thiophosphoric acids by a pseudomonad. Appl. Environ. Microbiol. 39, 463–465.
Coors, A., Vanoverbeke, J., De Bie, T., De Meester, L., 2009. Land use, genetic diversity and toxicant tolerance in natural populations of Daphnia magna. Aquat. Toxicol. 95, 71–79.
Cothran, R.D., Brown, J.M., Relyea, R.A., 2013. Proximity to agriculture is correlated with pesticide tolerance: evidence for the evolution of amphibian resistance to modern pesticides. Evolut. Appl. 6, 832–841.
Dada, N., Sheth, M., Liebman, K., Pinto, J., Lenhart, A., 2018. Whole metagenome sequencing reveals links between mosquito microbiota and insecticide resistance in malaria vectors. Sci. Rep. 8, 2084.
Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A., Callahan, B.J., 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6, 226.
Dumas, D.P., Caldwell, S.R., Wild, J.R., Raushel, F.M., 1989. Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*. J. Biol. Chem. 264, 19659–19665.
Duron, O., Labbé, P., Berticat, C., Rousset, F., Guillot, S., Raymond, M., Weill, M., 2006. High *Wolbachia* density correlates with cost of infection for insecticide resistant *Culex pipiens* mosquitoes. Evolution 60, 303–314.
Ebert, D., 2005. Ecology, Epidemiology, and Evolution of Parasitism in *Daphnia*. National Center for Biotechnology Information (US). Chapter 2, Introduction to Daphnia Biology, Bethesda (MD). ⟨https://www.ncbi.nlm.nih.gov/books/NBK2042/⟩.
Eckert, E.M., Pernthaler, J., 2014. Bacterial epibionts of *Daphnia*: a potential route for the transfer of dissolved organic carbon in freshwater food webs. ISME J. 8, 1808–1819.
Ejaz, M., Afzal, M.B.S., Shabbir, G., Serrão, J.E., Shad, S.A., Muhammad, W., 2017. Laboratory selection of chlorpyrifos resistance in an invasive pest, *Phenacoccus solenopsis* (Homoptera: Pseudococcidae): cross-resistance, stability and fitness cost. Pestic. Biochem. Physiol. 137, 8–14.
Feng, Y.E., Minard, R.D., Bollag, J.M., 1998. Photolytic and microbial degradation of 3, 5, 6-trichloro-2-pyridinol. Environ. Toxicol. Chem. 17, 814–819.
Fong, J.C., De Guzman, B.E., Lamborg, C.H., Sison-Mangus, M.P., 2019. The mercury-tolerant microbiota of the zooplankton Daphnia aids in host survival and maintains fecundity under mercury stress. Environ. Sci. Technol. 53, 14688–14699.
Foster, L.J.R., Kwan, B.H., Vancov, T., 2004. Microbial degradation of the organophosphate pesticide, Ethion. FEMS Microbiol. Lett. 240, 49–53.
Fox, J., Weisberg, S., 2011. An R Companion To Applied Regression. SAGE Publications, Inc, References 243 Thousand Oaks, California, USA.
Gorokhova, E., Rivetti, C., Furuhagen, S., Edlund, A., Ek, K., Breitholtz, M., 2015. Bacteria mediated effects of antibiotics on *Daphnia* nutrition. Environ. Sci. Technol. 49, 5779–5787.
Guha, A., Kumari, B., Roy, M.K., 1997. Possible involvement of plasmid in degradation of malathion and chlorpyrifos by *Micrococcus* sp. Folia Microbiol. 42, 574–576.
Houwenhuyse, S., Stoks, R., Mukherjee, S., Decaestecker, E., 2021. Locally adapted gut microbiomes mediate host stress tolerance. ISME J. 15, 2401–2414.
Jansen, M., Geldof, B.S., De Meester, L., Orsini, L., 2011a. Isolation and characterization of microsatellite markers in the water flea Daphnia magna. Mol. Ecol. Resour. 11, 418–421.
Jansen, M., Stoks, R., Coors, A., van Doorslaer, W., De Meester, L., 2011b. Collateral damage: rapid exposure-induced evolution of pesticide resistance leads to increased susceptibility to parasites. Evolution 65, 2681–2691.
Johnson, A.C., Donnachie, R.L., Sumpter, J.P., Jürgens, M.D., Moeckel, C., Pereira, M.G., 2017. An alternative approach to risk rank chemicals on the threat they pose to the aquatic environment. Sci. Total Environ. 599, 1372–1381.
Kamada, N., Seo, S.-U., Chen, G.Y., Nunez, G., 2013. Role of the gut microbiota in immunity and inflammatory disease. Nat. Rev. Immunol. 13, 321–335.
Kawecki, T.J., Lenski, R.E., Ebert, D., Hollis, B., Olivieri, I., Whitlock, M.C., 2012. Experimental evolution. Trends Ecol. Evol. 27, 547–560.
Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K., Fukatsu, T., 2012. Symbiont-mediated insecticide resistance. Proc. Natl. Acad. Sci. USA 109, 8618–8622.
Kim, K.D., Ahn, J.H., Kim, T., Park, S.C., Seong, C.N., Song, H.G., Ka, J.O., 2009. Genetic and phenotypic diversity of fenitrothion-degrading bacteria isolated from soils. J. Microbiol. Biotechnol. 19, 113–120.
Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glockner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, 1.
Kumar, S., Kaushik, G., Dar, M.A., Nimesh, S., Lopez-Chuken, U.J., Villarreal-Chiu, J.F., 2018. Microbial degradation of organophosphate pesticides: a review. Pedosphere 28, 190–208.
Labbé, P., Sidos, N., Raymond, M., Lenormand, T., 2009. Resistance gene replacement in the mosquito *Culex pipiens*: fitness estimation from long-term cline series. Genetics 182, 303–312.
Lapanje, A., Zrimec, A., Drobne, D., Rupnik, M., 2010. Long-term Hg pollution-induced structural shifts of bacterial community in the terrestrial isopod (*Porcellio scaber*) gut. Environ. Pollut. 158, 3186–3193.
Li, X.H., Jiang, J.D., Gu, L.F., Ali, S.W., He, J., Li, S.P., 2008. Diversity of chlorpyrifos-degrading bacteria isolated from chlorpyrifos contaminated samples. Int. Biodeterior. Biodegrad. 62, 331–335.

Lopez, L., Pozo, C., Rodelas, B., Calvo, C., Juarez, B., Martinez-Toledo, M.V., Gonzalez-Lopez, J., 2005. Identification of bacteria isolated from an oligotrophic lake with pesticide removal capacities. Ecotoxicology 14, 299–312.
Loria, A., Cristescu, M.E., Gonzalez, A., 2019. Mixed evidence for adaptation to environmental pollution. Evolut. Appl. 12, 1259–1273.
Lynch, J.B., Hsiao, E.Y., 2019. Microbiomes as sources of emergent host phenotypes. Science 365, 6460.
Macke, E., Callens, M., De Meester, L., Decaestecker, E., 2017. Host-genotype dependent gut microbiota drives zooplankton tolerance to toxic cyanobacteria. Nat. Commun. 8, 1608.
Mallick, B.K., Banerji, A., Shakil, N.A., Sethunathan, N.N., 1999. Bacterial degradation of chlorpyrifos in pure culture and in soil. Bull. Environ. Contam. Toxicol. 62, 48–55.
McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible analysis and graphics of microbiome census data. Plos One 8, 4.
Miner, B.E., De Meester, L., Pfrender, M.E., Lampert, W., Hairston, N.G., 2012. Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. Proc. R. Soc. B 279, 1873–1882.
Motiei, A., Brindefalk, B., Gorkohova, E., 2020. Disparate effects of antibiotic-induced microbiome change and enhanced fitness in *Daphnia magna*. Plos One 15, e0324833.
Nikoh, N., Hosokawa, T., Oshima, K., Hattori, M., Fukatsu, T., 2011. Reductive evolution of bacterial genome in insect gut environment. Genome Biol. Evol. 3, 702–714.
OECD (2004) OECD Guideline for the Testing Of Chemicals: Test No. 202: Daphnia sp. Acute Immobilisation Test. 1–12.
Oksanen, J., Blanchet, F.G., …, Wagner, H. (2020) Package Vegan. Community Ecology Package Version 2.5–7.
Pelissie, B., Chen, Y.H., Schoville, S.D., 2022. Genome resequencing reveals rapid, repeated evolution in the Colorado Potato Beetle. Mol. Biol. Evol. 39, msac016.
Phugare, S.S., Gaikwad, Y.B., Jadhav, J.P., 2012. Biodegradation of acephate using a developed bacterial consortium and toxicological analysis using earthworms (*Lumbricus terrestris*) as a model animal. Int. Biodeterior. Biodegrad. 69, 1–9.
Qi, W., Nong, G., Preston, J.F., Ben-Ami, F., Ebert, D., 2009. Comparative metagenomics of *Daphnia* symbionts. BMC Genom. 10, 21.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, 590–596.
Rahman, H.U., Asghar, W., Nazir, W., Sandhu, M.A., Ahmed, A., Khalid, N., 2021. A comprehensive review on chlorpyrifos toxicity with special reference to endocrine disruption: Evidence of mechanisms, exposures and mitigation strategies. Sci. Total Environ. 755, 142649.
Ramya, S.L., Venkatesan, T., Murthy, K.S., Jalali, S.K., Verghese, A., 2016. Detection of carboxylesterase and esterase activity in culturable gut bacterial flora isolated from diamondback moth, *Plutella xylostella* (Linnaeus), from India and its possible role in indoxacarb degradation. Braz. J. Microbiol. 47, 327–336.
Russell, R.J., Scott, C., Jackson, C.J., Pandey, R., Pandey, G., Taylor, M.C., Coppin, C.W., Liu, J.-W., Oakeshott, J.G., 2011. The evolution of new enzyme function: lessons from xenobiotic metabolizing bacteria versus insecticide-resistant insects. Evolut. Appl. 4, 225–248.
Senderovich, Y., Halpern, M., 2013. The protective role of endogenous bacterial communities in chironomid egg masses and larvae. ISME J. 7, 2147–2158.
Seo, J.S., Keum, Y.S., Harada, R.M., Li, Q.X., 2007. Isolation and characterization of bacteria capable of degrading polycyclic aromatic hydrocarbons (PAHs) and organophosphorus pesticides from PAH-contaminated soil in Hilo, Hawaii. J. Agric. Food Chem. 55, 5383–5389.
Serdar, C.M., Gibson, D.T., Munnecke, D.M., Lancaster, J.H., 1982. Plasmid involvement in parathion hydrolysis by *Pseudomonas diminuta*. Appl. Environ. Microbiol. 44, 246–249.
Singh, B.K., Walker, A., 2006. Microbial degradation of organophosphorus compounds. FEMS Microbiol. Rev. 30, 428–471.
Singh, B.K., Walker, A., Morgan, J.A.W., Wright, D.J., 2003a. Effect of soil pH on the biodegradation of chlorpyrifos and isolation of a chlorpyrifos-degrading bacterium. Appl. Environ. Microbiol. 69, 5198–5206.
Singh, B.K., Walker, A., Morgan, A.W., Wright, D.J., 2003b. Role of soil pH in the development of enhanced biodegradation of fenamiphos. Appl. Environ. Microbiol. 69, 7035–7043.
Singmann, H., Bolker, B., Westfall, J., Aust, F. (2016) Afex: analysis of factorial experiments. R package version 0.16–1.
Sison-Mangus, M.P., Mushegian, A.A., Ebert, D., 2015. Water fleas require microbiota for survival, growth and reproduction. ISME J. 9, 59–67.
Stehle, S., Schulz, R., 2015. Pesticide authorization in the EU - environment unprotected? Environ. Sci. Pollut. Res. 22, 19632–19647.
Stenersen, J., 2004. Chemical Pesticides: Mode Of Action And Toxicology. CRC Press, Boca Raton, Florida, USA.
United Nations Environment Program (UNEP) (2004) Screening Information DataSet: initial assessment report ethanol cas N°: 64–17-5.
Van Doorslaer, W., Stoks, R., Duvivier, C., Bednarska, A., De Meester, L., 2009. Population dynamics determine genetic adaptation to temperature in Daphnia. Evolution 63, 1867–1878.
Wang, G.-H., Berdy, B.M., Velasquez, O., Jovanovic, N., Alkhalifa, S., Minbiole, K.P.C., Brucker, R.M., 2020. Changes in microbiome confer multigenerational host resistance after sub-toxic pesticide exposure. Cell Host Microbe 27, 213–224.
Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.
Xu, G., Zheng, W., Li, Y., Wang, S., Zhang, J., Yan, Y., 2008. Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by a newly isolated *Paracoccus* sp. strain TRP. Int. Biodeterior. Biodegrad. 62, 51–56.
Zhang, Y.H., Xu, D., Liu, J.Q., Zhao, X.H., 2014. Enhanced degradation of five organophosphorus pesticides in skimmed milk by lactic acid bacteria and its potential relationship with phosphatase production. Food Chem. 164, 173–178.