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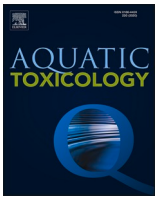

Toxicogenomic differentiation of functional responses to fipronil and imidacloprid in *Daphnia magna*

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ABSTRACT

Active substances of pesticides, biocides or pharmaceuticals can induce adverse side effects in the aquatic ecosystem, necessitating environmental hazard and risk assessment prior to substance registration. The freshwater crustacean *Daphnia magna* is a model organism for acute and chronic toxicity assessment representing aquatic invertebrates. However, standardized tests involving daphnia are restricted to the endpoints immobility and reproduction and thus provide only limited insights into the underlying modes-of-action. Here, we applied transcriptome profiling to a modified D. magna Acute Immobilization test to analyze and compare gene expression profiles induced by the GABA-gated chloride channel blocker fipronil and the nicotinic acetylcholine receptor (nAChR) agonist imidacloprid. Daphnids were expose to two low effect concentrations of each substance followed by RNA sequencing and functional classification of affected gene ontologies and pathways. For both insecticides, we observed a concentration-dependent increase in the number of differentially expressed genes, whose expression changes were highly significantly positively correlated when comparing both test concentrations. These gene expression fingerprints showed virtually no overlap between the test substances and they related well to previous data of diazepam and carbaryl, two substances targeting similar molecular key events. While, based on our results, fipronil predominantly interfered with molecular functions involved in ATPase-coupled transmembrane transport and transcription regulation, imidacloprid primarily affected oxidase and oxidoreductase activity. These findings provide evidence that systems biology approaches can be utilized to identify and differentiate modes-of-action of chemical stressors in *D. magna* as an invertebrate aquatic non-target organism. The mechanistic knowledge extracted from such data will in future contribute to the development of Adverse Outcome Pathways (AOPs) for read-across and prediction of population effects.

1. Introduction

Daphnia magna is used as a non-target aquatic invertebrate model species in guideline tests for ecotoxicological hazard and risk assessment of chemical compounds. These include active substances of plant protection products, biocides or pharmaceuticals, as well as industrial chemicals. The Organization for Economic Cooperation and Development (OECD) has developed two test guidelines using Daphnia as a test organism: the Daphnia sp. Acute Immobilization test (OECD TG 202) (OECD, 2004) and the D. magna reproduction test (OECD TG 211) (OECD, 2012). While the former test aims at the assessment of acute toxicity after 48 hours exposure using immobility as a lethal endpoint, the latter test is applied to identify chronic toxicity over a period of 21 days using reproduction and lethality as endpoints. Therefore, the readout of both guideline tests is limited in terms of mode-of-action classification of tests substances. This can be overcome by the

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application of novel systems biology approaches, which enable a detection of gene expression changes at the whole transcriptome level. Recently, different studies combined acute or chronic toxicity testing in *D. magna* with gene expression profiling at the RNA level, either using *D. magna* custom microarrays or next generation sequencing of RNA in order to assess the molecular modes-of-actions of a variety of stressors. Song et al. (2016) (Song et al., 2016) used microarray-based transcriptomics to gain mechanistic insights into acute toxicity of the insecticide emamectin benzoate in *D. magna* and Poynton et al. (2012) (Poynton et al., 2012) applied a similar approach for the toxicity assessment of silver nanomaterials. More recently, Fuertes et al. (2019) analyzed the molecular effects of chronic exposures of single and combined neuroactive drugs in *D. magna*, including the positive allosteric modulator of GABA-gated chloride channels diazepam. In 2016, the *D. magna* reference transcriptome became available, allowing a detection of gene expression changes via next generation sequencing of RNA (Orsini et al., 2016). In the same study, differential gene expression induced by the acetylcholinesterase inhibitor carbaryl was assessed in *D. magna*. More recent studies applied RNA sequencing in *D. magna* for identifying toxicogenomic responses to anticancer drugs (Russo et al., 2018) and microplastics (Coady et al., 2020). Taken together, these previous studies contributed to the successful establishment of transcriptomics as a tool for toxicogenomic studies of chemical and environmental stressors in *D. magna*, which provides the basis for characterizing modes-of-action, identifying biomarkers, and developing and refining AOPs.

In this study, we aimed at answering the questions whether and how the modes-of-action of two different nerve- and muscle targeting insecticides can be distinguished at the molecular level by integrating RNA sequencing in a modified version of the D. magna Acute Immobilization test. We selected the widely used insecticides fipronil and imidacloprid as model substances in our study. Fipronil is an inhibitor of gamma-aminobutyric acid (GABA)-gated chloride channels, which causes reduced neuronal influx and finally results in a hyperexcitation of the nervous system, paralysis and death (Gunasekara et al., 2007; Wang et al., 2016). A study by Narahashi et al. (2010) has also identified fipronil as a blocker of glutamate-activated chloride channels in insects. In a recent study, Fuertes et al. (2019) analyzed gene expression changes in *D. magna* induced by the pharmaceutical diazepam. Diazepam is a positive allosteric modulator of GABA-gated chloride channels, whose interaction with the receptor promotes GABA binding, which in turn increases the receptor's conductivity for chloride ions and thereby results in hypoexcitation (Campo-Soria et al., 2006; Costa et al., 1978). Therefore, diazepam acts in an opposing manner when compared to fipronil both at the molecular level and at the level of resulting neurotransmission.

Unlike fipronil, the neonicotinoid imidacloprid acts as a competitive modulator of the nicotinic acetylcholine receptor (nAChR) (Matsuda et al., 2001). Since imidacloprid is not degraded by acetylcholinesterases, it acts more potently than the natural ligand acetylcholine (Cox, 2001; Nagata et al., 1998). The imidacloprid-nAChR interaction results in a permanent opening of the nAChR channel pore, which induces a permanent ion flux and thus causes membrane depolarization. The resulting excitatory post-synaptic potential finally leads to paralysis of the insect, and consequently to death. A recent study by Orsini et al. (2016) analyzed transcriptome-wide gene expression changes in *D. magna* after exposure to the insecticide carbaryl. Carbaryl belongs to the chemical class of carbamates, acting as a slowly reversible inhibitor of acetylcholinesterase (Fukuto, 1990). Such an inhibition interferes with the naturally rapid degradation of acetylcholine, resulting in an accumulation of the neurotransmitter. As a consequence, nAChRs are more frequently and longer activated than under normal physiological settings, which results in an excitatory post-synaptic potential. Thus, despite acting via different primary molecular mechanisms, carbaryl and imidacloprid both trigger an excitatory signaling as a downstream effect.

Fipronil and imidacloprid both induce similar adverse effects in *D. magna*, which are triggered by different molecular initiating events (MIEs), making these insecticides ideal model substances for our experimental approach. Previously published effects of diazepam and carbaryl, two compounds that target similar key molecular events as fipronil and imidacloprid, should add further weight of evidence to the current study. The ability to differentiate MIEs via gene expression fingerprints in a *D. magna* short term test would allow the identification of the primary mode-of-action of unknown test substances during environmental hazard assessment.

2. Materials and methods

2.1. Test substances

Fipronil (CAS 120068-37-3, $\geq$ 95 % purity) and imidacloprid (CAS 138261-41-3, $\geq$ 98 % purity) were purchased from Merck KGgA (Darmstadt, Germany). Imidacloprid test and control solutions were prepared using copper-reduced tap water. For fipronil, a stock solution was prepared in acetone. The same final volume of acetone was added to all vials of test and control solutions. Acetone was evaporated by aeration, before test and control solutions were prepared using copper-reduced tap water. The pH of all test solutions was 8.1 $\pm$ 0.1. Further analytical parameters of the tap water are listed in Table S1.

Data used for comparison of effects of diazepam and carbaryl were obtained from previous studies, which analyzed gene expression effects induced in juvenile *D. magna* after a four days exposure to 100 ng/L diazepam (CAS 439-14-5) (Fuertes et al., 2019) or a two days exposure to 8 µg/L carbaryl (CAS 63-25-2) (Orsini et al., 2016).

2.2. Test organism

The test organisms were 4 – 24 hours old, juvenile *D. magna*. Cladocerans were obtained from the German Federal Environment Agency, Institut für Wasser-, Boden- und Lufthygiene (Dessau, Germany). Adult daphnia were fed daily with an algal suspension (Desmodesmus subspicatus) and ArtemioFluid (JBL GmbH & Co. KG). Prior to test start, at least 3 weeks old adult daphnia were separated from the stock population by sieving, and batches of 30 to 50 animals were held at room temperature in approximately 1.8 L copper-reduced tap water. At test start, newborn *D. magna* were separated by sieving, and individuals applied in the test were transferred with a wide bore Pasteur pipette a few hours after sieving to ensure that only healthy organisms were used.

2.3. Test design

For detecting substance-induced gene expression changes at low effect concentrations, an Acute Immobilization Test with *D. magna* was performed similar to the corresponding OECD guideline test (OECD, 2004). To ensure sufficient material for downstream transcriptome analysis, the main difference to the guideline test was the use of 50 daphnids per replicate and three replicates per condition. In order to identify two low effect concentrations (approximately the EC5 and the EC20), range finding exposure experiments were performed in this setting for both test substances, based on published literature and the ECHA registration dossiers. For fipronil, previously reported EC50 values ranged from 14.6 to 190.0 µg/L (Chevalier et al., 2015b; Hayasaka et al., 2012; Stark and Vargas, 2005). The maximal acceptable toxicant concentration (the geometric mean of LOEC and NOEC) was reported as 14 µg/L (McNamara 1990a, b). For imidacloprid, previously reported EC50 values ranged from 43 to 94 mg/L (Chevalier et al., 2015b; Sanchez-Bayo and Goka, 2006; Tisler et al., 2009). The ECHA registration dossier reported a 48 hours NOEC of 42 mg/L. Concentration-response curves were obtained from the range finding exposure experiments by plotting the percentage of immobilized daphnids after 48 hours exposure against the test substance concentration

(Fig. S1). Data analysis and calculation of effect concentrations were performed by probit analysis using a linear maximum likelihood regression model (ToxRat v.3.0.0 software; ToxRat Solutions GmbH, Alsdorf, Germany). For the detection of gene expression changes induced by low effect concentrations of each test substance, the identified EC5 (low exposure, LE) and the EC20 (high exposure, HE) (see Fig. S1) as well as a non-treated (imidacloprid)/solvent-treated (fipronil) control per test substance were analyzed in triplicate.

The test was conducted at 21.0 ± 0.3°C with a 16:8 h light/dark cycle. Prior to test start, 250 mL glass beakers were pre-saturated with each test solution. Then 50 juvenile daphnids per condition and replicate were added to each of the saturated glass beakers containing 150 mL of fresh, aerated test solutions. At test start, the oxygen saturation of all test solutions was 97 ± 1 % (8.25 ± 0.16 mg/L). Further water quality parameters are listed in Table S1. The oxygen saturation at test end was 91 ± 3 % (7.67 ± 0.23 mg/L). During the test, all glass beakers were loosely covered with a plexiglass plate to minimize evaporation. At 24 and 48 hours after test start, immobile daphnids were removed and counted as well as abnormal behavior and appearance were recorded.

For the detection of substance-induced gene expression changes at 48 hours after test start, the mobile daphnids of each test condition were transferred to 1.5 mL reaction tubes containing a mixture of sterile glass beads of 1 and 5 mm diameter. The supernatant was removed and the daphnids were euthanized immediately in liquid nitrogen. After the addition of 350µl lysis buffer (RNA/protein extraction kit, Macherey&Nagel), the daphnids were homogenized mechanically at 5 m/s for 10 s at room temperature using a FastPrep-24© homogenizer (MP Biomedicals, Irvine, USA). Total RNA was isolated using an RNA/protein extraction kit as recommended by the manufacturer (Macherey&Nagel). RNA was quantified using a Nanodrop system (Thermo Fisher Scientific, Waltham, USA) and quality was assessed with a 2100 Bioanalyzer system (Fig. S2) on the basis of the 28S/18S ratio (Agilent, Santa Clara, USA) prior to RNA-Seq library preparation.

2.4. Chemical analysis

Nominal test concentrations for fipronil were 16 µg/L and 32 µg/L, those for imidacloprid were 42 mg/L and 64 mg/L, corresponding to the EC5 and EC20 of each compound, respectively. The measured concentrations of both test substances in the aqueous solutions were determined by chemical analysis (Table 1). A detailed description of the analytical method is given in the supporting information section. Briefly, the aqueous samples were diluted with acetonitrile and were analyzed by high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). Both methods were successfully validated according to the EU guideline SANCO/3029/99 at a limit of quantification (LOQ) of 0.5 µg/L (fipronil) and 0.1 mg/L (imidacloprid), respectively (European Commission, 2000).

2.5. Transcriptomics

For transcriptome library preparation, RNA samples with a 28S/18S ratio > 2.0 were selected, assuring the analysis of high quality RNA. Poly (A)+ RNA was purified from total RNA and subjected to library preparation, using the TruSeq RNA Library Prep Kit v2 as recommended by the manufacturer (Illumina, San Diego, USA). Libraries were sequenced on an Illumina HiSeq 4000 System (Illumina, San Diego, USA) in 50 bp single read mode, producing approximately 30 million raw reads per sample. Adapter sequences were removed from demultiplexed fastq files by trimmomatic (Bolger et al., 2014), and sequence quality was assured via FastQC (Andrews, 2010). Sequences were aligned to the *D. magna* reference genome (daphmag2.4, GCA_001632505.1), containing a total of 27,350 genes, with STAR aligner v.2.5.2a (Dobin et al., 2013), resulting in alignment rates between 86.4% and 95.4%. Feature mapped reads were counted in feature-Counts v1.5.0-p1 (Liao et al., 2014). Mapped read tables were merged into a single count matrix for each substance and analyzed in R (R Core Team, 2019) via RStudio (Loraine et al., 2015). After removing genes with 0 counts in at least one of the analyzed samples, count normalization and differential expression analysis was performed in DESeq2 v1.26.0 (Love et al., 2014). This was based on three biological replicates per condition applying pairwise Wald's t-test with independent hypothesis weighting (IHW) (Ignatiadis et al., 2016). P-values were corrected after Benjamini-Hochberg. To measure the effect between the conditions while controlling replicate differences, a multifactor model design was used, including replicate as random factor to account for the variability among the independent biological replicates. Biological effect size cut off was determined for each treatment comparison as the 90 % quantile of the absolute non-shrunk $\log_2$-fold change values, focusing only on the top 10% with largest fold change values. Log_2-fold changes were then shrunk with apeglm (Zhu et al., 2019). The final set of differentially expressed genes (DEGs) was selected after statistical significance (padj < 0.05) and the individual effect size cut off. Raw and processed data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.uk/arrayexpress) (Athar et al., 2019) under accession numbers E-MTAB-9829 (fipronil) and E-MTAB-9830 (imidacloprid). Data quality assessment is shown in Figs. S3–S5. The DEG analysis script is publicly available under: https://github.com/hreinwal/DESeq2Analysis (Reinwald et al., 2021).

Genes of the compound-specific signatures (common subsets of DEGs by LE and HE for each test compound) were functionally annotated using BLASTX (Sayers et al., 2021). Briefly, coding sequences from Ensembl Biomart (Howe et al., 2021) were subjected to a BLASTX search against the non-redundant protein sequence database. If available, the hit among the characterized proteins with the lowest E-score was assigned to the respective gene. Assigned protein hits were functionally annotated manually via literature research.

To compare the results of this study with previous data obtained by Orsini et al. (2016), we identified DEGs induced by carbaryl in *D. magna* by applying an analysis pipeline similar to the above mentioned one to the carbaryl data of the NCBI BioProject with the accession number PRJNA284518. For comparative assessment of data obtained in a previous study after exposure of *D. magna* to diazepam (Fuertes et al., 2019), statistically differentially ($p \leq 0.05$) expressed genes after diazepam exposure were identified by the GEO2R (Barrett et al., 2013) on the diazepam-treated and control samples of the GEO dataset with the accession number GSE131587 (Fuertes et al., 2019). Agilent-066414 D. magna 180k v2 microarray probe IDs were converted to *Daphnia pulex* gene IDs via the corresponding JGI IDs obtained from the Agilent-066414 D. magna 180k v2 microarray annotation file (Campos et al., 2018)(GEO accession number GPL22721). *Daphnia pulex* gene IDs were then converted to the corresponding uniquely mapped *D. magna* (daphmag2.4) homologues with high orthology confidence using ENSEMBL Biomart (Kinsella et al., 2011).

2.6. Gene set enrichment analysis for gene ontology and functional classification

For gene set enrichment analysis (GSEA) on gene ontology (GO) and functional classification, the PANTHER classification system (Mi et al., 2013, 2019) was used. As this system supports *Daphnia pulex* gene IDs, but not *D. magna* gene IDs, the latter ones (daphmag2.4) were first converted to the corresponding *D. pulex* (V1.0) homologues using ENSEMBL Biomart (Kinsella et al., 2011). Only genes with a one-to-one mapping and high orthology confidence (orthology confidence score = 1) between *D. magna* and *D. pulex* were considered for GSEA. This gene set contained a total of 10,323 genes. To obtain comparable results between both substances, only the common set of transcripts detected in both exposure experiments (fipronil & imidacloprid) were used for the analysis. GSEA was performed via the statistical enrichment test of the PANTHER classification system (Mi et al., 2013, 2019), using *Daphnia pulex* gene IDs and the corresponding DESeq2-generated apeglm shrunk

log_2-fold change values as input. The three main GO types biological process (BP), molecular function (MF), and cellular component (CC) were analyzed separately. P-values were corrected for multiple testing following Benjamini−Hochberg and ontologies considered as significantly enriched for padj ≤ 0.01. Ontologies and conditions were clustered by Euclidean distance in R (R Core Team, 2019) via RStudio (Loraine et al., 2015). Functional classification of gene sets was performed on the basis of the corresponding converted *Daphnia pulex* gene IDs, using the PANTHER classification system (Mi et al., 2013, 2019).

3. Results

3.1. Selection of low effect concentrations of fipronil and imidacloprid in D. magna

For fipronil, we obtained an EC50 of 70 μg/L, an EC20 of 32 μg/L and an EC5 of 16 μg/L (Fig. S1). Based on these effect concentrations, we selected nominal exposure concentrations of 32 μg/L and 16 μg/L as high (HE) and low exposure concentrations (LE) for gene expression profiling (measured concentrations of 25.7 μg/L (HE) and 11.4 μg/L (LE), respectively) (Table 1). For imidacloprid, we obtained an EC50 of 97 mg/L, an EC20 of 64 mg/L and an EC5 of 42 mg/L (Fig. S1). Therefore, we selected nominal exposure concentrations of 64 mg/L as HE and 42 mg/L as LE for gene expression profiling using imidacloprid (measured concentrations of 63.9 mg/L and 42.5 mg/L, respectively) (Table 1).

To validate the choice of low effect concentrations for both substances, we recorded immobility after 48 hours exposure for each test condition (Table 2). While the low exposure concentration in both cases did not induce immobility (one-way ANOVA) relative to the corresponding control condition, the high exposure concentration caused significant immobility by 15 and 19 percent in the case of fipronil and imidacloprid, respectively.

3.2. Transcriptome response to fipronil

Exposure to the LE of fipronil resulted in a total of 83 differentially expressed genes (DEGs, padj ≤ 0.05), of which 8 genes were up- and 75 genes were down-regulated, while the exposure to the HE caused 875 DEGs, of which 74 genes were up- and 801 genes were down-regulated (Fig. 1A). Seventy-nine genes (95% of DEGs after exposure to the LE) were statistically significantly dysregulated under both exposure conditions (Fig. 1B). Without exception, these genes were simultaneously dysregulated by exposure to the LE and HE, showing a strong positive correlation (R = 0.85) when comparing the log_2-fold change values for both conditions (Fig. 1C). For these reasons, we defined this common DEG subset of LE and HE as fipronil-specific gene expression signature in *D. magna*. Using BLASTX sequence homology searches for the coding sequences, 61 (77%) of these genes could be linked to known proteins (Table S2). Twenty of these (33%) were related to the cuticle and to proteins involved in chitin synthesis and metabolism, such as cuticular proteins (Marcu and Locke, 1998), endochitinase (Koga et al., 1983) or keratin-associated proteins. Eight proteins (13%) were linked to organelle and cellular architecture, including spidroin, extensin, cell wall components and the coiled-coil domain containing protein 9. Another larger proportion of 7 proteins (11%) was related to lipid metabolism, such as the SEC14 protein 2, phospholipase D1 or carboxylesterase 3. Smaller protein sets were involved in functions such as digestion, immune defense, RNA processing, gene expression regulation, acid-based regulation, movement, transport, and stress response. We identified 4-aminobutyrate aminotransferase, an enzyme involved in GABA catabolism, as a significantly down-regulated member of the annotated DEGs of the fipronil-specific signature (Madsen et al., 2008).

The remaining genes, affected in a statistically significant manner by only one of the exposure conditions, showed a high degree of positive correlation (R = 0.80), even though not statistically significantly regulated in the respective other condition. Globally, we observed a concentration-dependent regulation of DEGs, showing generally higher log_2-fold change values after exposure to the HE than after exposure to the LE (Fig. 1C). To identify biological processes affected by fipronil, we performed functional classification analyses with all fipronil-responsive genes. For this, we used only unique genes from both treatment groups. While 20.5% of the genes were assigned to general cellular processes, almost equal proportions ranging from 7.5 to 14.2% were assigned to multi-organism processes, biological regulation, response to stimulus, signaling, developmental processes, multicellular organismal processes, metabolic processes, and immune system processes (Fig. 1D). Only 1 and 2.4% of the genes were involved in cellular component organization or biogenesis and localization, respectively.

3.3. Transcriptome response to imidacloprid

In the case of imidacloprid, exposure to the LE resulted in a total of 35 DEGs, of which 32 genes were up- and 3 genes were down-regulated (Fig. 2A). Ninetyfour DEGs were observed after exposure to the HE, of which 66 genes were up- and 28 genes were down-regulated. Nineteen DEGs of the LE condition (54%) were also significantly differentially expressed after exposure to the HE (Fig. 2B). As it was the case for fipronil, the common subset of DEGs induced by the LE and the HE of imidacloprid – the imidacloprid-specific signature – was highly positively correlated (R = 0.96) (Fig. 2C). Three genes of this signature coded for known or putative proteins of the class of aminopeptidase N. BLASTX sequence homology searches revealed linkage to known proteins for 13 genes (68%) of the imidacloprid-specific signature (Table S3). Including the aminopeptidase N-related proteins, the largest set of five of these proteins was involved in digestive functions, while the smaller protein sets had functions involved in movement and immune defense. Imidacloprid exposure led to a significant up-regulation of neuropeptide-like protein 31, which belongs to a class of proteins involved in neuronal plasticity and immune regulation, among other functions (De Fruyt et al., 2020; Urbanski and Rosinski, 2018).

The remaining genes, representing DEGs in only one of the exposure conditions, were also highly positively correlated relative to the corresponding other condition (R = 0.88). For imidacloprid, the concentration-dependence of the log_2-fold change values was less pronounced than for fipronil. Reflecting the generally lower number of DEGs in comparison to fipronil, the imidacloprid target genes represented a lower number of biological processes (Fig. 2D). While the largest proportion of 28.6% of these genes was assigned to general cellular processes, almost equal fractions of 14.3 to 17.9% were involved in response to stimulus, signaling, metabolic processes, and biological regulation. Only 7.1% of the imidacloprid targets were assigned to processes associated with localization.

3.4. Differentiation of molecular changes induced by fipronil and imidacloprid

For assessing specificity at the gene expression level, we compared the identified gene expression signatures of each treatment for both test substances (Fig. 3A).

While the common subsets of LE and HE of each single substance were highly significant, there was no common subset of the LE treatments of both substances. As a consequence, the compound-specific signatures were unique. A minor set of 6 genes was located in the common subset of both HE treatments, of which 3 genes were also significantly affected in the LE treatment of imidacloprid. A set of 3 genes was also commonly regulated when comparing the HE treatment of fipronil and the LE treatment of imidacloprid. Of these 9 genes in the common subset of both substance exposures, 4 genes were regulated in a similar direction and 5 genes were regulated in an opposing direction when comparing fipronil and imidacloprid (Fig. S6). Seven genes (78%) of this intersection could be linked to known proteins using BLASTX

sequence homology searches (Table S4). Dehydogenase/reductase SDR family member 4 (dhrs4) was down-regulated under the HE condition of fipronil and up-regulated under the comparable condition of imidacloprid, whereas decaprenyl diphosphate synthase subunit 2 (pdss2) was down-regulated and C1q and tumor necrosis factor-related protein 3-like protein (ctrp3) was up-regulated in both cases. The transcription regulatory protein LGE1-like and the keratin-associated protein 19-2-like were both down-regulated under the HE condition of fipronil and up-regulated under the LE condition of imidacloprid. A cysteine-rich protein and mucin-2-like isoform X1 were up-regulated by both compounds.

Nevertheless, the vast majority of fipronil and imidacloprid target genes was either affected by the corresponding substance only, or the direction of regulation allowed for a discrimination between both substances. The compound-specific gene expression signatures, containing the intersecting DEGs of each LE and HE condition (79 genes for fipronil and 19 genes for imidacloprid), separated well between both substances (Fig. 3B).

Given the high degree of specificity of each gene expression signature, we further aimed at a functional classification of the observed responses. We performed a gene set enrichment (GSE) analysis of each treatment condition and each test substance for the gene ontology types, biological process (BP), molecular function (MF), cellular component (CC), as well as for PANTHER pathways.

As the organism *D. magna* is not supported in the PANTHER classification system (Mi et al., 2013, 2019), analysis was performed based on the converted uniquely mapping *D. pulex* gene orthologs. For all gene ontologies and for PANTHER pathways, we observed a significant agreement between up- and down-regulation when comparing LE and HE exposure for both test substances (Figs. 4 and S7). As for the gene expression signatures, the ontologies MF and BP and the PANTHER pathways allowed for a clear differentiation between the test substances (Figs. 4A and B and S7A), while the ontology CC did not discriminate between fipronil and imidacloprid, suggesting that both substances affect similar cellular components (Fig. S7B).

A prominent proportion of molecular functions affected by fipronil exposure was associated with DNA binding and transcription regulation (Fig. 4A). A second major fraction of fipronil-responsive molecular functions was involved in ATPase-coupled transmembrane transport of protons (Fig. 4A). In terms of pathway regulation, fipronil exposure affected a number of metabolic routes including ATP synthesis and cholesterol biosynthesis (Fig. 4B). Furthermore, fipronil exposure, significantly impaired metabotropic glutamate receptor signaling and a pathway associated with the Alzheimers disease-presenilin pathway in mammals (Fig. 4B).

In contrast, imidacloprid mainly impaired oxidase and oxidoreductase molecular functions as well as CoA ligase activity (Fig. 4A). Imidacloprid exposure also affected a number of metabolic pathways, which differed from those impaired by fipronil exposure (Fig. 4B). Among the most significantly repressed pathways were those associated with the Parkinson disease pathway and vasopressin synthesis in mammals. Furthermore, imidacloprid exposure also impaired GABA synthesis and aminobutyrate degradation (Fig. 4B).

3.5. Assessment of mode-of-action specificity using previously published results

For comparative gene expression analysis of fipronil and diazepam in *D. magna*, we identified DEGs ($p \leq 0.05$) after diazepam exposure by analyzing previous data obtained by Fuertes et al. (2019). Comparing these diazepam target genes with the target genes of the exposure to the HE of fipronil (padj ≤ 0.05), we observed a total of 20 assignable genes in the common subset of both substances (Fig. S8). Plotting their expression changes after exposure to each substance in a scatter plot reveals a negative correlation (QCR = -0.50) (Fig. S8).

For comparing imidacloprid- and carbaryl-induced gene expression changes, we identified DEGs (padj ≤ 0.05) after carbaryl exposure from data obtained in the study by Orsini et al. (2016), and compared them to DEGs observed after exposure to the HE of imidacloprid. Out of 58 carbaryl target genes, a highly significant (hypergeometric distribution p-value = 2.78E-8) proportion of 9 genes was also statistically significantly regulated by imidacloprid. Plotting their expression changes after exposure to each substance in a scatter plot revealed a highly positive correlation (QCR = 1.00) (Fig. S9).

4. Discussion

Our study aimed to determine whether an extension of the Acute Immobilization test with *D. magna* to include transcriptome analyses after exposure to low effect concentrations would allow characterization and differentiation of modes-of-action at the molecular level. Effect concentrations in our study were within the range of the ECHA registration dossier (EC50 = 85 mg/L) and previous literature (EC50 = 94 mg/L) (Chevalier et al., 2015a), especially for imidacloprid. In the case of fipronil, the EC50 value we determined was also in the range of some previous studies (88.3 µg/L) (Hayasaka et al., 2012), but on average higher than previously determined values (Chevalier et al., 2015a). This discrepancy may be related to the nominally determined effect concentrations in our study, since the measured fipronil concentrations in the main test were only about 70-80% of the nominal concentrations, while for imidacloprid measured and nominal concentrations matched well.

A first indication of differentiability of fipronil and imidacloprid with our approach was provided by the direction of regulation of the respective target genes. While in the case of fipronil the majority of genes were down-regulated, imidacloprid preferentially caused activation of its target genes, which could be explained by the downstream signaling of the respective receptors involved. Whereas fipronil blocks the GABA receptor, causing a prevention of GABA-induced downstream processes, imidacloprid permanently activates the nAChR and thus specifically triggers downstream signaling pathways.

Our results suggest that fipronil can induce early effects with potential consequences for molting, supporting previous studies, which reported fipronil-dependent effects on molting in the juvenile brown shrimp *Farfantepenaeus aztecus* (Al-Badran et al., 2018). Carbonic anhydrase 1 and 14, which play important neurophysiological roles in pH and ion regulation (Emameh et al., 2014), were dysregulated by fipronil exposure, representing a possible response to blocked chloride ion currents. Fipronil-induced changes in carboxylesterase 3 expression may represent a defense mechanism, as this enzyme has been previously shown to play a role in pesticide detoxification (Wheelock et al., 2005). A larger proportion of DEGs regulated by fipronil could be linked to lipid metabolism. A study by Enell et al. (2010) (Enell et al., 2010) has shown that GABA can inhibit insulin signaling in its regulation of metabolism, stress and life span in the brain of *Drosophila melanogaster*. This provides a possible linkage between GABA receptor blocking and lipid metabolic processes, as insulin was shown previously to play an important role in lipid metabolism (Saltiel and Kahn 2001). The observed downregulation of 4-aminobutyrate aminotransferase, an enzyme involved in the degradation of GABA, may represent a feedback mechanism designed to counteract the low activity of the blocked GABA receptors (Cocco et al., 2017; Zheng et al., 2021).

On the other hand, imidacloprid dysregulated the expression of aminopeptidase N and related proteins, which play roles in dietary protein digestion and as receptors during Cry toxin-induced pathogenesis (Ningshen et al., 2013). While the relationship between nAChR activation and aminopeptidase N expression still remains elusive, the observed imidacloprid-induced upregulation of neuropeptide-like protein 31 may represent a response to permanent activation of nAChRs, as certain neuropeptides have been shown to reduce acetylcholine release in humans (Herring et al., 2008; Wiley et al., 1990). Though some neuropeptides have also been identified and characterized in

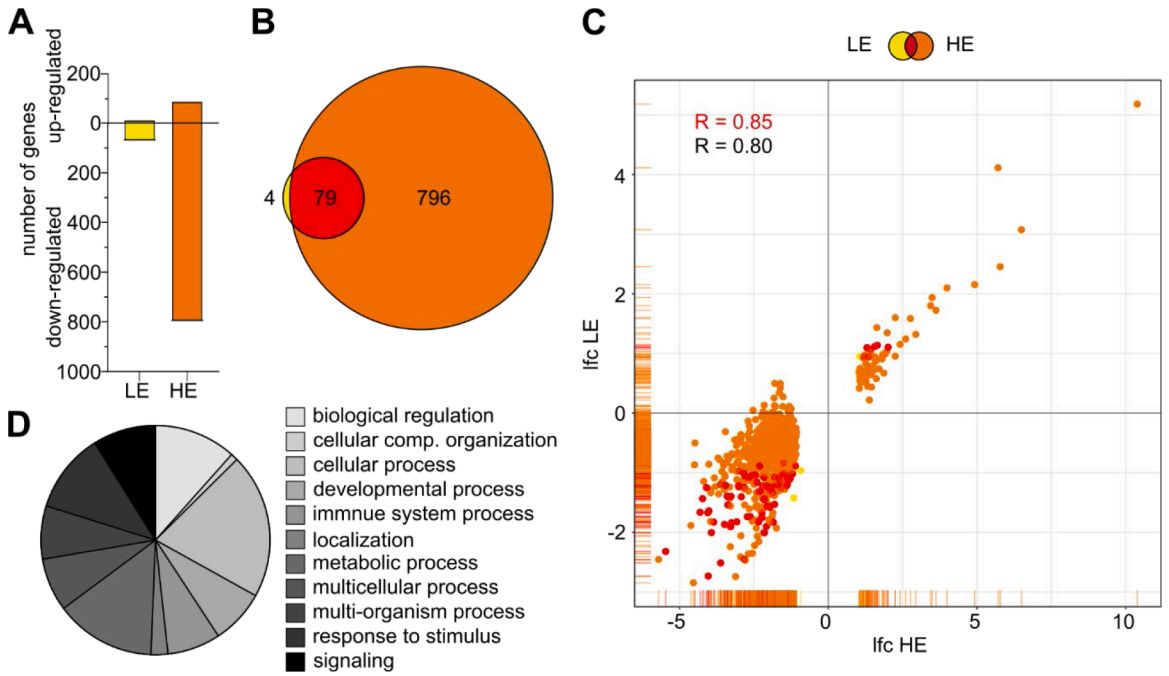

Fig. 1. Transcriptome response of *D. magna* to exposure to the LE and HE of fipronil after 48 hours. (A) Gene numbers of statistically significant (padj $\leq$ 0.05) up- and down-regulated genes after exposure to 11.4 µg/L (LE, yellow) and 25.7 µg/L (HE, orange). (B) Venn diagram showing differentially expressed genes (DEGs) after exposure to 11.4 µg/L (yellow) and 25.7 µg/L (orange) fipronil as compared to the non-treated controls. Displayed circles and the intersection size correspond to the numbers of DEGs. (C) Scatter plot comparing $\log_2$-fold change (lfc) values of DEGs observed after low and high exposure to fipronil. The common subset of both exposures is colored in red and considered as substance-specific signatures. (D) Circle plot showing a functional classification analysis of all fipronil-induced DEGs. Biological processes associated with these genes are indicated.

invertebrates (Xu et al., 2016), their physiological roles in *D. magna* require further research. A member of the imidacloprid target family of dhrs proteins is also a target of the ecdysone response in honeybee caste development (Guidugli et al., 2004), providing a linkage to impaired molting.

Among the few gene targets of both test substances, pdss2 was down- and ctrp3 was up-regulated in both cases. For defective mutations of pdss2, Parkinson's-like neuromuscular defects were observed in mice in

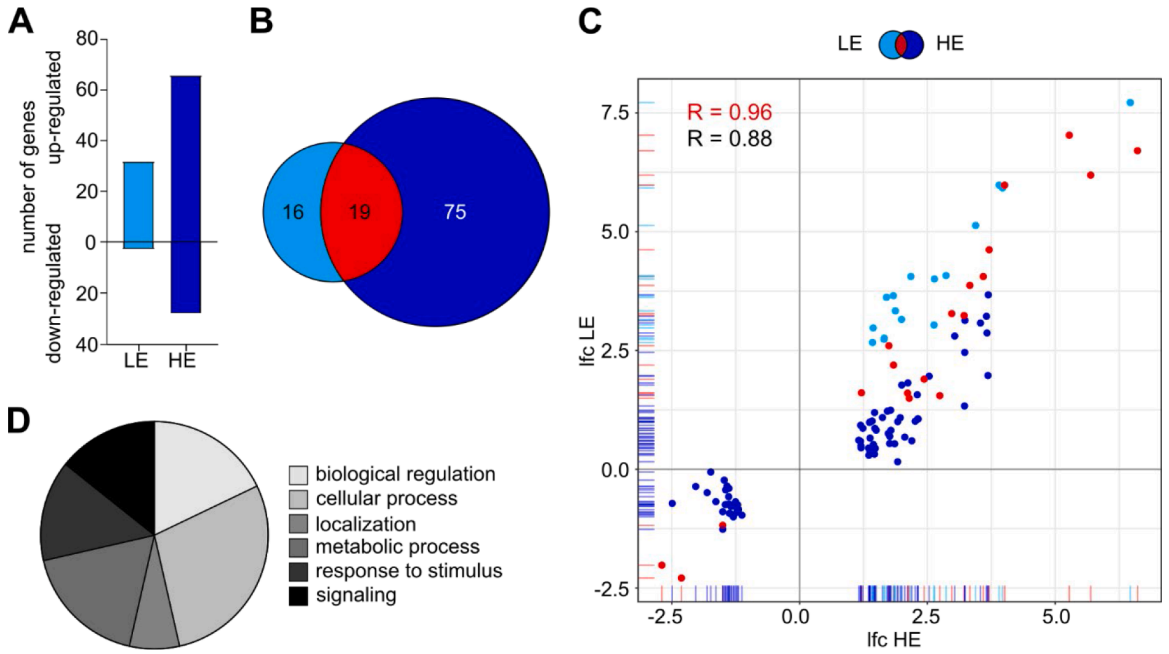

Fig. 2. Transcriptome response of *D. magna* to exposure to the LE and HE of imidacloprid after 48 hours. (A) Gene numbers of statistically significant (padj $\leq$ 0.05) up- and down-regulated genes after exposure to 42.5 mg/L (LE, light blue) and 63.9 mg/L (HE, dark blue). (B) Venn diagram showing differentially expressed genes (DEGs) after exposure to 42.5 mg/L (light blue) and 63.9 mg/L (dark blue) imidacloprid as compared to the non-treated controls. Displayed circles and the intersection size correspond to the numbers of DEGs. (C) Scatter plot comparing $\log_2$-fold change (lfc) values of DEGs observed after low and high exposure to imidacloprid. The common subset of both exposures is colored in red and considered as substance-specific signatures. (D) Circle plot showing a functional classification analysis of all imidacloprid-induced DEGs. Biological processes associated with these genes are indicated.

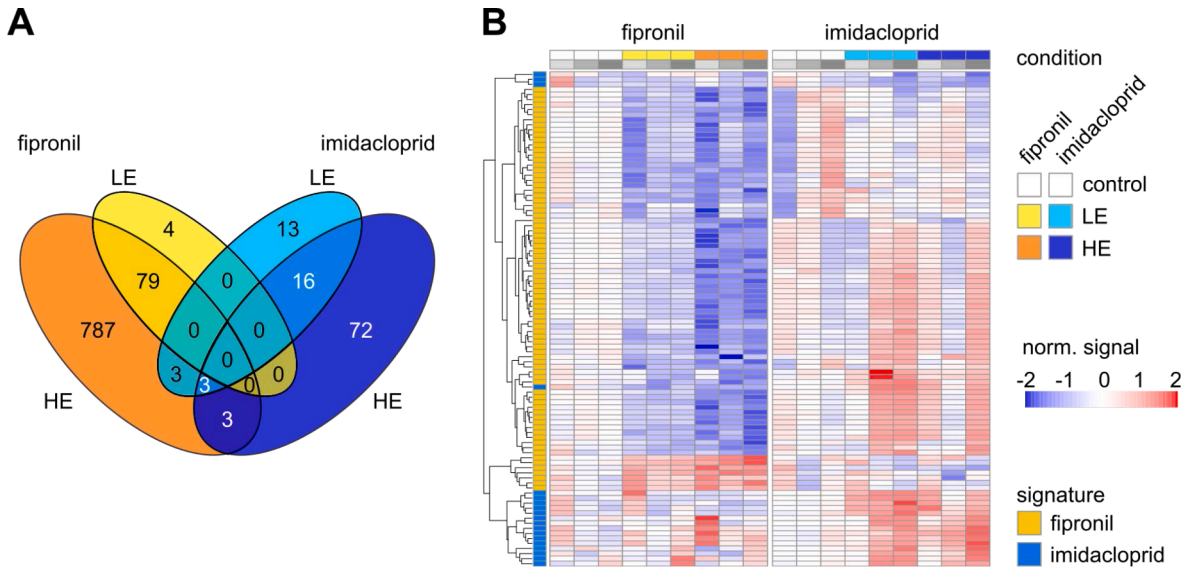

Fig. 3. Comparative functional genomics of fipronil and imidacloprid in *D. magna*. (A) Venn diagram of DEGs in the low (LE, yellow) and high exposure condition (HE, orange) for fipronil and the low (LE, light blue) and high exposure condition (HE, dark blue) for imidacloprid. Numbers indicate the DEG count for the corresponding common subset. (B) Heatmap containing the signature genes of each test substance, which are indicated in red in Figure 1 and Figure 2. The relative signal normalized to the control within each treatment of each gene (rows) is shown for each sample (columns). As compared to the mean of each non-treated control, an enhanced expression is indicated in red and a suppressed expression is indicated in blue. Genes are clustered after maximum distance measure with average linkage. The association with a fipronil or an imidacloprid signature is indicated in yellow and light blue, respectively.

a previous study (Ziegler et al., 2012), while ctrp3 has been previously associated with neuromuscular disorders in mice (Rehorst et al., 2019), directly linking both gene products to proper neuronal function in mammals. Dhrs4, which was down-regulated by fipronil and up-regulated by imidacloprid, is involved in retinoic acid synthesis in mammals (Endo et al., 2009). In line with these findings, neonicotinoid treatment has been previously shown to interfere with the retinoid system in bees (Gauthier et al., 2018), while Sarti et al. (2013) have shown that retinoic acid reduces GABA signaling in mammals by inducing receptor internalization (Sarti et al., 2013). In the case of fipronil, suppression of dhrs4 could lead to decreased retinoic acid synthesis, which could provide a feedback mechanism for GABA receptor blocking.

Taken together, at the gene level, the observed signatures for fipronil and imidacloprid separate well between both substances without major overlaps, and therefore, may be utilized in future ecotoxicogenomic studies to discriminate the corresponding modes-of-action in *D. magna*.

At the functional level, an interconnection of ligand-gated chloride channel activity with transcription regulatory pathways, such as the Wnt signaling pathway, was shown in previous studies in humans (Rapeti-Mauss et al., 2020). Therefore, fipronil-induced changes in transcription regulation may result from signaling cascades downstream of GABA-gated chloride channel activity, such as Wnt signaling, which is common in invertebrates and vertebrates (Holstein, 2012). We observed fipronil-induced effects in ATPase-coupled transmembrane transport of protons, which supports the results of a previous study, in which fipronil was shown to reduce mitochondrial activity by interfering with bioenergetic functioning in honeybees (Nicodemo et al., 2014). While effects on ATP synthesis are in line with the above mentioned interference with mitochondrial activity, fipronil-induced effects on cholesterol and saturated fatty acid synthesis have also been observed in a previous study in zebrafish embryos (Yan et al., 2016). Besides its antagonistic activity on GABA-gated chloride channels, fipronil has also been shown to sensitively block glutamate-activated chloride channels in insects (Narahashi et al., 2010), providing a link to glutamate signaling. GABA signaling has been previously discussed as a drug target for treatment of Alzheimer's disease in mammals (Solas et al., 2015), suggesting conserved mechanisms within the Alzheimer's disease pathway to be targeted by fipronil in *D. magna*.

Imidacloprid predominantly impaired oxidase and oxidoreductase function, which is in line with previous observations of oxidative stress induction in *D. magna* (Jemec et al. 2007; Qi et al., 2018). Acetyl-CoA ligases, whose expression was impaired by imidacloprid exposure in our study, are known to be involved in fatty acid synthesis (Wakil et al., 1983), and thus, our findings may explain chronic effects of imidacloprid on fatty acid synthesis, which were previously observed in bumblebees (Erban et al., 2019). Here, imidacloprid exposure also significantly impaired the expression of genes involved in the Parkinson's disease pathway and in vasopressin synthesis in mammals. In line with these observations, drugs targeting nAChRs have been previously suggested as a therapeutic treatment of Parkinson's disease (Quik and Wonnacott, 2011). Furthermore, an activation of vasopressin synthesis has been observed after activation of central nicotinic cholinergic receptors in humans (Cavun et al., 2004), which (once again) suggests common mechanisms in invertebrates. A study by Taylor-Wells et al. (2015) (Taylor-Wells et al., 2015) has shown antagonistic effects of imidacloprid on the fipronil-insensitive GABA receptor mutant Rdl in insects. Therefore, the observed effects on GABA synthesis and aminobutyrate degradation may possibly result from feedback mechanisms (Fig. 1 and Table 1).

Comparison of our data for fipronil with those previously recorded for the positive allosteric modulator of GABA-gated chloride channels diazepam (Fuertes et al., 2019) showed a negative correlation in expression changes between common target genes of both compounds, consistent with their opposing modes-of-action. A similar observation was made when comparing our data for imidacloprid with those previously published for the acetylcholinesterase inhibitor carbaryl (Orsini et al., 2016). This consistency, especially considering the different gene expression platforms and experimental settings used to obtain these results, underscores both the validity of our approach as well as of our results. In particular, these cross-platform and cross-substance consistent gene expression fingerprints likely contain genes that respond directly downstream of GABA-gated chloride channel blocking/activation (fipronil/diazepam) or activated/suppressed acetylcholine signaling (imidacloprid/carbaryl) and can be linked to the corresponding physiological changes. Thus, these are promising

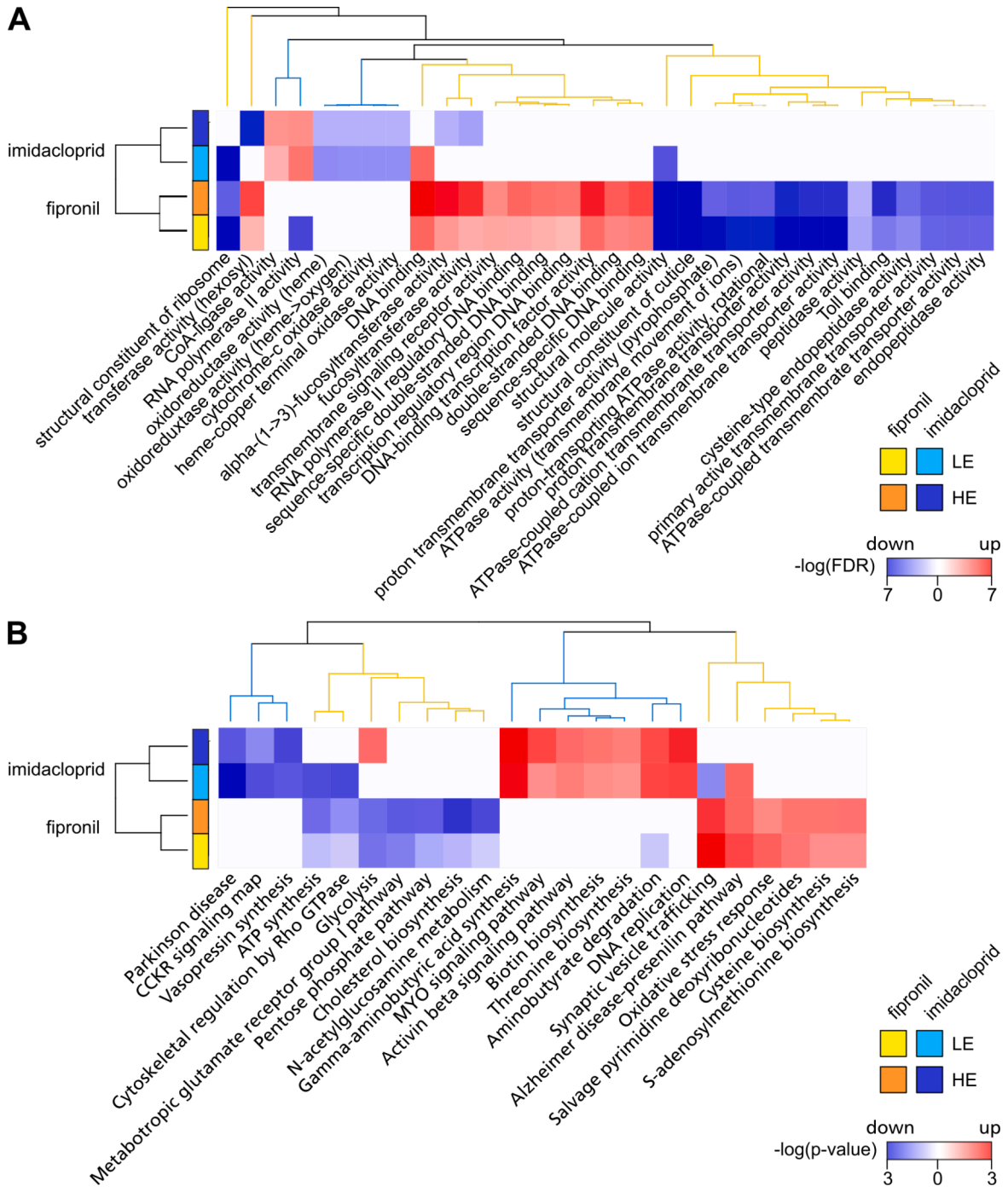

Fig. 4. GSE analysis after exposure to fipronil and imidacloprid based on the log_2-fold change values. (A) Heatmap of gene ontologies (molecular function) statistically significantly (FDR $\leq$ 0.01) enriched in the low and high exposure condition of each substance as identified by gene set enrichment analysis. –log(FDR) values are displayed as a color code. Up-regulation is indicated in red and down-regulation is indicated in blue. Non-significant as well as no regulation is colored in white. Gene ontologies and conditions are clustered by Euclidean distance based on the –log(FDR) change values. Clusters are colored by test substance as applied for signatures in panel B of Figure 3. (B) as in (A), but for PANTHER pathways statistically significantly (p-value $\leq$ 0.05) enriched in the low and high exposure condition. –log(p) values are displayed as a color code.

Table 1
Nominal and measured substance concentrations of fipronil and imidacloprid used for transcriptomics. Measured concentrations are given as the mean of fresh and aged (48 hours) solution. LOQ = limit of quantification.

	fipronil		imidacloprid	
condition	nominal concentration	measured concentration	nominal concentration	measured concentration
control	0 µg/L	< LOQ	0 mg/L	< LOQ
LE (~EC5)	16 µg/L	11.4 µg/L	42 mg/L	42.5 mg/L
HE (~EC20)	32 µg/L	25.7 µg/L	64 mg/L	63.9 mg/L

Table 2
Immobility observed in the different test conditions. For each exposure condition the immobility is given as the mean ± standard error of three replicates in %. Statistical significance compared with the control was calculated using a one-way ANOVA. n.s. = non statistically significant; * $p_{adj} \leq 0.05$

	fipronil		imidacloprid			
condition	concentration	immobility [%]	significance	concentration	immobility [%]	significance
control	0 µg/L	5 ± 1		0 mg/L	7 ± 3	
LE	11.4 µg/L	7 ± 2	n.s.	42.5 mg/L	13 ± 2	n.s.
HE	25.7 µg/L	15 ± 6	*	63.9 mg/L	19 ± 2	*

biomarker candidates that can be used in short-term ecotoxicological assays with *D. magna*, e.g., by RT-qPCR or other targeted approaches, to assess the appropriate mode-of-action. Future expansion of such toxicogenomic fingerprinting to a broader range of modes-of-action will strengthen weight-of-evidence approaches and facilitate prediction of environmental hazards of unknown chemicals to aquatic invertebrates in the future.

5. Conclusion

While high exposure concentrations of both test substances induced similar paralytic effects, exposure to the LE and HE of each substance in our study resulted in highly distinct gene expression patterns. Similarly, the expression of the common subset of fipronil target genes, with the previously obtained targets of the positive allosteric GABA-gated chloride channel modulator diazepam was significantly negatively correlated when comparing both substances. Given that fipronil and diazepam act in an opposing manner on GABA-gated chloride channels, this observation strongly suggests a highly specific and consistent gene expression signature of fipronil. Similarly, we found a positive correlation between imidacloprid targets and those observed previously for the acetylcholinesterase inhibitor carbaryl. In this case, the inhibition of acetylcholinesterase is believed to induce an accumulation of acetylcholine at presynaptic sites, which (in turn) results in a hyperactivation of nAChRs, similar to the direct activity of imidacloprid. Taking into account that our observed gene expression changes were specific for each test substance and consistent between exposure conditions, these target gene sets may be considered as biomarker candidates for the corresponding modes-of-action in *D. magna*.

Furthermore, by applying functional classification analysis to the identified gene sets, we identified specific molecular functions and pathways affected by each of the model substances, part of which validated previous studies. A significant proportion of impaired molecular functions and pathways was consistent with the substances's mode-of-action, which further validates the specificity of our findings. Therefore, our study proves that an integration of transcriptomics in a modified version of the D. magna Acute Immobilization test can be used to differentiate modes-of-action of test substances of interest when applying low effect concentrations. Prospectively, such a combination of systems biology methodologies with ecotoxicological test guidelines will help to understand the underlying molecular mechanisms of adverse effects on invertebrate organisms and populations, which will provide weight-of-evidence for ecotoxicological hazard assessment.

Authors' statement

Julia Pfaff performed the investigation and the formal analysis and contributed to manuscript writing. Hannes Reinwald wrote analysis scripts in R and contributed to formal data analysis and data curation. Steve U. Ayobahan contributed to formal data analysis. Julia Alvincz contributed to experimental investigation. Bernd Göckener performed chemical validation of test substance concentrations. Orr Shomroni performed formal RNA-Seq raw data analysis. Gabriela Salinas was involved in data curation for RNA sequencing data. Rolf-Alexander Düring and Christoph Schäfers contributed to study conceptualization. Sebastian Eilebrecht conceptualized and supervised the study, contributed to formal data analysis and wrote, reviewed and edited the original draft the manuscript.

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Declaration of Competing Interest

The authors declare to have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.105927.

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