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Exploring gene expression as a sublethal endpoint in gammarids exposed to pesticides: insights from next-generation sequencing

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Pesticide residues are frequently detected in surface waters, with several compounds known to adversely affect aquatic organisms. Gammarids are particularly suitable indicator organisms for assessing the sublethal effects of such contaminants due to their high sensitivity and their central ecological role in freshwater ecosystems. While behavioral endpoints and feeding rates have been commonly used to evaluate sublethal pesticide effects, gene expression changes have received comparatively little attention, despite their proven value in other ecotoxicological contexts. This study investigates the potential of gene expression as a sensitive sublethal endpoint in gammarids collected from natural populations. A laboratory exposure experiment was conducted using the model pesticides azoxystrobin and acetamiprid, both of which are regularly detected in surface waters. Gammarids collected from the wild were exposed under controlled conditions to sublethal concentrations of the test substances. Subsequently, RNA sequencing (RNA-seq) was performed to characterize genome-wide transcriptional responses. Two independent exposure and sequencing experiments were carried out, resulting in the identification of 145 and 326 differentially expressed transcripts per experiment when comparing exposed animals to controls. Gene ontology (GO) term enrichment analyses revealed significant effects on metabolic processes, cell proliferation, and cell differentiation. Notably, the two experimental runs yielded distinct transcriptional profiles, with minimal overlap in differentially expressed transcripts despite the use of gammarids from the same population and the short interval (12 days) between experiments. The study demonstrates the applicability of transcriptomic analyses for detecting sublethal pesticide effects in field-collected gammarids and provides a practical workflow for the evaluation of RNA-seq data in non-model organisms. At the same time, it highlights important limitations, including high genetic variability within wild populations and incomplete transcriptome annotation, which together contribute to inconsistencies across repeated experiments.

Keywords Gammaridae, Pesticides, Sublethal exposure, Gene-expression, And next generation sequencing

In recent years it has become increasingly evident that surface waters are contaminated with various chemicals, due to agriculture and treated wastewater from residential and industrial sources[1,2]. When considering not only substance occurrence but also resulting environmental risk, the issue becomes even more evident: 74% of the samples contained micropollutants (MP), like pesticides, at concentrations which posed a risk of chronic adverse effects and 18% posed a risk for acute toxicity in aquatic organisms[1]. Pesticides can be introduced into surface waters through runoff events and lead to brief concentration peaks[3], through direct spray drift[4], indirect spray drift, drainage[5] or even accidental spillage[6]. When reaching different environmental zones they can affect non-target organisms, causing individual stress and impacting populations in their ecosystems[7,8].

For some MP, prospective and retrospective hazard assessment is conducted to estimate the risk of single substances to organisms in aquatic environments. This hazard assessment is performed with representative standard organisms and involves contaminant exposure experiments to assess both acute toxicity and chronic effects[9]. Gammarids, a diverse collection of amphipod crustaceans in the Gammaridae family[10], are prominent model organisms for such studies, involving both laboratory and field-based strategies due to their ecological importance[11–15]. They play a crucial role in the detritus cycle, including litter breakdown processes, and serve

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as a significant component of food chains by acting as prey for secondary consumers. Moreover, gammarids are renowned for their sensitivity to a broad spectrum of chemical stressors[16].

A variety of sublethal endpoints have successfully been assessed to quantify the impact of MP on gammarids. These include behavioural endpoints such as feeding rate, movement, respiration and amplexus formation[17,18]. Even though these endpoints are well suited to reflect the impact of MP on gammarids, they are difficulty to assess in an *in-situ* field application. Therefore, other sublethal endpoints would help to indicate micropollutant impact,for example enzymatic activity (fundamental to growth, moulting, digestion and cellular stress) has been tested[18]. However, the latest developments in the field of gene expression research are revealing new opportunities. Methods such as qPCR focus on specific selected transcripts[19,20], but more comprehensive approaches exist, such as next generation sequencing (NGS), specifically RNA-sequencing or RNA-seq, which are holistic approach as they analyse all transcriptional changes[21].

The latter successfully detected a transcriptional response in *Gammarus fossarum* exposed to abiotic stressors such as salinity increase, flow velocity reduction and fine sediment deposition[22]. The sublethal transcriptional response observed indicated stress by adapting to a changing abiotic environment. Such interesting results raise the question of whether and how transcriptional changes can be also induced by sublethal exposure to MP, and if those transcriptional changes could be detected and then help in future risk assessment.

As an initial exploration of this topic, this study examined the effects of sublethal concentrations of two pesticides azoxystrobin and acetamiprid on gene expression in gammarids. Azoxystrobin concentrations in surface waters have been reported to range from 0.06 to 11.1 mg/L in industrialized countries such as United States, France, Germany, Greece, China and Brazil with maximum concentrations observed up to 29.7 mg/L[23]. Additionally, Acetamiprid concentrations of up to 1 mg/L have been detected in surface and groundwater in the Netherlands[24].These model compounds were selected not only because they are frequently detected in small streams across European surface waters, but also because they were identified as contributing the highest toxic pressure among pesticides detected in *Gammarus pulex* populations in such environments[14]. In Switzerland, both substances have been implicated in instances where chronic or acute ecotoxicological quality criteria were exceeded, as reported by the Swiss Centre for Applied Ecotoxicology[1,25,26].

To avoid inducing acute toxicity, exposure concentrations were chosen to be well below lethal thresholds, guided by previously reported LC_{50} values for gammarid species. While most published gene expression studies in gammarids employ extended exposure durations spanning several days, previous research has demonstrated that even short-term exposures can elicit detectable changes in gene expression, as shown using both qPCR[27,28] and next-generation sequencing (NGS) methods[29].

In this experiment, we aimed to expand upon existing exposure studies by employing gene expression as a sublethal endpoint. RNA sequencing (RNA-seq) was used to assess transcriptome-wide changes following 24 h of laboratory exposure of wild-caught gammarids to azoxystrobin and acetamiprid. Gammarids were collected from the wild, as their cultivation and reproduction in the laboratory is considerably more difficult and labor-intensive compared to other species such as Daphnia. Although some studies have shown that gammarids can be maintained and even reproduce in laboratory settings over several months, this remains a complex process and is not yet established as a standard procedure . The objectives were twofold: first, to establish and validate a laboratory workflow and data analysis pipeline for transcriptomic studies in gammarids; and second, to explore the potential of gene expression profiling for detecting sublethal pesticide effects under controlled conditions. Ultimately, this approach is intended to be exploratory for future transfer to field-based applications, for instance, to compare water systems with differing contamination levels or to evaluate the impact of specific point sources of pollution.

Material and Methods

Sampling and acclimatization of the Gammarids

In the river systems of the Upper Rhine near Basel, on the German–Swiss border, *Gammarus fossarum* and *Gammarus pulex* are the most commonly occurring species of the genus *Gammarus*[30]. For the exposure, only male gammarids were collected to reduce the biological variability on June 16th and 28th 2021, at a small stream in Höllstein, Germany (47° 38′ 14.35″ N 7° 45′ 25.36 E), near the Swiss border. The site exhibited a high abundance of gammarids in prae-copula formation, comprising a mixture of two species (*Gammarus pulex* and *Gammarus fossarum*), and was characterized by minimal agricultural activity. Given its location directly at the stream source, low to negligible contamination was assumed. The specimens collected for the experiment likewise represented a mixture of both species. For acclimatization, the gammarids collected were kept in an aquarium in the laboratory for seven days. Within five days, the amount of artificial pond water (APW, according to the Artificial Pond Water Protocol by Cold Spring Harb Protocol[31] was increased from 30 to 70% compared to original stream water. The gammarids were kept at 16 °C with a 16/8 h light/dark cycle. Oxygen concentrations were checked regularly. The gammarids were fed ad libitum with leaves from the sampling sites and supplemented once a day with ground Tetra Micro Granules (Tetra GmbH, Germany)[32].

Pesticides

Both compounds were purchased from Sigma-Alrich (Sigma-Aldrich, Buchs, Switzerland): Acetamiprid (CAS: 190604–92-3, order number: 39246, purity: ≥ 98.0%), azoxystrobin (CAS: 131860–33-8, order number: 31697, purity: ≥ 98.0%). Stock solutions, which were 100-times more concentrated than the highest exposure concentration, were prepared in artificial pond water[31]. LC_{50} values of *Gammarus pulex* were found to be 50 µg/L for acetamiprid and 270 µg/L for azoxystrobin[33]. Other studies found an LC50 of 1920 µg/L for azoxystrobin in *Gammarus lacustris*, and 148.4 µg/L for acetamiprid in *G. fossarum*[15,34]. In *Gammarus fasciatus*, the LC50 for acetamiprid was reported as 80 µg active ingredient/L[35]. The exposure concentrations were chosen to be at least around ten times lower than reported LC50 values to ensure gammarid survival. Experimental design was based on published LC50 values, and no preliminary range-finding test was conducted in order to minimize unnecessary animal use in accordance with ethical considerations.

Exposure

After gammarid acclimatization, two exposure experiments (June 22nd to 23rd 2021 and July 4th to 5th 2021) were conducted. Each experiment consisted of 3 groups of 6 1L glass beakers with 600 mL of APW and 10 individuals each. One group was the control, one group was for azoxystrobin (32 µg/L) exposure, and one for acetamiprid (4 µg/L) exposure. Each beaker was aerated and contained green and black glass stones to mimic the riverbed environment and provide some cover. After 24 h exposure the 10 gammarids of each group were immediately transferred to storage in RNAlater (Sigma-Aldrich, Buchs, Switzerland) at −80 °C until further processing. As the methods established here were to be transferred to field experiments, RNAlater and not liquid nitrogen was used to fix the gammarids as a logistically simpler alternative. In addition, the prevention of RNA degradation by RNAlater has already been shown by[36]. A graphical overview of the NGS experimental setup is shown in Fig. 1.At the beginning and end of the exposure, composite water samples for each condition were sampled for later chemical analyses.

Water chemical analysis

1 mL aliquots of the 250 mL test beaker were put into a 2 mL tube, centrifuged for 5 min at 13,000 g and the supernatant was transferred to an HPLC glass vial. The samples were analysed on an Agilent 1260 Infinity II Prime HPLC system (Agilent Technologies, Basel, Switzerland). The system modules consist of a quaternary pump (G7104C), an autosampler (G7167A) and a column oven (G7116A). The mixer inside the pump was bypassed for a low delay volume. Inside the autosampler, a 1290 inline filter (0.3 µm, 1.3 µL delay volume) was mounted to hold back possible small particles from the sample preparation. For chromatography, a Zorbax Eclipse Plus C8 column (2.1 × 50 mm, 1.8 µm particle size) (Agilent Technologies) was used. The following mobile phase was used for gradient separation: mobile phase A, 1 mM ammonium fluoride in water; mobile phase B, acetonitrile. Pump flow was set to 0.4 mL/min. The gradient started at 0.5 min with 15% B, from 0.5–6.5 min, 95% B, from 6.5–7 min, 95% B, followed by re-equilibration for 4 min. The column compartment was maintained at 40 °C and injection volume set to 1 µL. The end of the LC column was connected to an Agilent 6465 Ultivo triple quadrupole mass spectrometer equipped with a classic electrospray ionization (ESI) source (G1948B). The ESI source was operated in positive mode with the following settings: nebulizer pressure, 35 psi; drying gas flow, 11 L/min; drying gas temperature, 350 °C; and capillary voltage, 1500 V.

The MS was run in multi-reaction monitoring mode (Table S1). For the precursor ion, resolution was set to wide at 1.3 m/z FWHM (full width at half maximum). For the product ion, 0.7 m/z FWHM unit resolution was selected. The LC–MS system was controlled using MassHunter Data Acquisition for Ultivo version 1.2 (Agilent Technologies). Raw data were analysed using the Quantitative MassHunter software version 10 and analysed data were collected using Microsoft Excel 365.

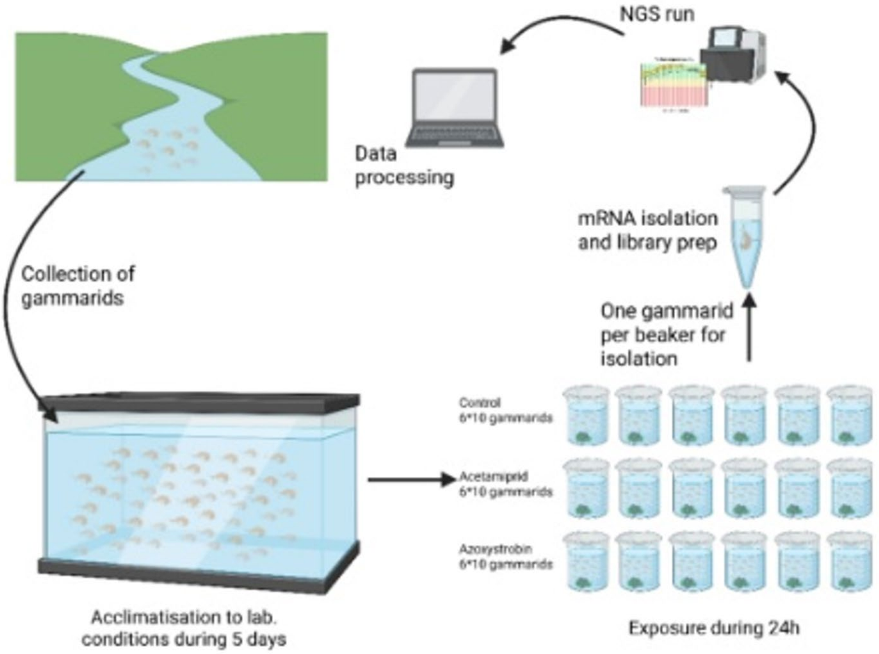

Fig. 1. Schematic overview of the experimental setup for the next-generation sequencing (NGS) study. The experiment was conducted in duplicate, resulting in two exposures for each pesticide. Created in BioRender. Züger, D. (2026) https://BioRender.com/e82p296.

The calibration curve was performed using eight points with a range of 1—100 pg/µL. The peak area was integrated with the Agile 2 integrator. For every sequence run, the calibration curve was freshly prepared. Additionally, for quality performance a blank sample, a method blank sample and two spiked samples with 10 pg/µL were run per sequence. A linear least-square fit through the data points and the origin was used. Linearity was considered acceptable when the correlation coefficient (R^2) was at least 0.995 and the accuracy of the calibration point was > 80%.

Gene expression analysis of NGS experiments

RNA extraction, library preparation and sequencing:

From each of the 6 beakers of both experiments, one random gammarid from the stored samples was thawed, resulting in 6 samples per condition. RNA was extracted using the TRIzol protocol (Thermo Fisher Scientific, Reinach, Switzerland). The concentration and quality of the isolated RNA-samples were verified using a NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific, Reinach, Switzerland). Using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (Bioconcept, Allschwil, Switzerland) the mRNA was isolated according to the manufacturer's instructions. For each sample, 5 µg of isolated mRNA was used to prepare the library needed for the NGS-run. The libraries were created using the Swift RNA Library Kit (Swift BioSciences, now IDT, Coralville, Iowa, United States). The libraries were then quantified by quantitative PCR (Collibri Quantification Kit, Thermo Fisher Scientific, Reinach, Switzerland). They were first sequenced with a MiSeq Reagent Nano Kit v2 on a MiSeq (Illumina, San Diego, California, United States) to ensure that contamination with adapter sequences was low and data quality was high before the actual sequencing was performed with the NextSeq 500/550 High Output Kit v2.5 on a NextSeq (Illumina, San Diego, California, United States). In total, sequencing of the unstranded library on the NextSeq with paired end sequencing yielded approximately 370 million reads. Total reads per sample are in SI2.

Data processing

Quality control, read cleaning and transcriptome assembly

The raw data was processed using tools integrated in the web-based platform useGalaxy.eu[37]. FastQC v0.74 + galaxy0-2[38] and MultiQC v1.11 + galaxy1[39] were used for quality control. Trimming was performed using Trimmomatic v0.38.1[40] in paired-end mode, adapter sequences were cut off using ILLUMINACLIP and reads averaging a quality lower than 25 over four bases were cut off using SLIDINGWINDOW. Reads shorter than 50 bases were also removed. Foreign transcripts within our data were identified using Kraken2 v2.1.1 + galaxy1[41], transcripts identified as non-amphipod, eg. human, prokaryotic or viral were removed, as the whole organism was examined and foreign transcripts from bacteria or parasites could be present. In each sample fewer than 1.5% foreign transcripts were identified, unidentified transcripts were retained. The transcriptome was assembled using Trinity v2.15.1 + galaxy1[42] based on all samples, with Minimum count for K-mers to be assembled set at 2, to get longer sequences and less redundancy. Assembly quality was determined with Trinity Stats v2.15.1 + galaxy0[42] and Busco 5.5.0 + galaxy0[43] running on the Eukaryota (2024–03-23) database with the lineage *arthropods* selected.

Annotation and enrichment

Transdecoder 5.5.0 + galaxy2[44] was used to identify 48,328 open reading frames (ORFs). The resulting amino acid sequences were then fed into the eggNOG-mapper[45,46] which resulted in annotation of proteins includinh GO-Terms[47,48]. 16,631 transcripts were annotated with GO-Terms, which were then used to perform a GO-Enrichment using the R-package TopGO v2.54.0[49]. eggNOH-mapper results are in the SI2 in the table eggNOG Mapper Results. For each condition, positively and negatively regulated transcripts were used as a different input to perform the GO-Enrichment analysis, to be able to differentiate between up and down regulated pathways as TopGO does not account for the foldchange. The biological process terms were the main interpretation focus of the enrichment results. For the significant GO-Terms, their ancestor terms in the ancestor chart were evaluated as well, to group them into similar categories or fields where an effect was visible.

Read counting and differential expression analysis

The trimmed and filtered reads were then used with the galaxy tool Align reads and estimate abundance v2.15.1 + galaxy0[42]. For each sample, reads were aligned to transcripts in the previously assembled transcriptome and counted. The salmon algorithm was selected for the abundance estimation[50]. The counts were assembled into one matrix for downstream analysis. Differential expression analysis was conducted for each experiment repetition, where exposures to each pesticide (n = 6) were compared to control (n = 6). Analysis was performed using DESeq2 2.15.1 + galaxy0[51]. To only display significant results, all differentially expressed transcripts with an adjusted p-value higher than 0.05 were filtered out.

Data visualization

Heatmaps were created using R v4.3.2[52] with the package ComplexHeatmaps v2.18.0[53],significant GO-Terms and PCA were visualized using ggplot2 v3.5.0[54].

Results

NGS experiment

Chemical analysis and mortality

Measured pesticide concentrations in the exposure were close to the nominal concentrations. For each condition a slight to moderate decrease of 2–22% after 24 h was detected (Table S2). Mortality stayed low as intended, between 0 and 2 specimens out of 60 for each exposure and control. This indicated that sublethal concentrations had been selected (Table S2).

Sequencing/assembly results

During sequencing with the Illumina NextSeq 550, 370,589,758 reads were sequenced from 36 samples. The reads were mainly of good quality with Phred quality score up to 35 and an average above 30. After eliminating 14% of poor-quality reads using trimmomatic and filtering for foreign transcripts using Busco, 318,666,614 reads remained. Final assembly with trinity was composed of 190′867 transcripts. After filtering the transcriptome for low expression transcripts, the mapping rate for Busco was 81.4%. Sample remapping rates were always above 80% and the Ex90N50 transcript length was 1903 bp.

Significant changes in gene expression after exposure to pesticides

As a result of differential expression analysis, transcripts with significant changes in expression compared to the control were detected. For Acetamiprid in the first repetition 326 and in the second 251 transcripts were significantly up or down regulated. For azoxystrobin 145 in the first and 323 in the second repetition were significantly changed. In the first experiment more transcripts were up- than downregulated. In the second however, for both pesticides a similar number of transcripts were up- and downregulated, the number of significantly up and downregulated transcripts are displayed in Fig. 2. The pattern of the regulated transcripts differed considerably between the two exposure experiments. Also there were almost no similarly expressed transcripts found for the same conditions when comparing experiment repetitions.

Exposures to acetamiprid only 12 days apart resulted in the induction of just three transcripts with consistent regulation patterns, whereas azoxystrobin induced seven transcripts with the same pattern. The overlap is

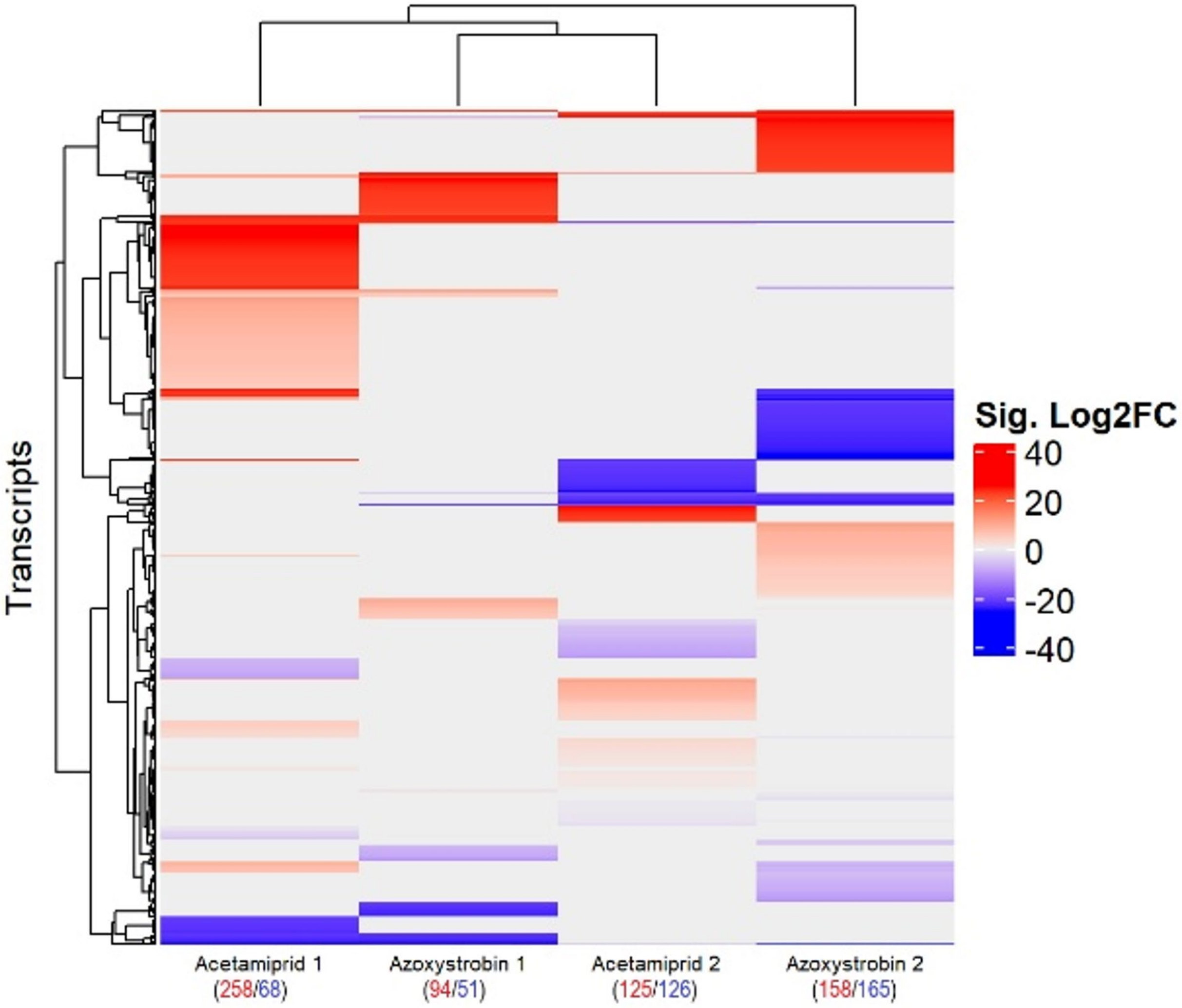

Fig. 2. Heatmap of the log2 Fold Change (Log2FC) of the significantly differentially expressed transcripts of gammarids after exposure to acetamiprid and azoxystrobin of two repeated experiments (1 and 2).

illustrated in Fig. 3 (Venn diagram), and the limited concordance in induction patterns is further visualized in the heatmap shown in Fig. 2.

For an overview, Principal component analysis (PCA) were performed on the data and clustering each experiment repetition separately showed deviations from the control for each pesticide exposure but no clear separations between conditions and the control (Fig. 4 and 5).

GO-Enrichment

Assessing metabolic influences after acetamiprid and azoxystrobin exposure, a higher number of GO-Terms suggested metabolic suppression rather than induction. In the case of acetamiprid 1, higher amino sugar metabolism was detected. However, a reduction in responses to simple sugars was also found, suggesting a potential shift in metabolic pathways. Additionally, reduced developmental processes related to cell formation and differentiation were identified in both acetamiprid 1 and 2 exposure. Conversely, in azoxystrobin, the first of the two experiments indicated increased cuticle development. The second acetamiprid experiment displayed upregulated inflammatory responses and regulation of viral symbiotic processes. Furthermore, a downregulation of responses to chemical stimuli, particularly known for paraquat, as well as reduced energy-coupled proton transport, were seen. Figure 6 and Fig. 7 show details of the regulated transcripts. An additional GO term analysis using a *p*-value threshold of 0.5, for an explorative view due to limited GO-annotation in the analysed transcriptome, revealed no overlapping GO terms between experiment 1 and experiment 2 following acetamiprid exposure. In contrast, three overlapping GO terms were identified under azoxystrobin exposure, two of which are related to mitochondrial metabolism and one to the myelin sheath (SI, Overlapping GO-Terms).

Discussion

Changes in gene expression

Using NGS, a transcriptional response of gammarids to sublethal concentrations of the pesticides azoxystrobin and acetamiprid was detected, with between 145 and 326 transcripts significantly differentially expressed in each

B: The amount of significantly expressed transcripts for each condition and the overlapping amount of significantly expressed transcripts between the different conditions within the same experiment repetition.

Fig. 3. (**A**) The amount of significantly expressed transcripts for each condition and the overlapping amount of significantly expressed transcripts between the same condition of an experiment repetition. (**B**) The amount of significantly expressed transcripts for each condition and the overlapping amount of significantly expressed transcripts between the different conditions within the same experiment repetition.

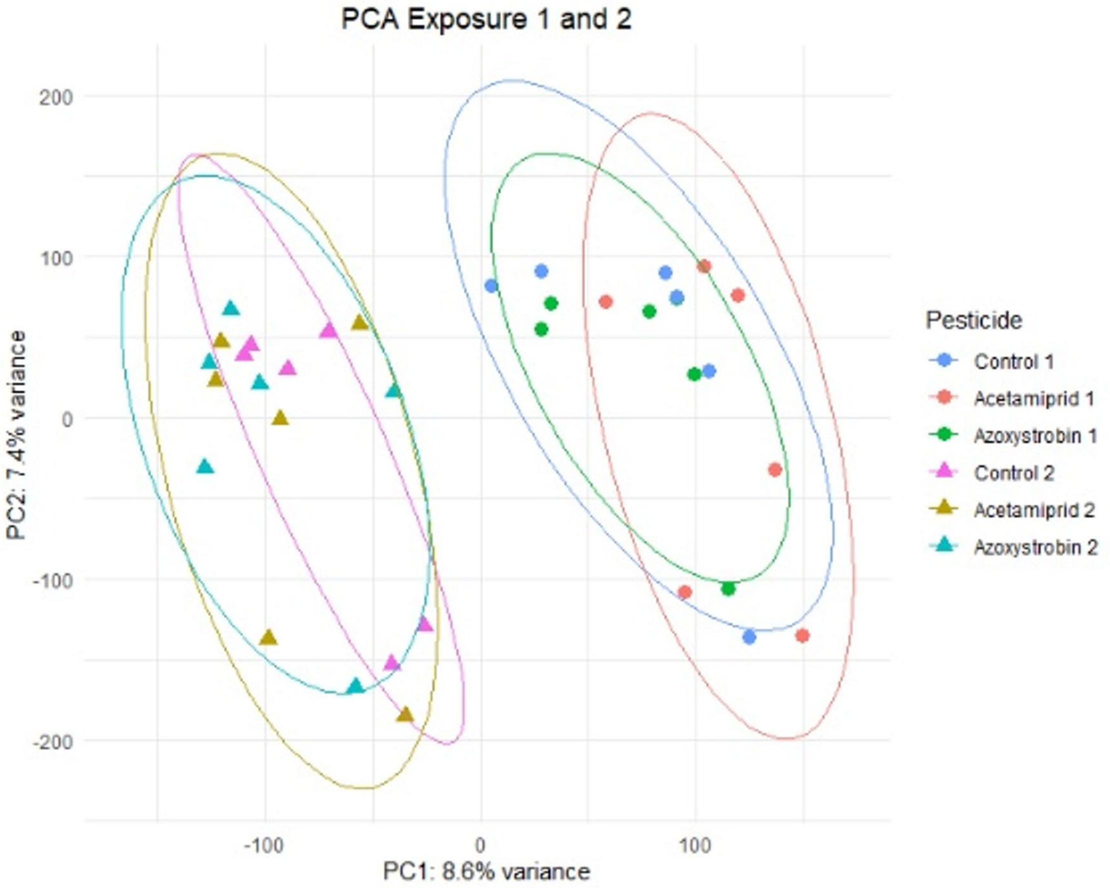

Fig. 4. Principal component analysis of the whole dataset where each symbol represents one sample of the respective condition, both experiment repetitions (1 and 2) are shown for each pesticide and control.

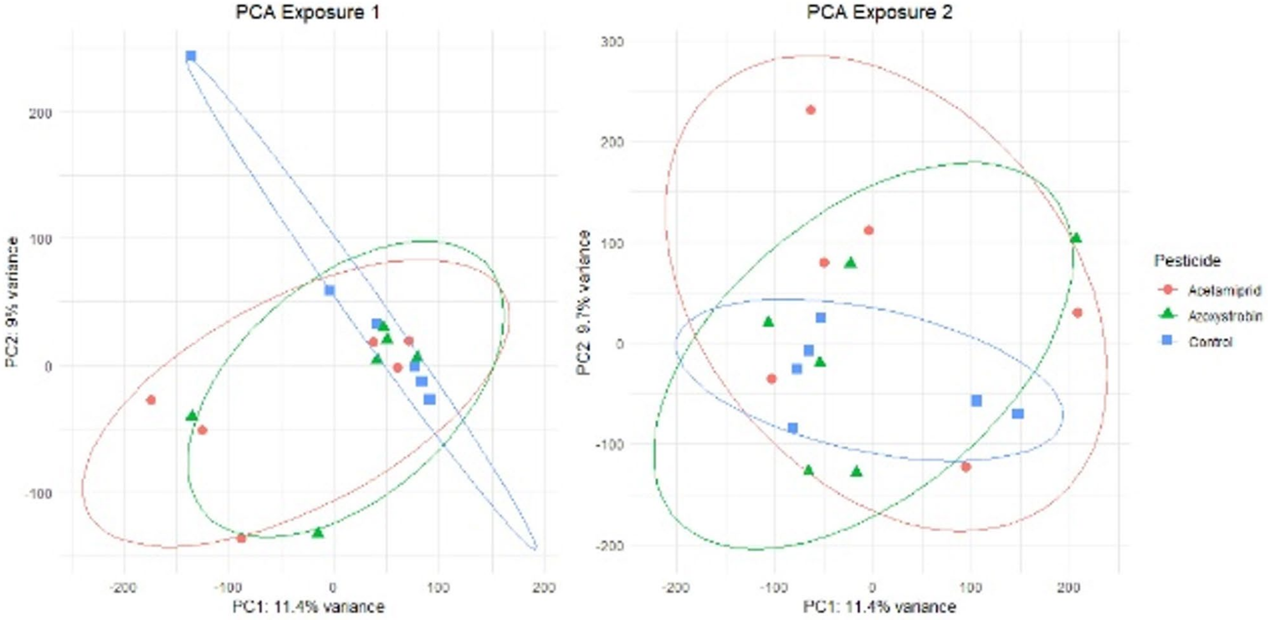

Fig. 5. Principal component analysis of each exposure experiment where each symbol represents one sample of the respective conditio.

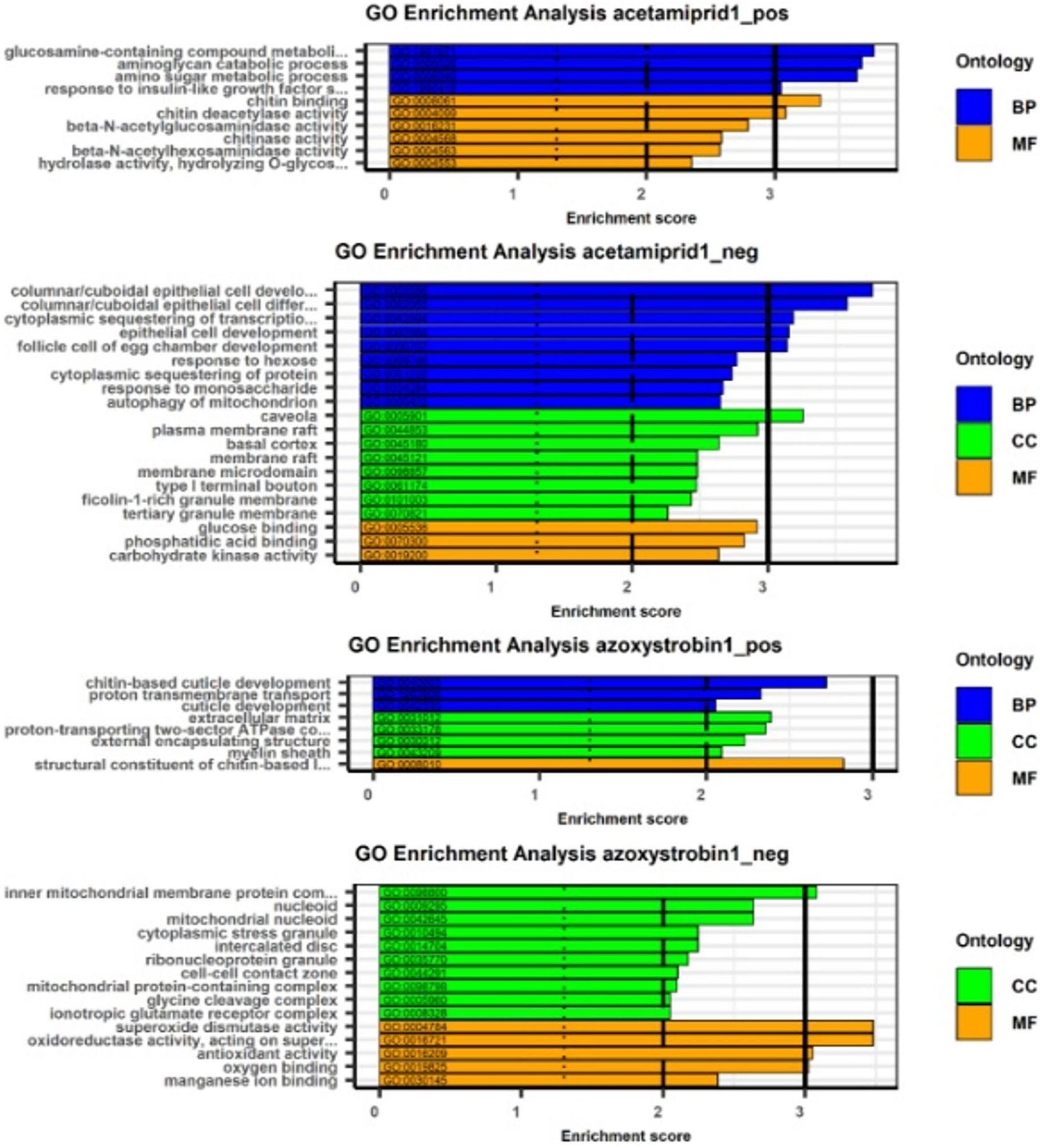

Fig. 6. The GO-Enrichment results of the first experiment. Only GO-Terms with a p-value lower than 0.01 from the enrichment analysis are displayed. Pos means positively enriched Terms, neg means negatively enriched Terms. BP = Biological processes, CC = Cellular component, MF = Molecular function Cutoff lines drawn at equivalents of $p = 0.05$, $p = 0.01$, $p = 0.001$. Both experiment repetitions (1 and 2) are shown for each pesticide.

experiment compared to the control. Transcriptional change after exposure is expected, as differential expression responses enable organisms to cope with stressors[55]. Transcriptional changes are also consistent with several sublethal behavioural responses associated with pesticide exposure and directly observable effects, namely feeding rate, locomotion, respiration, and amplexus formation[17,18]. This type of stress, manifested initially in differential gene expression, can be associated with adaptation to stressors such as pesticides. This adaptation comes at the expense of reduced fitness when having to cope with additional stressors such as heat, which have been found to act increasingly synergistically in adapted populations[56]. In the long term, adaptation under constant exposure to pesticides has also been associated with a reduction in population-level genetic diversity[57].

After stressor exposure both to model substances tested in this study and other contaminants, transcriptional responses are expected to occur in pathways connected with detoxification, growth suppression and change in

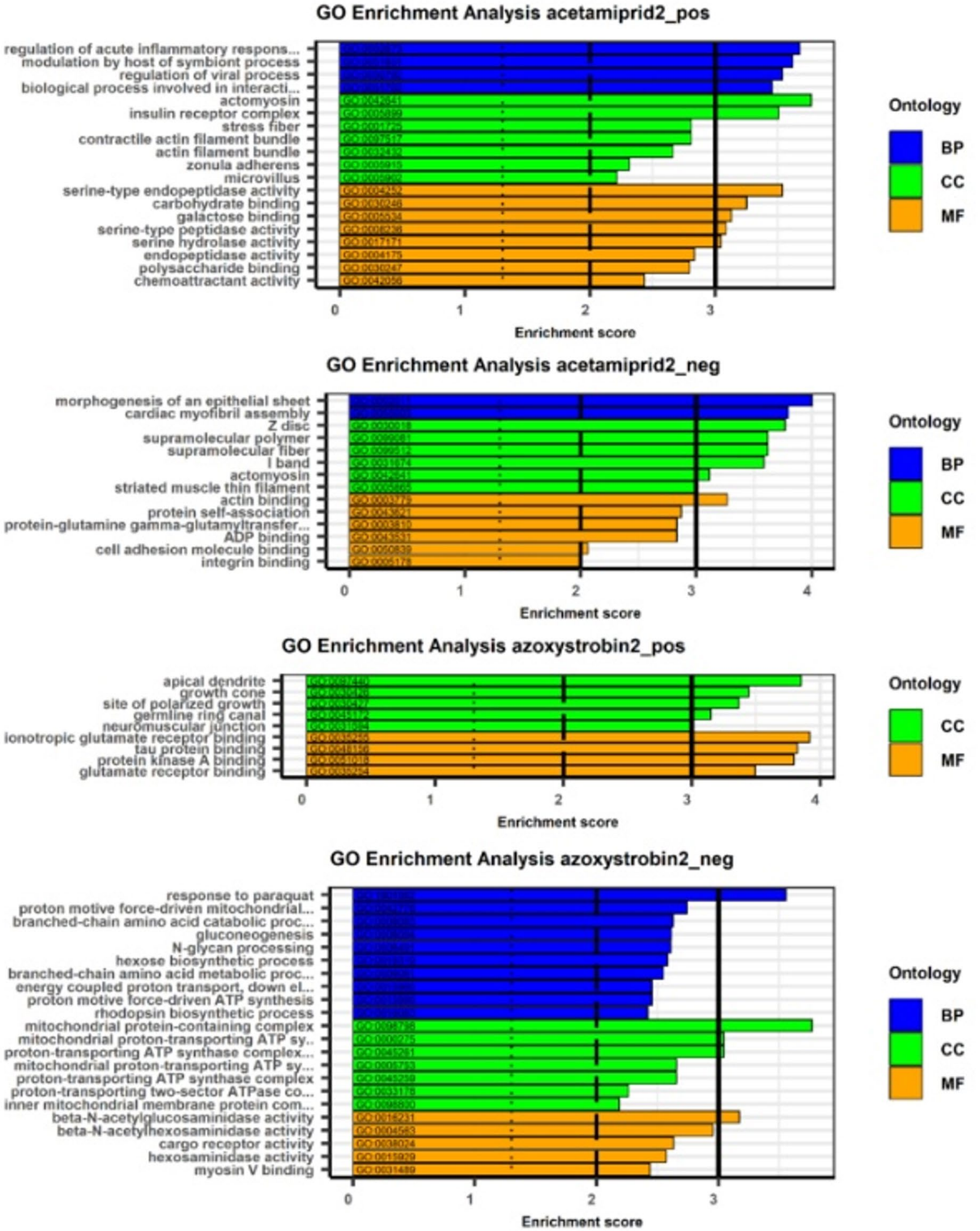

Fig. 7. The GO-Enrichment results of the second experiment. Only GO-Terms with a p-value lower than 0.01 from the enrichment analysis are displayed. Pos means positively enriched Terms, neg means negatively enriched Terms. BP = Biological processes, CC = Cellular components, MF = Molecular function. Cutoff lines drawn at equivalents of p = 0.05, p = 0.01, p = 0.001. Both experiment repetitions (1 and 2) are shown for each pesticide.

metabolism[58]. Identification of distinctively affected biological processes in this study proved to be difficult due to varying observations between experiment repetitions. Even when performing the GO term analysis with a *p*-value threshold of 0.5, there was little overlap between the two experiments. Only three GO terms were shared between experiment 1 and experiment 2 under azoxystrobin exposure, whereas no overlapping GO terms were observed for acetamiprid (SI2 Table GO-Terms overlapping).

Nevertheless, GO enrichment displayed metabolic suppression in some cases, consistent with experiments by Brasseur et al. Although they tested different stressors, metabolic suppression represented a shift in which resources are redirected from vital processes under stress[21]. In both acetamiprid experiments, processes connected to development, cell formation and differentiation were reduced, which are linked to endpoints commonly used as stress indicators[59] and can be interpreted as reallocation of resources to stress responses. Gene Ontology (GO) term analysis indicated that transcripts associated with muscle contraction exhibited altered expression following acetamiprid exposure in experiment 2. Such effects on muscle function and locomotor activity have been documented in experimental studies across different species, including reduced swimming velocity and thoracic limb activity in *Daphnia magna* as well as impaired movement in cockroaches[60].

Another indication is that acetamiprid exposure in one experiment led to initialization of inflammatory response processes, which is a direct link to a heightened stress response. No other commonalities were found between the experiments.

In azoxystrobin experiment 1, GO terms associated with mitochondrial function were affected, whereas in experiment 2, GO terms related to carbohydrate metabolism were altered. Both processes can influence energy production. Effects of azoxystrobin on the energy metabolism of gammarids have already been demonstrated in a study by Konschak et al., where chronic exposure to 15 and 30 mg/L azoxystrobin resulted in negative impacts on energy metabolism[61]. A study by Wüthrich et al. also reported significant effects on energy metabolism following azoxystrobin exposure. Specifically, reduced food intake and decreased activity of the electron transport chain were observed, both of which negatively impact energy metabolism in [62]. These findings are consistent with our observations, where GO terms related to mitochondrial function (experiment 1) and carbohydrate metabolism (experiment 2) were affected. Both processes play a central role in energy production, suggesting that azoxystrobin may interfere with metabolic pathways at multiple levels.

Principal component analysis (PCA) of individual samples (Fig. 4) did not reveal clearly distinct response patterns specific to each pesticide. However, replicates exposed to pesticides exhibited noticeable deviations from their respective controls. When both experimental repetitions were included in a combined PCA (Fig. 5), a separation between replicates from the two experiments became apparent. This indicates substantial biological variability among gammarids collected just two weeks apart from the same field site—a variability that was not mitigated by the laboratory acclimatization period or the subsequent sublethal pesticide exposure.

The different outcomes observed in the NGS experiment repetitions show the difficulty of providing general conclusions on specific reactions or the molecular mode of action in Gammarids when exposed to the selected pesticides at the chosen sublethal concentrations. Also they illustrate the challenges of working with wild populations collected in the field. Since the maintenance and breeding of gammarids in the laboratory still pose a significant challenge, it is necessary to work with specimens collected from the wild. Alther and colleagues were able to successfully keep gammarids for several months, and reproduction did occur. However, this required substantial effort, as factors such as food composition and quality, as well as aquarium conditions, have a major impact on the survival and well-being of gammarids[63]. Robust, standardized protocols for laboratory maintenance and breeding of gammarids have yet to be established.

Further investigation is needed to determine what caused the variability, as there are many factors at play. Like intra-population differences in the organisms, since a certain genetic diversity would be expected in wild populations. However, it is known that in gammarids, the genetic structure varies significantly between populations from different rivers, but not within a population from the same river[64]. On organism level different methylation patterns or the age of an individual, could cause general variability in gene expression[65]. While next-generation sequencing (NGS) inherently involves a degree of technical variability, such effects are expected to be minimal in this study, as all samples were processed and sequenced within the same experimental run. To further reduce biological variability, future studies may consider pooling multiple individuals per sample—even though a single organism provided sufficient RNA for analysis in the present case[66]. Given the observed biological variability, it is possible that NGS was not sensitive enough to reliably detect sublethal effects at the tested concentrations. Employing higher pesticide concentrations could potentially increase the number of significantly differentially expressed transcripts and reveal more distinct transcriptional patterns.

In addition to biological variability, differences between the two exposure experiments were evident (Fig. 5). One possible explanation for these discrepancies could be increased environmental contamination due to rainfall events. Between the first and second sampling time points of the gammarids, there was a period of heavier rainfall. This may have led to the leaching of pesticides from agricultural fields located further away from the sampling site into the groundwater, potentially contaminating the spring as well. The relationship between rainfall events and pesticide inputs into surface and groundwater has been described by Chow and colleagues[67] Such environmental influences may not have been fully mitigated by the seven-day acclimatization period in the laboratory. However, several studies have shown that pesticide or chemical residues in gammarids are eliminated within a few days, suggesting that a 7-day period in clean water is sufficient for depuration[10,68]. Nevertheless, the extent to which field-derived conditions persisted remains unclear, as baseline gene expression at the time of collection was not measured due to limited analytical capacity. A more detailed understanding of the effects of transferring organisms from the field to the laboratory, particularly with respect to the role of acclimatization, would help to better interpret the observed gene expression patterns. This also applies to potential differences resulting from the short time interval between the two experimental repetitions. Without baseline expression data for each collection, it is difficult to disentangle effects caused by treatment from those introduced by sampling variability or environmental fluctuations at the collection site.

Other limitations

As gammarids are a non-model organism, the limited annotation data exacerbates biological interpretation of the signals observed. Just 8.7% of the transcriptome's transcripts were annotated with GO-Terms, suggesting that only highly conserved regions were identified. Low abundance and more importantly species-specific genes/transcripts are likely lost, preventing deeper biological insights and understanding. However, these problems will diminish in coming years as annotation efforts and possibilities continue to grow.

Future of transcriptomics in risk assessment and biomarker identification.

The present study aimed to substantially expand knowledge on transcriptomic responses in gammarids and thereby contribute to the future integration of omics approaches into ecological risk assessment. In line with previous work, our findings indicate that transcriptomics, although highly informative, still faces limitations that currently preclude its use as a stand-alone regulatory tool. In particular, incomplete genomic annotation for many non-model species restricts functional interpretation and cross-study comparability[69,70]. Nevertheless, transcriptomic data provide valuable insights into sublethal effects, stress responses, and adverse outcome pathways, thereby supporting a more mechanistic understanding of toxicologi-cal processes. Strengthening the contribution of transcriptomics to risk assessment will require improved genomic resources as well as integrative strategies combining transcriptomics with proteomics and targeted qPCR validation.

Beyond effect-based applications, NGS approaches may gain additional relevance in ecological risk assessment through their potential to detect sequence-defined stressors in environmental samples. A prominent emerging example is RNA interference (RNAi)-based pesticides, which consist of double-stranded RNA molecules designed to silence essential genes in target pests. To overcome the inherent instability of naked dsRNA, these products are increasingly formulated with nanomaterials or other carrier systems that enhance environmental persistence, uptake efficiency, and biological activity[71]. Importantly, different classes of nanomaterials confer distinct application routes and exposure profiles. Polymeric carriers such as chitosan derivatives, star polycation polymers, and guanylated polymers rely primarily on electrostatic interactions with dsRNA, improving stability against nucleases and enabling oral delivery and foliar spraying. Guanylated polymers, in particular, introduce guanidinium functionalities that enhance membrane interaction and cellular translocation, thereby increasing delivery efficiency but potentially also prolonging bioavailability in non-target organisms. Lipid-based nanoparticles and cationic liposomes encapsulate dsRNA within amphiphilic structures and are mainly applied via foliar sprays or feeding-based exposure, where pH-responsive release mechanisms facilitate intracellular delivery following uptake. Peptide-based systems, including branched amphiphilic peptide capsules and cell-penetrating peptides, represent biologically inspired carriers that promote direct membrane crossing and intracellular delivery, although their environmental stability and large-scale applicability remain less well characterized. Carbon-based nanomaterials, such as carbon and graphene quantum dots, combine small particle size, surface tunability, and in some cases intrinsic antimicrobial activity, making them particularly effective for foliar application and surface-associated delivery. Inorganic carriers, notably layered double hydroxides and mesoporous silica nanoparticles, provide exceptional protection against UV radiation and wash-off, thereby extending dsRNA persistence on plant surfaces and increasing the likelihood of transport into adjacent environmental compartments, including surface waters. A comprehensive overview of the physicochemical properties, delivery mechanisms, and application routes of the different nanomaterial classes used for RNAi-based pesticide formulations has been provided in a recent review by Liu et al.[72].

As a consequence of these formulation strategies, RNAi-derived sequences may persist longer in aquatic environments and become bioavailable to non-target organisms[73]. In this context, NGS-based approaches could, in principle, be used to detect RNAi-related sequences directly in water samples or exposed organisms, complementing conventional exposure assessment methods that primarily focus on chemical analytics. Integrating such sequence-specific exposure information with transcriptomic effect profiling may enable a more comprehensive and mechanistically in-formed assessment of emerging pesticide classes, particularly where formulation-dependent differences in stability and bioavailability are expected. Ultimately, combining NGS-based detection of RNAi-derived molecules with transcriptomic biomarkers could support the development of formulation-aware risk assessment frameworks that better capture the complexity of nanoparticle-assisted pesticide exposure in aquatic ecosystems.

Conclusion

This study demonstrates the feasibility of applying next-generation sequencing (NGS) to pesticide exposure assessments in gammarids, while emphasizing the limitations associated with field-collected populations. Although transcriptomic responses to sublethal pesticide exposure were observed, high variability and limited annotation constrained the identification of consistent stress markers. The divergence in transcriptional profiles between temporally close replicates highlights the influence of genetic or environmental variability. Overall, the findings underscore the need for further research to define robust molecular endpoints and improve mechanistic understanding for future risk assessments using wild populations of gammarids. With improvements in experimental design and the use of more potent stressors, NGS has the potential to yield more pronounced and consistent transcriptional responses.

Data availability

The raw data of the NGS sequencing experiments and the assembled transcriptome are deposited in the European Nucleotide Archive under the project accession number PRJEB97523.

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Author contributions

D.Z. and V.C. did the experiment and wrote the main manuscript. B.K. assisted with the execution of the NGS analysis and the subsequent data analysis. T.H. did the chemical analysis. M.L. supported the writing of the manuscript and carried out proofreading.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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