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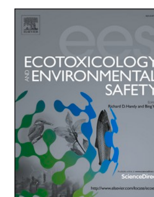

Contamination of aquatic environment with anticancer reagents influences Daphnia magna – Ecotoxicogenomics approach

Damian Mielecki [a,b], Elżbieta Grzesiuk [a], Anna Bednarska [d], Damian Garbicz [a,c], Bianka Świderska [a], Malgorzata Grzesiuk [a,d,e,*]

[a] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland
[b] Department of Immunohematology, Centre of Postgraduate Medical Education, Marymoncka 99/103, 01-813 Warsaw, Poland
[c] Maria Sklodowska-Curie National Research Institute of Oncology, Roentgena 5, Warsaw, Poland
[d] Department of Hydrobiology, Faculty of Biology, University of Warsaw, Poland
[e] Department of Biochemistry and Microbiology, Institute of Biology; Warsaw University of Life Sciences (SGGW), Warsaw, Poland

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A B S T R A C T

Pharmaceuticals used in human medicine contaminate freshwater ecosystems. Chemotherapeutics applied in cancer treatment are found in freshwaters at low concentrations (in the range of ng L^{-1}) which, however, can be toxic or mutagenic to aquatic organisms. The aim of this study was to determine the impact of the alkylating/crosslinking anticancer agents, cyclophosphamide (CP) and cisplatin (CDDP), at the concentration detected in water, on *Daphnia magna* life history, transcriptome, and proteome. This filter feeding cladoceran is an important member of the aquatic food webs controlling algal biomass and forming basic food for planktivorous fish. Here, observations of the *D. magna* growth rate, age at first reproduction, and the number of eggs produced were performed in the presence of CP or CDDP. The *D. magna* proteins and RNA were isolated and analysed by mass spectrometry and the mRNA-seq method, respectively. Five generations of contact with the pharmaceuticals in question significantly influenced the *D. magna* life history parameters with the growth rate and number of laid eggs decreased, whereas age at first reproduction was increased. A decrease in survivorship was observed when daphnids were exposed to CP. These changes are the result of modifications in the gene/transcript expression followed by differences in the proteome profile in comparison to the untreated control. The proteome changes were generally in accordance with the modified transcriptome. The ecotoxicogenomics approach makes it possible to get closer to a complete picture of the influence of CP and CDDP on *Daphnia*. We have gathered evidence that animals in the presence of anticancer pharmaceuticals attempt to cope with permanent stress by changing their proteome and transcriptome profile. Additionally, our analyses indicate that CDDP showed a stronger effect on tested organisms than CP.

1. Introduction

Pharmaceuticals are commonly present in aquatic environments as waste from human activity connected with medical treatments. In the 1970 s, clofibric acid was detected as being the first pharmaceutical in sewage water in the United States (Tabak and Bunch, 1970). Since then, the use of all kinds of medicines has dramatically elevated, followed by their increasing presence in wastewater, ground, and surface water (WHO 2012). Medicines used in the treatment of civilisation diseases – medicines such as antibiotics, antidepressants, cardiovascular pharmaceuticals, analgesics, anticancer substances etc., have recently been on the rise in the aquatic ecosystem (Patel et al., 2019). Active pharmaceutical ingredients are a global problem that is likely negatively affecting the freshwater ecosystem (Bouzas-Monroy et al.,).

Alkylating and crosslinking agents are frequently use in anticancer therapy; thus their production, consumption, and as a result, input to the freshwater ecosystems has increased (Zhang et al., 2013). Cyclophosphamide (CP) belongs to oxazaphosphorines that through its metabolite phosphoramide mustard, creates DNA crosslinks at the N7 position of guanine. The damage is irreversible, and the blockage of replication finally leads to apoptosis. Next, cisplatin (CDDP) is a platinum-derived pharmaceutical and is classified as an alkyl-like chemical, despite

* Corresponding author at: Department of Biochemistry and Microbiology, Institute of Biology; Warsaw University of Life Sciences (SGGW), Warsaw, Poland.
E-mail address: malgorzata_grzesiuk_bieniek@sggw.edu.pl (M. Grzesiuk).

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lacking an alkyl group, so not inducing the alkylation reaction. CDDP is a highly reactive crosslinker making DNA-DNA and DNA-protein cross-links that cause inhibition of cell growth and lead to cell apoptosis (Kartalou and Essigmann, 2001a, 2001b). The pharmaceutical interferes with DNA replication; thus it shows high cytotoxicity, especially in relation to fast proliferating cells and also those perceived as cancerous. This is why CDDP serves as an anticancer compound used in several types of cancer treatment (Drugs of choice for cancer, 2003).

Consumed pharmaceuticals are excreted in unchanged form or as metabolites thereof. Some of the metabolites, including anticancer pharmaceuticals, remain active and may show comparable or higher toxicity than the original substance. In developed countries, sewage from households and hospitals is treated in sewage treatment plants (STP); however, the efficiency of purification is low; thus, pharmaceuticals become serious contaminants of the water environment (Sosnowska et al., 2009). Pollutants found in freshwater are usually present at relatively low concentrations, e.g. about 36 pM (10 ng L^{-1}) and 333 pM (100 ng L^{-1}) of CP and CDDP, respectively (Kosjek and Heath, 2011). Pharmaceuticals, by design, should exert their biological effects at low doses, and usually act on physiological pathways, evolutionarily conserved across taxa (Arnold et al., 2014). Moreover, it should be noted that pharmaceuticals are delivered constantly and are accumulating in the aquatic ecosystem causing chronic exposure of organisms inhabiting these environments.

In the previous studies (Grzesiuk et al., 2018, 2019), we used the primary consumer of genus *Daphnia* as a model organism. *Daphnia* is a filter feeding cladoceran occupying a central position in freshwater trophic webs as a consumer of algal biomass (Sommer, 1986) and as prey for planktivorous fish (Lampert, 2006). Changes in fitness induced by pharmaceuticals influence not only planktonic crustaceans but may have a cascade effect on the functioning of all aquatic food webs (Sodré and Bozelli, 2019). In those experiments, the animals were cultured until first reproduction (about 11 days), which can hardly be seen as chronic exposition. Nevertheless, we have found that CDDP, but not CP treatment, both at environmental concentrations, decreased the survival and population growth rate of *Daphnia pulex* and *Daphnia pulicaria*. On the contrary, the individual growth rate was affected only by CP and solely in *D. pulicaria*. CP as well as CDDP exposure resulted in decreased fecundity (Grzesiuk et al., 2019).

Many of the traditional methods used to assess the potential impact of a chemical on aquatic organisms depend on analysing the organism responses, e.g. mortality, growth, reproduction of generally sensitive indicator species at constant concentration, and deriving 'endpoints' based on observed parameters, e.g. median lethal concentrations, not observed effect concentrations, etc. Such an approach is crucial, especially when examining acute toxicity, i.e. substances at high concentrations. However, when looking at environmentally relevant, usually low, concentrations of chemicals, e.g. pharmaceuticals, we may overlook a toxic effect at the organismal level. Furthermore, organism response analysis does not provide insight into the mechanism of chemical toxicity. Understanding the mechanism underlying a toxicological outcome makes it possible to predict the impact of chemical stressors like pharmaceuticals on organisms inhabiting polluted environments. The holistic approach used here makes it possible to link the molecular events with changes in the life history parameters at the organismal level.

The aim of the present study was to evaluate the CP and CDDP influence on *Daphnia magna* clones during chronic exposition (70 days, at least five generations) to environmental concentrations of mentioned alkylators. In the natural environment, the lifespan of *Daphnia* ranges from a few days to a couple of weeks (see e.g Lampert, 1993), so regarding the chronic effect of pharmaceuticals, permanent exposure should cover several subsequent *Daphnia* generations. Here, we studied the influence of CP or CDDP, at environmental concentrations, on *D. magna* transcriptome and proteome and further, the impact on the animal's life history and population parameters. An analysis of the cellular functions brought us closer to understanding the temporal dynamics of potential toxicity and adaptive response pathways for *D. magna* exposed to CP and CDDP in the natural environment.

2. Materials and methods

2.1. Pharmaceuticals

Two pharmaceuticals administered in cancer chemotherapy – cyclophosphamide monohydrate (CP; SIGMA C7397–1 G) and cis-platin (CDDP; TCI Europe, D3371) – were used. Stock solutions were prepared by suspending 1 mg of CP and CDDP in 1 ml of Milli-Q water and vortexing the solutions until the pharmaceuticals were completely dissolved. Stock solutions were stored at 4 °C in the dark. The working concentrations were as follows: 35.8 pM (10 ng L^{-1}) of CP and 333 pM (100 ng L^{-1}) of CDDP. These were in the range of those found in freshwater ecosystems (Kosjek and Heath, 2011).

2.2. Daphnia magna clones

We used three *D. magna* clones, referred to as D, N and S, taken from three separate ponds in the same region in order to obtain different genotypes while keeping environmental conditions relatively constant. The clones came from three ponds in the suburbs of Ceske Budejovice, Czech Republic in May 2016: D was obtained from Domin (49.00 N, 14.43E), N was isolated from Novy Vrebensky Rybnik (49.00 N, 14.44E) and S from Stary Vrebensky Rybnik (49.01 N, 14.43E). Experimental monocultures were established by isolating a single female of each clone from the clone library of the Department of Hydrobiology (University of Warsaw, Poland). Second clutch neonates of each subsequent generations were used for further culture and in the experiment. To standardise the pre-experimental conditions, animals of each clone were cultured for at least three generations in the laboratory prior to the experiment. Both pre-experimental and experimental daphnides were cultured under constant conditions: in the temperature-controlled water bath (20 °C ± 0.5 °C), summer photoperiod (16 L:8D; with dim light, 0.30 ± 2 μmol s-1 m-2), fed daily with green algae, *Acutodesmus obliquus* (SAG 276–3a) at the non-limiting growth concentration of 1 mg C_{org} L^{-1} (Lampert, 1987); *Daphnia* were fed daily, culture medium was changed every second day. Lake water originating from Szczęśliwice (Warsaw, Poland) was used. To minimise the possibility of the unintentional exposure of the daphnids to pharmaceuticals which could theoretically be present in the lake water, the water was filtered through 0.45 μm prior the use, then extensively aerated and passed, in a closed circuit, through a biological filter for at least 4 weeks. Prior to use, the water was filtered through a finer pore sized filter, i.e. a 0.2 μm capsule filter (Sartobran® P) to clear out the bacteria.

2.3. Experimental setup

The experiment was performed with five subsequent generations of *Daphnia*, lasting about three months. The animals were treated with 35.8 pM (10 ng L^{-1}) CP and 333 pM (100 ng L^{-1}) CDDP added to the medium. A medium devoid of pharmaceuticals served as a control. The experimental media were prepared with a fresh dose of CP or CDDP to avoid the problem of CP/CDDP decay. During the experiment, for each treatment there were 15 replicates of animals from which to observe life history parameters. Five out of the 15 replicates were taken to determine body mass for further growth rate calculations. Additionally, 5 replicates of 20 individuals were designated for the proteome and 5 replicates of 30 individuals for transcriptome analysis. Daphnides for which the life history parameters were determined were kept individually in 100 ml glass vessels while each replicate for proteome and transcriptome analyses consisted of 30 individuals in 3000 ml vol. of medium. Proteome and transcriptome analyses were performed with five-day-old daphnides in fifth generation. This age, at 20 °C, corresponds to pre-adult instar in

which females are sexually mature but there are no eggs in the brood chamber. We performed proteome and transcriptome analyses at this life stage intentionally; the animals were old enough, so the effect of CP and CDDP should already be manifested. Additionally, because there was no egg in the brood chambers the results of proteome and transcriptome were not biased by the developing embryos. Individuals for life history analysis were cultured until they released their second clutch offspring. As mentioned, second clutch neonates constituted individuals of the next generation. All three clones were tested simultaneously.

2.4. *D. magna* life history parameters

For each treatment and clone, five 5-day old daphnides were dry-weighted (24 h in 60 ºC) in order to calculate their individual juvenile somatic growth rate (Trubetskova and Lampert, 1995; Lampert and Trubetskova, 1996). Nine remaining individuals from each clone were further observed to determine the age at first reproduction (AFR) and size at first reproduction (SFR) and the number of eggs. *Daphnia* were photographed and measured using the NIS Nikon System.

Dry mass increase was converted to juvenile somatic growth rates (g_i) according to formula $gi = (\ln[W_{t1}] - \ln[W_{t0}]) \times \Delta t^{-1}$ were (Wt_0) - weight of animals at the beginning of the experiment < 12 h old, Wt1 5-day-old daphnid, Δt – time (in days) between the beginning of the experiment and the point at which the animals were harvested. The dry body mass in t_1 was obtained by weighing the 5-day-old *Daphnia* individually. The animals were weighed using an Orion Cohn C-35 Microbalance Thermo Scientific (exact to within 0.1 µg) device.

2.5. Total protein isolation and mass spectrometry data analysis

After the indicated time point, 20 *Daphnia* individuals, 5 days old, were collected and placed in liquid nitrogen. The animals were then homogenised in a buffer containing 25 mM Tris-HCl pH 7.5, 8 M urine, and 2% CHAPS. The homogenate was centrifuged (30,130 x g, 10 min, 4 °C) and the supernatant analysed for protein content using the Bradford assay (Bradford, 1976). The protein extracts were subjected to a mass spectrometry analysis performed in the Mass Spectrometry Laboratory (IBB PAS, Warsaw, Poland). Samples were prepared according to SP3 protocol described previously (Grzesiuk et al., 2019) with minor modifications. Briefly, cysteines were reduced in 20 mM tris(2- carboxyethyl)phosphine (TCEP) followed by an incubation with 50 mM s-methylmethanethiosulfonate (MMTS). Proteins were cleaned on 160 µg of SP3 bead mix by rinsing with 80% ethanol, acetonitrile and an additional wash with isopropanol. Proteins were digested overnight with 0.8 µg of trypsin/LysC enzyme mix (Promega). After digestion, peptide concentrations were determined using Pierce Quantitative Colorimetric Peptide Assay (Thermo Scientific). 2 µg of each sample were measured on QExactive mass spectrometer coupled to nanoAcquity UPLC (Waters) with parameters described previously (Grzesiuk et al., 2019). A substantial part of the mass spectrometry data analysis was performed in PEAKS Studio 8.5 (Ma et al., 2003). Generally, the default settings were used, with data refinement with Correct Precursor by Mass only. Identifications were carried out with Error Tolerance of Precursor mass at 15 ppm, using monoisotopic mass and Fragment ion at 0.5 Da. The cleavage was set to Trypsin with Lys C proteases. Only one non-specific cleavage was allowed at the end of a peptide, with a maximum of 3 missed cleavages. The peptide modifications were checked for beta-methylthiolation (45.9877 Da monoisotopic mass) and oxidation (15.9949 Da monoisotopic mass), with three maximum variable PostTranslational Modifications per peptide allowed. The quantification was performed with Mass Error Tolerance of 30 ppm, Retention Time Shift Tolerance of 6 min, FDR set at 1%, and Abundance Calculation Method as Area.

The identification was performed with the database prepared from proteins collected from UniProt as *D. magna* proteome UP000076858 (26,600 entries). Additionally, contaminant databases were used: 1) proteome of *Acutodesmus obliquus* UP000256970 (18,538 entries) and 2) manually curated human keratins database retrieved from UniProt (54 entries).

2.6. RNA isolation and mRNA-seq

Ten individuals were harvested in fifth generation, placed in stayRNA (A&A Biotechnology), kept for several minutes, and frozen in liquid nitrogen with the solution removed. After collecting all samples, mRNA was isolated with the Qiagen RNeasy Mini Kit with modifications. Namely, the individuals were crushed with the RNeasy Power Soil Total RNA kit: 0.5 ml Bead Solution, 50 µl SR1, 160 µl IRS, and 0.7 ml PCI were added to the frozen sample. The suspensions were vortexed for 15 min and centrifuged (2500 x g, 15 min, 4 °C). The aqueous phase was mixed with an equal volume of 70% ethanol. The following procedure was done according to the RNeasy Mini kit protocol, with on-column DNA digestion with RNase-free DNase set (Qiagen). RNA was eluted twice with the same eluent (40 µl). The following procedures were performed at Genomed SA (Poland): mRNA was isolated with the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB), and libraries prepared with the NEBNext Ultra Directional RNA Library Prep kit (Illumina), in PE150 mode. Raw sequences were trimmed and filtered with Cutadapt 1.18 (Martin, 2011). The resulting sequencing depths are presented in Supplementary File 2.

2.7. Filtering, alignment, and read counts

The quality was assessed and the reads were trimmed and filtered with fastp 0.20.0 (Chen et al., 2018). The reads corresponding to rRNA and mitochondrial mRNAs were filtered out with Tagdust 2.33 (Jacques and Darío, 2020). For the mapping of reads, two assemblies of *D. magna* (Taxid ID: 35525) were used, daphmag2.4 (NCBI Assembly: GCA_001632505) of clone Xinb3, and ASM399081v1 (NCBI Assembly: GCF_003990815) of clone SK. The annotation files available from NCBI ftp for these two clones were cured and modified with AGAT 0.5.1 scripts suit (Jacques and Darío, 2020), if needed. Reads were then aligned, with or without mitochondrial DNA (NCBI Accession: NC_026914.1), with STAR 2.7.7a (Dobin et al., 2013). The intron lengths, for STAR alignment, were retrieved with the publicly available script (https://gist.github.com/sp00nman/e9adb3c7e207c0de03d7) and a custom one. The alignments with STAR were performed manually in two-pass mapping (–sjdbFileChrStartEnd flag), with SJ.out.tab files produced in the first run containing the information about splice junctions. The aligned reads were counted in regard to features (genes) with featureCounts 1.6.2 (Liao et al., 2014). Also, the count tables generated by STAR were used in subsequent analyses. On average, 60% of all paired reads were successfully extracted.

A differentially expressed transcripts (DET) analysis was performed with the following TSA transcriptomes: 1) *D. magna* Xinb3 (NCBI BioProject: PRJNA284518, Prefix: GDIP01), with 255355, 2) eugenes database (http://arthropods.eugenes.org/EvidentialGene, all transcripts: dmagset7finloc9b.puban.mrna.gz + dmagset7finalt9b.puban.mrna.gz, primary transcripts: dmagset7finloc9b.puban.mrna.gz) (Gilbert, 2013), and 3) *D. magna* SK (NCBI BioProject: PRJNA490613, Prefix: GGWI01) with 25895 contigs, and with the kallisto 0.46.1 (Bray et al., 2016). As with the differentially expressed genes (DEG) analysis, the indexes for kallisto were prepared without or with mitochondrial transcriptome, with k = 31. Simultaneously, an estimation of transcript abundances was performed with kallisto using the bootstrap analysis with 50 samplings.

Since the available transcript databases for *D. magna* Xinb3 and SK clones, and also eugenes database, contained sequences with N's (seqN) to a different extent than the ones that were redundant, the filtering was performed producing databases with a different degree of sequences with N's and redundant ones removed. First, the data were prepared with blast-all-vs-all results produced with an NCBI blast-2.11.0 + blastn

script (Camacho et al., 2009), with the following parameters: evalue 1e-100, strand plus, and perc_identity 100, with qcovs and qcovhsp included in the result table. The custom scripts were used to filter out the sequences with respect to the N's content and minimum sequence coverage of longer sequence in the alignment with no overhangs restriction, and a 4-nt shift allowed for a shorter sequence, as shown in Supplementary Table 1. In the case of the *D. magna* SK clone, the mmseqs package (Steinegger and Soding, 2018) was also used to cluster and remove redundant sequences, with the following parameters: easy-cluster, global-alignment, evalue 1e-100, and varied coverage.

Counts produced by featureCounts and STAR were corrected, accounting for GC bias and normalised with the EDASeq 2.24.0 R package (Risso et al., 2011) with default parameters. Only genes presenting an overall sample expression equal to or more than 10 were kept for further analysis. Normalised counts were used in a subsequent analysis performed with the DESeq2 1.30.1 R package (Love et al., 2014).

2.8. Statistical analysis

To analyse life history parameters, we used a three-way ANOVA followed by a Tukey post hoc (at $\alpha = 0.05$) test with Bonferroni correction to test the effect of the pharmaceuticals (CP and CDDP) on the *Daphnia* life history parameters. Analyses were performed using Statistix 9.0 (Analytical Software, Tallahassee, USA).

All statistical analyses and visualisations for transcriptome and proteome data were performed with R 4.0.5 project (Team, 2020), particularly with ggplot2 3.3.3 (Hadley, 2016), reader 1.0.6, and ggh4×0.1.2.1. For data modification, curation, and preparation, custom bash scripts were used. The statistical analysis of STAR alignments was performed with the samtools 1.10 package (Li et al., 2009), especially flagstat, stats, and idxstats. Venn diagrams were prepared with the VennDiagram R package (Chen and Boutros, 2011).

To analyse DEGs and its production in DESeq2 the corrected and normalized GC counts were subjected to the standard DESeq2 path. The likelihood-ratio test (LRT) was used and clone as a reduced factor. For dispersion estimation, the variance stabilising transformation (vst) was used with blind=TRUE. The statistical analysis and DETs production in sleuth the kallisto transcript abundance estimations were analysed with sleuth 0.30.0 (Pimentel et al., 2017). Two tests were used – LRT and Wald's test for comparisons – with samples divided according to treatments. The clone bias was also accounted with clone as a reduced factor. To analyse the DEGs and DETs group intersection, the DEGs and DETs groups produced by different methods were checked for common genes/transcripts with the SuperExactTest 1.0.7 R package (Wang et al., 2015). Statistical analysis of intersections and assigns corresponding p-values were also performed in this R package.

3. Results

3.1. Life history parameters

Pharmaceutical exposure significantly affected *Daphnia's* age at first reproduction (AFR) and the number of produced eggs; however, it showed no significant effect on growth rate and size at first reproduction (Table 1, Fig. 1). The post hoc analysis (Tukey HSD post hoc analysis at $\alpha = 0.05$) of the effect of pharmaceutical exposure revealed that CDDP caused a delay in reproduction and a decreasing number of eggs produced by the female when compared to the untreated control.

Further analysis of the Treatment×Generation interaction revealed that there was no significant difference in AFR between treatments in the first generation, and the observed adverse effect of CP and CDDP on AFR manifested only after chronic, i.e. five generations, exposure [~12.5 (clones D=10.6; N = 9.3; S=18) days in control females and ~11 (clones D=10; N = 9.1; S=14) and ~15 (clones D=16.5; N = 10.6; S=17.6) days in the CP and CDDP, respectively]. Similarly, there were no significant differences in fecundity between treatments in females in the first generation (~7.5 eggs on average, regardless of the treatment: clones D=7.3; N = 8.1; S=6.8), but the effect of the pharmaceutical was observed after exposure for a long time. In females from the fifth generation, the chronic exposition to CDDP resulted in decreased fecundity [~ 4 eggs on average (clones D=3.8; N = 3.3; S=4.6)], while there were no significant differences in the number of eggs produced by control and CP-exposed females [~5.5 eggs on average (clones D=6.8; N = 7.1; S=3.2)]. An analysis of the Treatment×Generation×Clone interaction revealed that in the first generation there were no significant differences between treatments in all clones in all observed life history parameters. However, chronic exposure led to a deleterious outcome in *Daphnia*. The most striking effect was the extinction of CDDP-exposed D clone animals at the beginning of the fourth generation. Other negative effects include delayed reproduction and a decreased growth rate observed in S-clone females and decreased fecundity of N- and S-clone animals. The exposition to CP did not caused increased mortality; it did, however, result in decreased fecundity in S-clone females and delayed reproduction of D- and S-clone animals.

Table 1
Two-way ANOVA testing the effect of genotype (D, N, and S clone) and pharmaceutical treatment (no pharmaceutical, cyclophosphamide, cisplatin) and effect on life history parameters: growth rate, age and size at first reproduction, number of eggs.

Variable	Factor	df	F	p-value
Growth rate				
n = 9	Clone (**C**)	2	26.55	**< 0.001**
	Treatment (**T**)	2	1.55	0.2159
	Generation (**G**)	1	131.78	**< 0.001**
	C x T	4	3.65	**< 0.01**
	G x T	2	1.69	0.1878
	C x G x T	4	2.58	**< 0.05**
	Error	127		
Size at first reproduction				
n = 9	Clone (**C**)	2	15.11	**< 0.001**
	Treatment (**T**)	2	2.97	0.7656
	Generation (**G**)	1	116.59	**< 0.001**
	C x T	4	4.57	0.0875
	G x T	2	4.32	0.7591
	C x G x T	4	1.01	**0.0637**
	Error	135		
Age at first reproduction				
n = 9	Clone (**C**)	2	81.92	**< 0.001**
	Treatment (**T**)	2	34.53	**< 0.001**
	Generation (**G**)	1	483.13	**< 0.001**
	C x T	4	10.52	**< 0.001**
	G x T	2	33.03	**< 0.001**
	C x G x T	4	7.75	**< 0.001**
	Error	135		
Number of eggs				
n = 9	Clone (**C**)	2	3.85	**< 0.01**
	Treatment (**T**)	2	0.48	**< 0.05**
	Generation (**G**)	1	46.53	**< 0.001**
	C x T	4	3.37	**< 0.01**
	G x T	2	6.74	**< 0.01**
	C x G x T	4	1.08	**< 0.01**
	Error	135		

3.2. Numbers and characteristics of differently expressed genes (DEGs) and differently expressed transcripts (DETs)

The scaffolds coverage analysis was performed for an alignment based on *D. magna* Xinb3 and SK clones and the corresponding databases including the mitochondrial genome (Fig. S1). The Xinb3 assembly consists of 28801 scaffolds, but only 991 of them presented at least 20 aligned reads across all samples, with a maximum of 5% of all aligned reads. The inclusion of mitochondrial genes increased the number of these scaffolds to 1183. On the other hand, since the SK clone assembly contains 10 chromosome sequences, among 4192 of all scaffolds, the majority of reads (up to 20%) aligned to them, reducing the number of

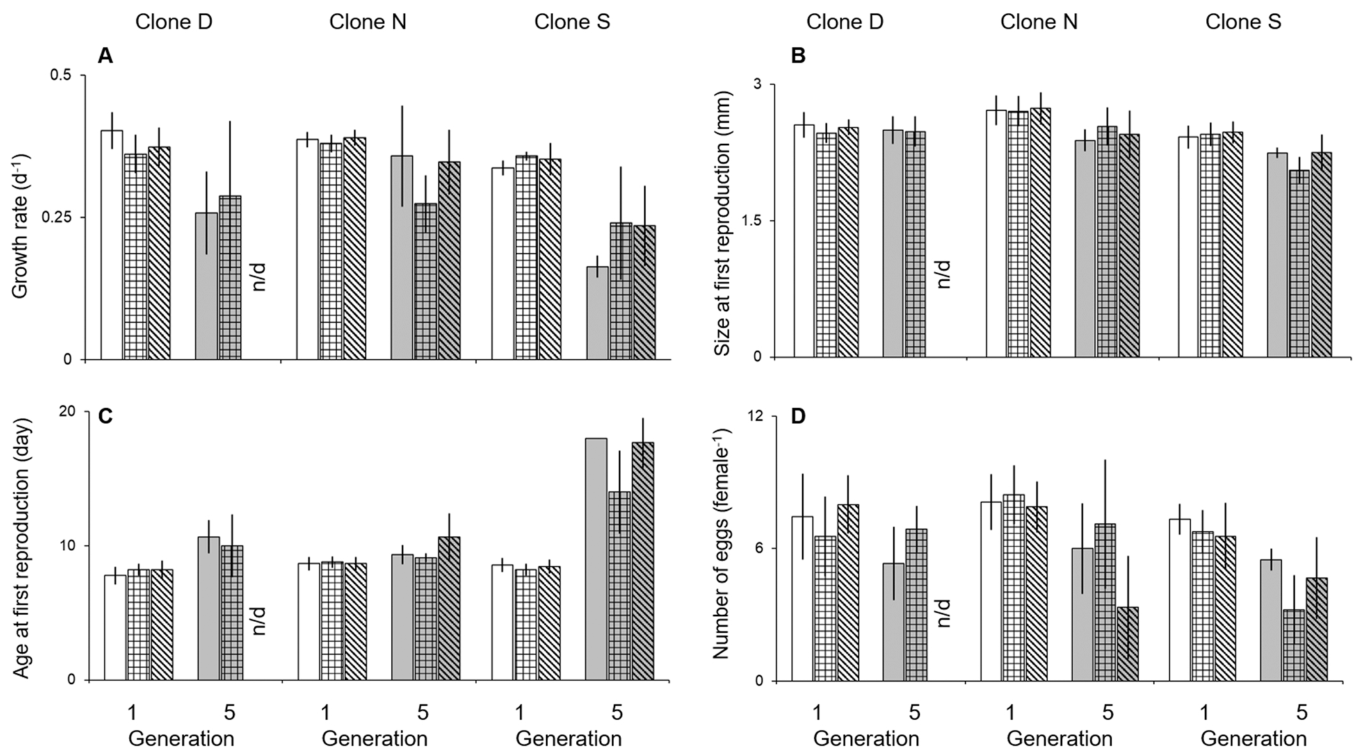

Fig. 1. *Daphnia magna* (D, N, and S clone) life history parameters. (A) Individual growth rate (mean ± SD), (B) size and (C) age at first reproduction (average ± SD), and (D) number of eggs per female (average ± SD) cultured without pharmaceutical (empty bars), in the presence of CP (checkered pattern bars), or CDDP (striped bars). Results are showed for animals exposed for one (bar with white filling) and over five (bar with grey filling) generations. n/d indicates no data.

sequences with at least 20 aligned reads to 242, and 276 with mitochondrial DNA included. The majority of reads (on average 95%) were aligned to the scaffold databases used, with the corresponding annotations, in the properly paired mode (Fig. S2). A minor portion of them (about 5%) presented a secondary alignment. There is no visible difference in the alignment statistics in regard to the source of scaffold, either Xinb3 or SK clone, or the presence of mitochondrial DNA in the alignment index.

In the case of kallisto, the fraction of mapped reads depends substantially on the database used (see Table S1). The majority of reads (at least 85%) mapped to the Xinb3 transcriptome, including the version with mitochondrial transcripts, except the databases with seqNs filtered out (Fig. S3). The use of SK transcripts reduced the fraction of mapped reads to about 75%, with no difference in regard to filtering, which is quite obvious considering that the filtering method did not substantially change the number of transcripts. Surprisingly, the inclusion of mitochondrial transcripts decreased the mapped reads to about 65%. The use of the eugenes databases presented the most divergent results. The database, consisting of all transcripts, made it possible to map about 80% of reads, and the removal of seqNs decreased this number by about 5%. In the case of finloc9b database, the depletion of seqNs decreased the mapping from about 80 to about 50%, being the lowest fraction of mapped reads.The use of eugenes databases with mitochondrial transcripts further decreased this number by about 5–10%. The filtering method dependent on the coverage (any or at least 80%) does not give any visible difference in the fraction of mapped reads.

The number and the character of regulation differed significantly according to the type of analysis chosen. In the case of genomic alignments, the majority of genes, after both CP and CDPP treatments, were downregulated, being 20–34 when the Xinb3 genome was used, and 42–55 in the case of the SK genome. There was no substantial difference according to the featureCounts method used (e.g. uniquely or multiply mapping reads), with or without the mitochondrial genome. There were only a couple of genes upregulated in any case. The numbers of genes dysregulated after treatments were similar for both chemicals used (Fig. 2).

On the other hand, the characteristics for DETs were very different. There were far fewer DETs after CP treatments, up to 76, and compared to CDPP, up to 580, and they were mainly downregulated. Also, these numbers differ according to the transcriptome database type and filtering degree. The use of Xinb3 transcriptome made it possible to detect the lowest number of DETs, and with the filtering of seqNs substantially improving the counts (Fig. 3).

In the case of the analysis with SK transcriptome, there were no substantial differences in the number of DETs according to the filtering method used, and these numbers are some of the highest. When the eugenes database was used, the results were quite different based on the filtering method. The highest number of DETs were obtained when the finloc9b database was used, regardless of the filtering method, i.e. up to 317 upregulated and 263 downregulated genes. Generally, the inclusion of mitochondrial transcriptome slightly increased the number of DETs detected (Fig. 3). Considering the distribution of adjusted p-values in the groups of DEGs and DETs, there is no visible bias, with p-values being more or less evenly distributed, with a slight tendency to higher DET values after CDPP treatment (Figs. S4 and S5). The same applies for the fold change distribution according to the mean of normalised counts (Figs. S6 and S7) and adjusted p-values vs. fold changes (Figs. S8 and S9).

3.3. DEG and DET intersections

The existence of collected common genes was investigated among five groups of genes/transcripts with a statistically significant change in expression of *D. magna* analysis based on 1) the Xinb3 assembly, 2) SK assembly, 3) Xinb3 transcriptome, 4) SK transcriptome, and 5) eugenes transcriptome. In the case of the analysis based on *D. magna* Xinb3 assembly, almost all of the genes (19) were common regardless of the treatment (Fig. 4). Among these groups, eight showed at least a 1.5-fold

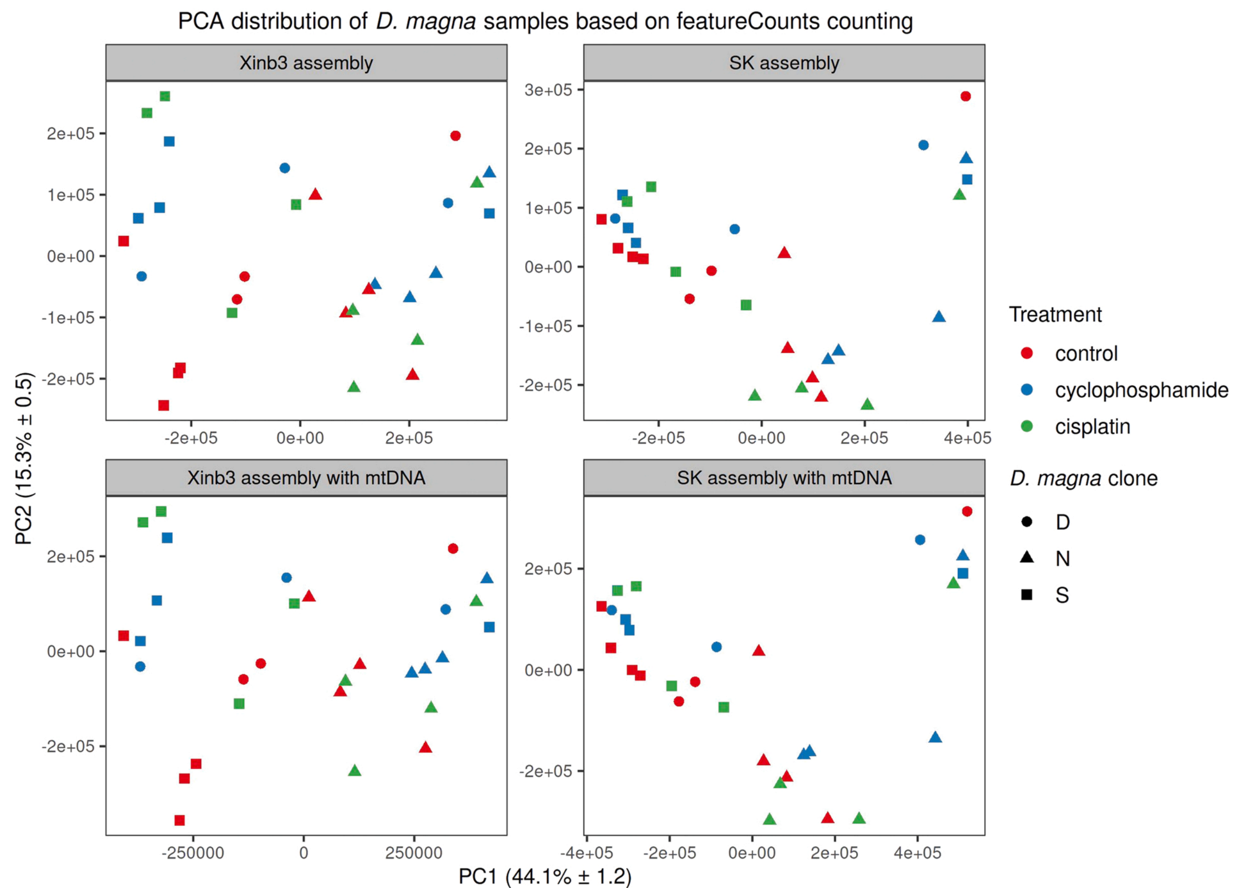

Fig. 2. The numbers of DEGs (padj < 0.1) according to the reference assembly used (*D. magna* Xinb3, SK, without or with mitochondrial genome), treatment (CP, CDDP), and counting method used: featureCounts with uniquely mapping reads (fC_unique), with uniquely mapping reads and that with both ends successfully aligned (fC_unique_bothendsaligned), with multi-mapping reads allowed (fC_multiple), or with multi-mapping reads allowed and these having both ends successfully aligned (fC_multiple_bothendsaligned), or STAR counting (STAR_counting).

change in expression, being only nuclear (Table 2). When the *D. magna* SK genome was used as a reference, these numbers were much higher, namely, 41 and 20, respectively (Fig. 5, Table 2). Although, the DET analysis generally showed higher numbers of differentially appearing transcripts, all being nuclear, there were fewer common mRNAs between organisms treated with CP and the ones treated with CDPP (Fig. 6). When the *D. magna* Xinb3 transcriptome was used as a reference, there were no common transcripts between these two treatments. There was only one common mRNA in the case of the eugenes database. An analysis based on the *D. magna* SK transcriptome showed as many as 33 transcripts common to both chemicals. When the 1.5-fold change was considered, the analysis with the *D. magna* SK transcriptome showed only four transcripts common for both treatments (Table 2). The distribution of the fold changes among the gene/transcript groups presented in Table 2 does not diverge significantly and stays between − 5–5-fold, with the majority of genes/transcripts downregulated. The expression of GDIP01014878.1, an uncharacterised protein, increased by 7.48-fold, and the expression of GGWI01013762.1, putative lysozyme-like, and GGWI01018931.1, extensin-like, decreased by 15.54 and 20.63-fold, respectively (Fig. 7, Supplementary File 1). The presence of mitochondrial genome or transcriptome had no visible impact on the calculated fold changes of DEGs/DETs.

3.4. Description of DEGs and DETs identified with at least a 1.5-fold change in D. magna treated with CP or CDPP

In *D. magna* treated with CP or CDPP, the majority of the genes found were downregulated, with counterparts identified in other analysis pathways. Many of them are connected with glucose metabolism, such as galactose-3-O-sulfotransferase 2 and glucose dehydrogenase. Three apolipoproteins D-like, clustered in the genome sequence, are also present. In humans, these proteins are responsible for, e.g. the response to drug and reactive oxygen species, and tissue regeneration (Najyb et al., 2017; Martínez-Pinilla et al., 2015; Bajo-Grañeras et al., 2013). There are several proteases, such as brachyurin-like (with a chitin binding property) (Roy et al., 1996), trypsin alpha-like and chemotrypsin-like proteins, the F-box only protein 30, being a substrate-recognition component of the SCF (SKP1-CUL1-F-box protein)-type E3 ubiquitin ligase complex (Sartori et al., 2013), and also chitinase-like and prisilkin-39 (chitin binding protein) (Kong et al., 2009). Calphotin is a protein responsible for signal transduction connected with Ca^{2+} in *Drosophila melanogaster* (Martin et al., 1993). Also, transcript-producing protein involved in egg development in, e.g. the fruit fly, chorion peroxidase-like, was differentially expressed (Tootle and Spradling, 2008). The protein with strong antimicrobial activity, acanthoscurrin-2-like, was downregulated (Lorenzini et al., 2003). Additionally, the heme-binding protein 2, able to promote cell necrosis in humans, was downregulated in the response to cisplatin (Szigeti et al., 2006). Interestingly, the expression of one long non-coding RNA was downregulated in *D. magna* treated with alkylating agents tested. There are several DEGs/DETs having homologs in different analysis pathways, such as glucose dehydrogenase, brachyurin-like, and some uncharacterised proteins. Although not directly found by blast, some of the functions are represented by different DEGs/DETs, e.g. extensins (Table 2).

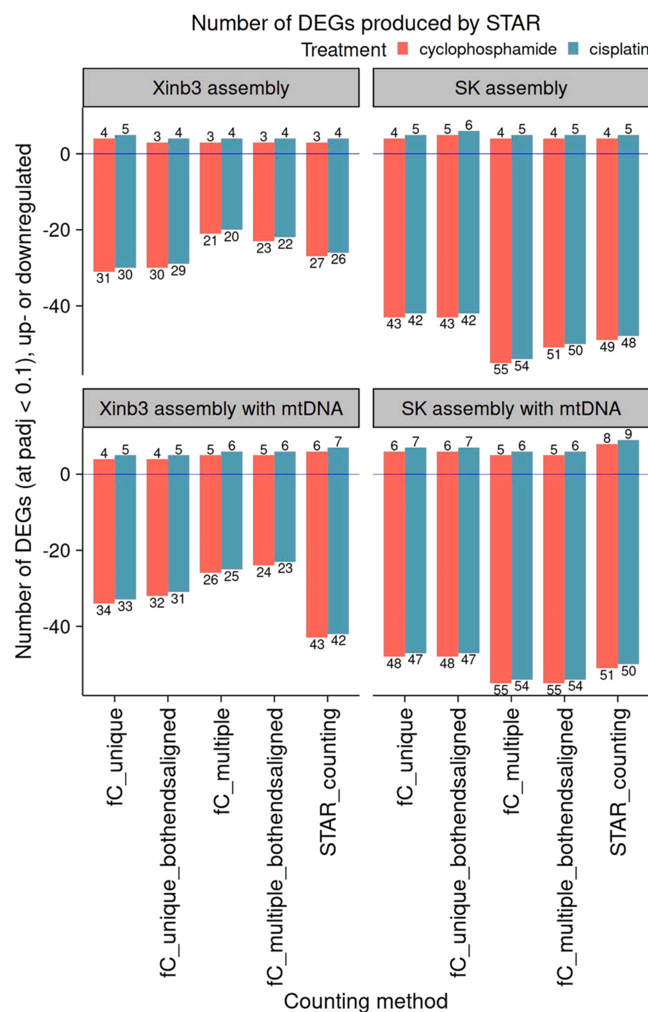

Fig. 3. The numbers of DETs (padj < 0.1) according to the reference transcriptome used (*D. magna* Xinb3, SK, or eugenes, without or with mitochondrial genome), treatment (CP, CDDP), and transcriptome database filtering method (see Table 2).

3.5. Differential proteins found by mass spectrometry

The proteome changes were in accordance with the modified transcriptome. The most robust answer was observed for the CDDP treatment, followed by CP, for the *D. magna* S clone, with the exception that almost all of these proteins increased in abundance (Fig. 3). The mass spectrometry data was identified with the *D. magna* proteome database, composed of Xinb3 clone proteins. The PCA grouping did not show any visible clustering of clones and/or treatments (Fig. 8). The treatment with chemicals led to a change in abundance of several proteins. The most profound effect was observed for CP or CDPP treated *D. magna* S, with an increased concentration of 46 and 75 proteins, respectively, and only one protein downregulated for both treatments. In the case of other clones, the effect was at the level of several proteins with a lower presence. For *D. magna* N treated with CP or CDPP it was four and six proteins, respectively, with only two proteins upregulated for the latter treatment, and for the *D. magna* D treated with CP, two proteins were downregulated and one upregulated (Fig. 9). The protein groups that changed with different treatments showed partial overlapping. Noticeably, the *D. magna* S clone in response to CP and CDPP shared 34 common proteins. The N clone individual responses to both chemicals shared two proteins. Treatment of individuals originating from N and S clones with CP caused the same protein change. Interestingly, there is: 1) one protein common for all the responses except the *D. magna* N clone treated with CP; 2) two proteins common for three treatments, *D. magna* S treated with both chemicals and *D. magna* N treated with CDPP; 3) one protein common for the response in the *D. magna* S and D clones (Fig. 9).

The common protein(s) mentioned above included: 1) calmodulin down- or upregulated; 2) poly(U)-specific endoribonuclease and beta-klotho; 3) uncharacterised protein (UniProt ID: A0A0P5SIK7). Additionally, in the groups of proteins changed by treating *D. magna* with the chemicals tested, there appeared several proteins functioning within stress response and cytoskeleton. For instance, *D. magna* N in the presence of CP responded with the lowered synthesis of seizure 6-like protein, superoxide dismutase [Cu-Zn], and putative fibronectin type-III domain-containing protein, and in the presence of CDPP – seizure 6-like protein and peptide transporter family 1. In the case of *D. magna* S, almost all of the proteins were upregulated. In the presence of CP, these included: putative cold-shock DNA-binding family protein (upregulated 13.94-fold), cofilin/actin-depolymerizing factor, troponin I, myosin regulatory light chain 2 smooth muscle, heat shock protein 83, angiotensin-converting enzyme, myosin heavy chain, DnaJ subfamily C member 13, vinculin, calreticulin, tubulin alpha chain, glucosylceramidase, ADP/ATP translocase, V-type proton ATPase, ferritin, putative RNA-binding protein 38, adenosine deaminase, failed axon connections, SWI/SNF complex subunit SMARCC1, protein Z-dependent protease inhibitor, lysosomal alpha-glucosidase, ribonuclease kappa, putative ovochymase-2, trithorax group osa-like protein, or DOMON domain-containing protein. Most of these proteins were common to both treatments.

The functions of proteins identified by mass spectrometry are generally consistent with the ones showed by transcriptome analysis, taking part in energy metabolism, cytoskeleton reorganisation, and more directly in response to DNA and protein stress, like cold-shock DNA-binding family protein and DnaJ protein involved in membrane trafficking through early endosomes.

3.6. Database

All the information about the project as well as the raw data files for all samples are available under the NCBI BioProject PRJNA749952.

4. Discussion

In this study, we demonstrate the impact of anticancer pharmaceuticals at environmentally relevant concentrations, on a non-target organism, *Daphnia magna*. To reflect the conditions prevailing in contaminated freshwaters, *D. magna* individuals were chronically exposed to CP or CDDP for five generations. Analysing *D. magna* reaction at transcriptomic, proteomic, and whole organism level, we derived a detailed picture of the disturbance in functioning of the organism under conditions resembling a contaminated environment. The growth rate, age at the first reproduction, number of eggs and, marginally ($p = 0.06$), the size at first reproduction were significantly affected by one or both of the tested pharmaceuticals (Table 1, Fig. 1). *Daphnia* originating from D clone cultured in the presence of CDDP died at the beginning of the fourth generation, before the animals reached maturity. A pattern was observed in our previous studies, where exposing *Daphnia pulex* to CDDP resulted in 12.5% mortality until the first reproduction (Grzesiuk et al., 2019). Observed mortality can be caused by a highly cytotoxic CDDP effect, including inhibition of DNA replication. This anticancer pharmaceutical can affect the clonal composition of the *Daphnia* population and lead to clonal selection. On the contrary, the presence of CP at environmental concentrations did not increase animal mortality in the first generation (five days exposure) (Grzesiuk et al., 2019; Heath et al., 2016); however, in one of the tested *Daphnia* clones, the growth rate decreased after five generations of CP exposure (Table 1, Fig. 1A). These results indicate that exposure over a long period is more reliable in reflecting changes in the environment caused by pharmaceutical pollution and makes it possible to “catch” negative effects which may be

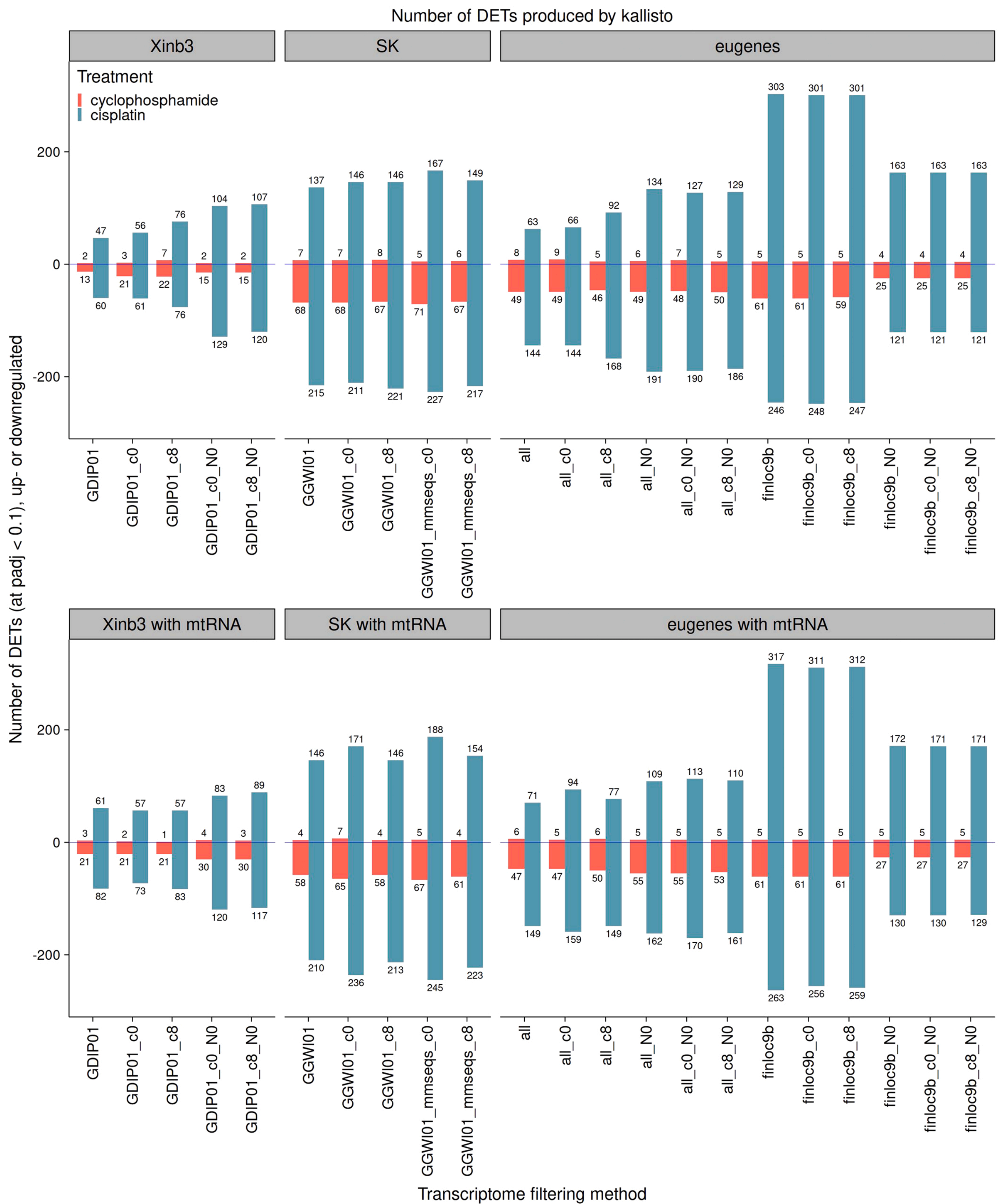

Fig. 4. The numbers of common DEGs (padj < 0.1) based on *D. magna* Xinb3 assembly reference database. Only four groups were used for clarity, based on counting method (featureCounts or STAR) and treatment (CP or CDPP). These groups were prepared by producing intersections from lower level groups e.g. the featureCounts, CP group was prepared from corresponding groups but differing from featureCounts counting method.

Table 2
DEGs/DETs identified in *D. magna* treated with CP or CDPP, with the following analysis pathways: Xinb3 or SK assembly, or Xinb3, SK, or eugenes transcriptome, with the expression changed at least 1.5-fold. Many of them have homologs found in other pathways (bold). Most of DEGs/DETs are downregulated, except of several upregulated (red). These groups were prepared by producing intersections from lower level groups. E.g. the *Xinb3 assembly* group was prepared from corresponding groups differing by counting method (featureCounts with different settings or STAR counting).

Database:	DEGs/DETs:
Xinb3 assembly	**APZ42_014895**, **APZ42_017540**, **APZ42_017321**, **APZ42_022288**, **APZ42_018848**, **APZ42_028696**, **APZ42_017127**, **APZ42_017527**
SK assembly	LOC116927196, LOC116926887, LOC116926898, **LOC116935604**, **LOC116926351**, LOC116930324, **LOC116916100**, **LOC116927046**, **LOC116929538**, LOC116931996, LOC116930322, **LOC116927868**, LOC116920699, **LOC116932977**, **LOC116930323**, LOC116923742, **LOC116927875**, **LOC116927090**, **LOC116918081**, LOC116918232
Xinb3 transcriptome, CP	GDIP01093162.1
SK transcriptome, CP	**GGWI01022628.1**, **GGWI01005376.1**, **GGWI01008215.1**, **GGWI01007660.1**, **GGWI01006212.1**
eugenes transcriptome, CP	–
Xinb3 transcriptome, CDPP	**GDIP01034727.1**, GDIP01130212.1, GDIP01014878.1, GDIP01093031.1, **GDIP01003730.1**, GDIP01121867.1, **GDIP01018060.1**, GDIP01200883.1, GDIP01199633.1, **GDIP01194876.1**
SK transcriptome, CDPP	GGWI01013762.1, **GGWI01005664.1**, **GGWI01009036.1**, **GGWI01009630.1**, **GGWI01005376.1**, **GGWI01012634.1**, **GGWI01007660.1**, **GGWI01007821.1**, GGWI01014890.1, GGWI01011340.1, **GGWI01022628.1**, **GGWI01010607.1**, **GGWI01018429.1**, **GGWI01008215.1**, GGWI01018560.1, GGWI01012292.1, GGWI01015184.1, **GGWI01007679.1**, **GGWI01003655.1**, GGWI01015810.1, GGWI01022959.1, **GGWI01010569.1**, GGWI01021177.1, **GGWI01001502.1**, GGWI01012143.1, GGWI01018931.1
eugenes transcriptome, CDPP	**Dapma7bEVm003379t1**, **Dapma7bEVm015434t1**

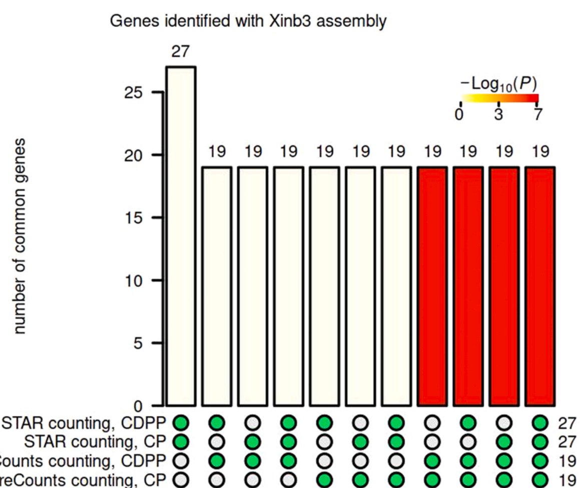

Fig. 5. The numbers of common DEGs (padj $<$ 0.1) based on *D. magna* SK assembly reference database. Only four groups were used for clarity, based on counting method (featureCounts or STAR) and treatment (CP or CDPP). These groups were prepared by producing intersections from lower level groups e.g. the featureCounts, CP group was prepared from corresponding groups differing by featureCounts counting method.

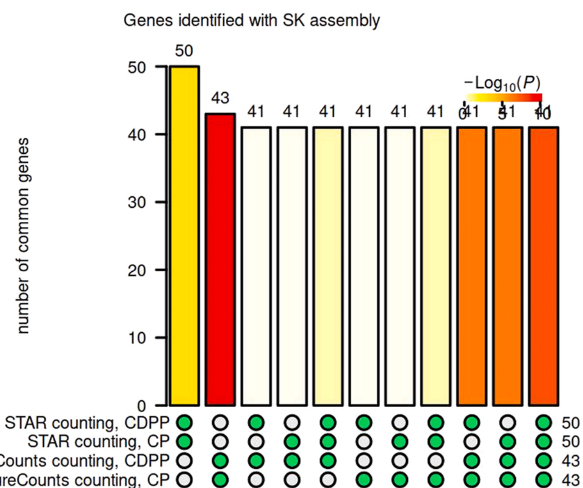

Fig. 6. The numbers of common DETs (padj $<$ 0.1) based on transcriptome database used (Xinb3, SK, or eugenes, transcriptome), and treatment (CP or CDPP). These groups were prepared by producing intersections from lower level groups e.g. the *Xinb3, CP* group was prepared from corresponding groups differing by transcriptome filtering method (see Supplementary Table 1).

overlooked during a short time of exposure.

The age at the first reproduction of all tested clones was affected by both agents, CP and CDDP (Table 1; Fig. 1C). For the first time, we report that *D. magna* exposed to CDPP delays the first reproduction. The size at first reproduction was not affected by alkylating agents (Table 1, Fig. 1B). Both results agree with our previous observations (Grzesiuk et al., 2018).

Our transcriptomic analysis showed that considering only at least a 1.5-fold gene/transcript expression changed, the lists of DEGs/DETs were inclusive for the Xinb3 and SK genome, and SK transcriptome, with the list of DEGs based on SK genome mapping, consisting of additional genes identified (Tables 2 and 3). Some of the functions were even represented by different DEGs/DETs. Among them, there were two proteins downregulated in response to both CP and CDDP treatments – the galactose-3-O-sulfotransferase 2 and glucose dehydrogenase, connected with sugar metabolism. This signifies that sugar metabolism is one of the key responses to environmental stress, evolutionary conserved (Kultz, 2005; Swart et al., 2019), and common among organisms under chemical stress (Borgatta et al., 2015; Lee et al., 2015).

In response to the pharmaceuticals tested, three D-like apolipoproteins were found. Their human homologs take part in, e.g. the response to reactive oxygen species, and tissue regeneration (Najyb et al., 2017; Martínez-Pinilla et al., 2015; Roy et al., 1996). Furthermore, their production is induced by some pharmaceuticals in order to obtain a therapeutic effect. Interestingly, in *D. magna*, few proteins connected with protein degradation (e.g. brachyurin and other proteinases) and/or chitin metabolism (prisilkin-39) were downregulated in response to both pharmaceuticals used. In particular, one of the extensins, GGWI01018931.1, was 20.63-fold downregulated (Fig. 7, Supplementary File 1). This phenomenon could imply a carapace remodelling, which in turn may allow organisms to recover energy. All proteins listed above are associated with energy allocations. Changes in the level of these proteins can cause late reproduction, decrease in growth rate and fecundity. Indeed, this is the case since the number of eggs produced at the first reproduction decreased in *D. magna* exposed to CDDP in comparison to non-treated individuals (Table 1, Fig. 1). This result is in agreement with our previous findings on *D. pulex* and *D. pulicaria* (Grzesiuk et al., 2019). In the present study we detected a lower number of eggs produced by animals exposed to CDDP but not to CP. *Daphnia* exposed to CP showed an upregulation of vinculin, a cytoskeletal protein playing an important role in the regulation of focal adhesions and embryonic development (Laneve et al., 2008). It can be speculated that

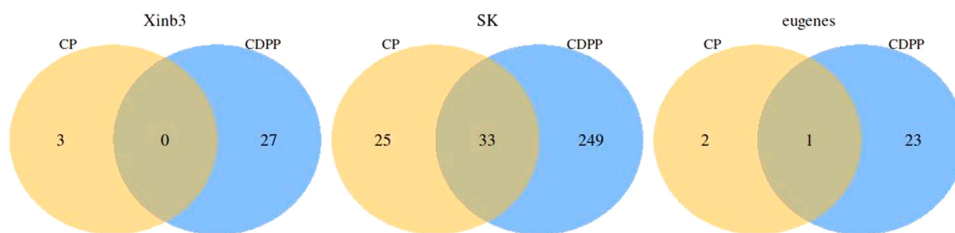

Fig. 7. Distribution of DEGs/DETs expression changes (boxplot) in the groups according to the database used for mapping (Xinb3 or SK assembly, or Xinb3, SK, or eugenes transcriptome, without or with mtDNA/RNA). Only genes with padj < 0.1 and |logFC| > 1.5 are shown. The statistics was prepared for particular counting methods (featureCounts and STAR) or database filtering method (see Table 2).

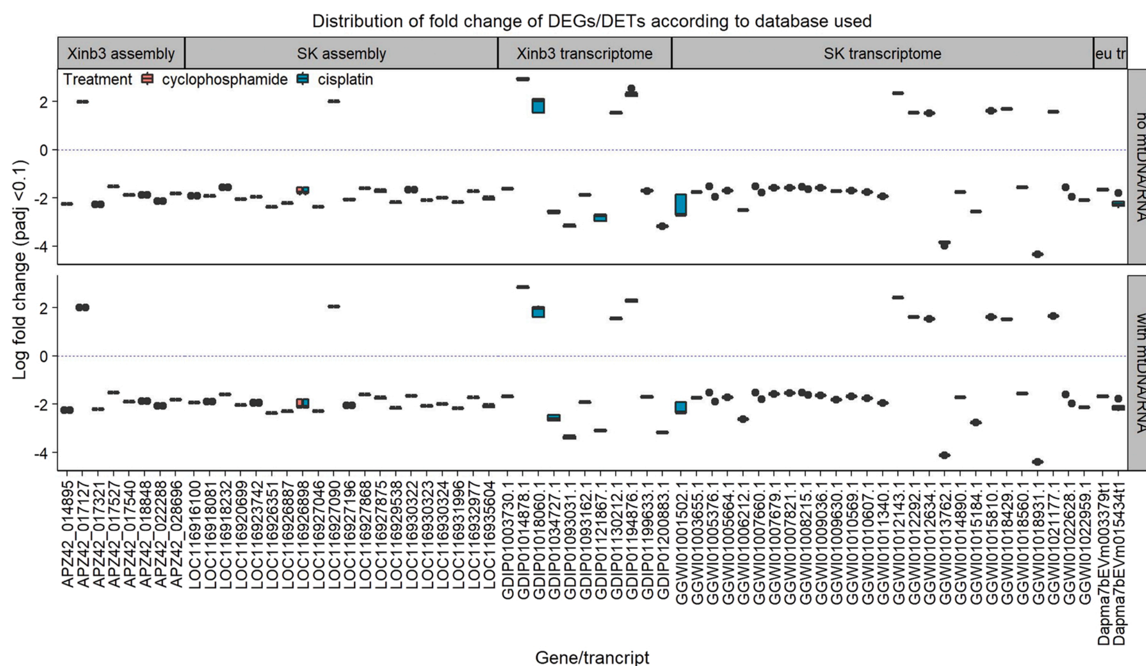

Fig. 8. Clustering of *D. magna* samples, according to the PCA based on the normalized areas produced by PEAKS (with quality score for peptides set at 10).

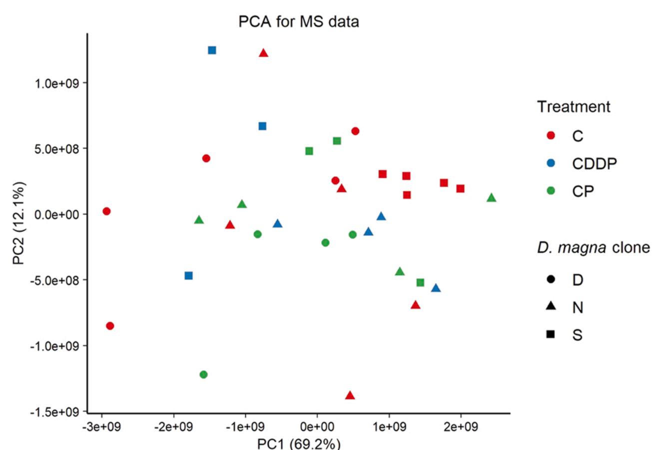

Fig. 9. Numbers of common DAPs (padj < 0.1) based on MS semi-quantitative analysis, based on the *D. magna* clone (N, S, D) and treatment (CP or CDPP).

vinculin upregulation allows CP-exposed daphnids to produce as many eggs as not-treated *Daphnia*.

In the proteome profile of *D. magna* originating from S clone, almost all proteins were upregulated in the presence of CP. These included the putative cold-shock DNA-binding family protein (upregulated 13.94-fold) and HSP83. Cold-shock proteins are multifunctional RNA/DNA binding proteins. Their biological activities range from the regulation of transcription, splicing and translation, to the orchestration of exosomal RNA content. HSP83 chaperon promotes maturation and structural protein maintenance. It is also considered as a biomarker in toxicology. Evidence links a skewed cold shock protein expression pattern with cancer and inflammatory diseases (Lindquist and Mertens, 2018). Alkylating agents such as CP and CDDP modify DNA, RNA, and proteins by alkylation, creating products that, if not repaired, are toxic or mutagenic to the cell and at high concentrations lead to cell death or result in cancer promotion. Here, we showed that even a low, environmental concentration of CP or CDDP causes proteome modifications similar to cancer transformation in human.

CDDP exposure caused much greater changes in the transcription profiles, with up to about 500 transcripts dysregulated, compared to 80 after CP treatment (Fig. 3). In the animals exposed to CDDP, the majority of transcripts were downregulated. This is reflected at the organismal level, where increased mortality is observed in animals exposed to CDDP but not to CP. The chronic treatment with low CD or CDDP doses caused a response in transcriptome similar to the one observed in *D. magna* treated with low concentrations of hydroxymethylfurfural (HMF). HMF – a plant-based chemical that potentially substitutes petroleum-based substances at the highest concentration changed the transcription levels of up to 1638 genes (Sartori et al., 2013; Swart et al., 2019).

The proteome changes were generally in accordance with

Table 3
Characteristics of genes/transcripts identified in *D. magna* treated with CP or CDPP, with the following analysis pathways: Xinb3 or SK assembly, or Xinb3, SK, or eugenes transcriptome, with the expression changed at least 1.5-fold (UP – uncharacterized protein).

Gene/transcripts:	Homolog:	Function:
APZ42_014895	LOC116935604	putative galactose-3-O-sulfotransferase 2
APZ42_017540	LOC116927875	UP
APZ42_017321	LOC116927046 GGWI01005376.1 GGWI01007660.1	UP
APZ42_022288	LOC116926351	UP
APZ42_018848	LOC116916100 GDIP01003730.1 GGWI01005664.1 Dapma7bEVm003379t1	UP
APZ42_028696	LOC116932977 GGWI01007821.1	glucose dehydrogenase
APZ42_017127	LOC116927090 GDIP01018060.1 GGWI01012634.1	UP/brachyurin-like
APZ42_017527	LOC116927868	UP
LOC116927196		UP
LOC116926887		UP
LOC116926898		lncRNA
LOC116930324	GGWI01008215.1	lazarillo protein-like, apolipoprotein D-like
LOC116929538	GGWI01006212.1	titin-like
LOC116931996	GGWI01022628.1 GGWI01009036.1	glucose dehydrogenase [FAD, quinone]-like
LOC116930322		apolipoprotein D-like
LOC116920699		UP
LOC116930323	GGWI01010607.1	apolipoprotein D-like
LOC116923742		extensin-like
LOC116918081		UP
LOC116918232		UP
LOC116918081	GGWI01007679.1	UP
GDIP01034727.1	Dapma7bEVm015434t1	UP
GDIP01130212.1		UP
GDIP01014878.1		UP
GDIP01093031.1		UP
GDIP01121867.1		UP
GDIP01200883.1		adventurous-gliding motility protein Z-like
GDIP01199633.1	GGWI01009630.1	extensin-like
GDIP01194876.1	GGWI01018429.1	calphotin-like
GGWI01013762.1		putative lysozyme-like
GGWI01014890.1		chorion peroxidase-like
GGWI01011340.1		acanthoscurrin-2-like
GGWI01018560.1		heme-binding protein 2-like
GGWI01012292.1		prisilkin-39-like/shematrin-like protein 1
GGWI01015184.1		titin homolog
GGWI01003655.1	GGWI01010569.1	trypsin alpha-like
GGWI01015810.1		chymotrypsin BI-like
GGWI01022959.1	GGWI01001502.1	F-box only protein 30-like
GGWI01021177.1		UP
GGWI01012143.1		acidic mammalian chitinase-like
GGWI01018931.1		extensin-like

transcriptome modifications. The most robust responses were observed for the CDDP treatment, followed by CP, for the *D. magna* individuals originating from the S clone. Almost all proteins were found to be elevated in pharmaceutical-exposed animals compared to non-treated ones (Fig. 10). On the other hand, in response to CP or CDDP, most of the *Daphnia* transcripts were either downregulated (analysed by regular aligners) or showed the equal bias of down- or upregulated ones (analysed with pseudoaligners). This phenomenon could reflect the different regulation of mRNA and proteins. Generally, most of the transcripts, especially those connected with energy metabolism, degradation and metabolism of proteins and chitin, could be downregulated, whereas proteins crucial for stress response – stabilised. Indeed, the functions of proteins identified by mass spectrometry are mostly consistent with the

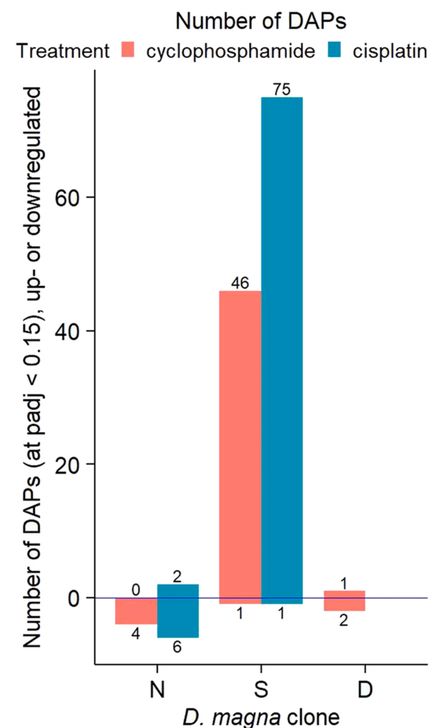

Fig. 10. Numbers of DAPs (padj < 0.1 or 0.15) appearing in *D. magna* based on MS semi-quantitative analysis, according to treatment (CP or CDPP).

functions of proteins encoded by mRNA taking part in energy metabolism, cytoskeleton reorganisation, and more directly in DNA and protein response to stress by inducing cold-shock DNA-binding proteins and DnaJ chaperone.

Interestingly, the expression of one long non-coding RNA was downregulated in *D. magna* when treated with pharmaceutical tested, as was with several other genes/transcripts with uncharacterised protein products. It should be taken into consideration that the abundance of several uncharacterised proteins was also changed during the treatments.

Calmodulin – a protein binding Ca^{2+}, poly(U)-specific endoribonuclease and beta-klotho novel β-glucuronidase are proteins generally involved in signal transduction as well as the response to stress (Laneve et al., 2008; Lee et al., 2021; Kuro-o et al., 1997; Olejnik et al., 2018). In the present study down- or upregulated calmodulin was common for all the responses except the *D. magna* N clones treated with CP. Both poly(U)-specific endoribonuclease and beta-klotho were common for three treatments: *D. magna* S treated with both chemicals and *D. magna* N treated with CDDP (Fig. 4). In our previous studies, CDDP exposition has led to the elevation of calmodulin level in *D. pulex*, whereas in *D. pulicaria* males, poly(U)-specific endoribonuclease prevailed (Grzesiuk et al., 2019). The proteins listed above are assumed to be important in the *Daphnia* response to CP and CDPP treatment.

Aquatic organisms developed defence systems against reactive oxygen species (ROS), including the induction of antioxidant enzymes such as superoxide dismutase (SOD). With the use of *D. magna*, in the response to oxidative stress, the enzymatic activities of ROS-induced SOD have been extensively employed as a biomarker for the evaluation of environmental stressors (Tang et al., 2011; Yu et al., 2009). In the present study, superoxide dismutase was downregulated in *D. magna* individuals from the N clone in the presence of CP. A similar response was obtained by Swart and co-workers (2019), when *Daphnia* was exposed to HMF (Lee et al., 2015).

Due to the rapid development of genomic technologies and their usefulness in ecotoxicological risk assessment, the term “ecotoxicogenomics” has emerged to describe the integration of various

techniques, i.e., transcriptomics, proteomics, metabolomics, and epigenomics into ecotoxicology. Ecotoxicogenomics is defined as the study of gene and protein expression in non-target organisms, and it provides insight into modes of action and helps in the evaluation of the potential toxicity of substances beyond the traditional endpoints of death and reproduction. Searching only for negative consequences of a substance is insufficient to fully describe the stress or adaptive response displayed by an organism to maintain homeostasis. Using this holistic approach, we observed the response of *Daphnia* to the alkylating type of pharmaceuticals, and mechanistic insights were provided into the pathways of genetic and biological processes underlying the shaping of the final phenotype.

5. Conclusions

Using *Daphnia* as a model organism, and with an ecotoxicogenomics approach, we have extended the study of the effects of anti-cancer drugs (CP and CDDP) polluting the environment of freshwater organisms. During the five-generation exposition to anticancer pharmaceuticals at a concentration detected in the environment, we observed changes in the *D. magna* life history parameters as well as proteome and transcriptome profiles. The reactions to anticancer agents observed at each level are complementary. Changes in the regulation of mRNA and proteins is key to understanding our results. In response to CP and CDDP, most of the *Daphnia* transcripts were either downregulated (analysed by regular aligners) or showed an equal bias of down- or upregulated ones (analysed by pseudoaligners). On the other hand, almost all proteins were found to be elevated in drug-exposed animals compared to non-treated ones. Most of the transcripts, especially related to energy metabolism, degradation, and the metabolism of proteins and chitin, could be downregulated, and proteins crucial for stress response can be stabilised. The functions of proteins are mostly consistent with the assignments of proteins encoded by mRNA participating in energy metabolism, cytoskeleton rearrangement, and more directly in DNA and protein response to stress.

The consequence of reorganisation at the molecular level is the allocation of energy, effecting life history parameters, i.e. a decrease in the number of eggs laid and a prolonged period of time at first reproduction. Observed mortality, together with other changes in life history, can lead to a reduction in the number of *Daphnia* individuals in the environment. Since the *Daphnia* genus controls algal biomass and also composes food for planktivorous fish, an environment contaminated with pharmaceuticals indirectly disturbs the functioning of aquatic food webs.

The ecotoxicogenomics approach makes it possible to get closer to a complete picture of the influence of CP and CDDP on *Daphnia* during long exposition in a laboratory which mimics conditions in contaminated freshwater. We have gathered evidence that animals in the presence of anticancer pharmaceuticals display the signs of stress, and that *Daphnia* attempt to cope with this permanent stress by changing their proteome and transcriptome profile. Additionally, our analyses show that CDDP had a stronger effect on the tested organisms than CP.

CRediT authorship contribution statement

Damian Mielecki: Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Elzbieta Grzesiuk**: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Anna Bednarska**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Damian Garbicz**: Investigation, Methodology, Writing – review & editing. **Bianka Świderska**: Data curation, Formal analysis, Validation. **Malgorzata Grzesiuk**: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Malgorzata Grzesiuk reports financial support was provided by National Science Centre.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.114372.

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