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Nickel and binary metal mixture responses in *Daphnia magna*: Molecular fingerprints and (sub)organismal effects

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ABSTRACT

The recent development of a custom cDNA microarray platform for one of thé standard organisms in aquatic toxicology, *Daphnia magna*, opened up new ways to mechanistic insights of toxicological responses. In this study, the mRNA expression of several genes and (sub)organismal responses (Cellular Energy Allocation, growth) were assayed after short-term waterborne metal exposure. Microarray analysis of Ni-exposed daphnids revealed several affected functional gene classes, of which the largest ones were involved in different metabolic processes (mainly protein and chitin related processes), cuticula turnover, transport and signal transduction. Furthermore, transcription of genes involved in oxygen transport and heme metabolism (haemoglobin, δ-aminolevilunate synthase) was down-regulated. Applying a Partial Least Squares regression on nickel fingerprints and biochemical (sub)organismal parameters revealed a set of co-varying genes (haemoglobin, RNA terminal phosphate cyclase, a ribosomal protein and an "unknown" gene fragment). An inverse relationship was seen between the mRNA expression levels of different cuticula proteins and available energy reserves. In addition to the nickel exposure, daphnids were exposed to binary mixtures of nickel and cadmium or nickel and lead. Using multivariate analysis techniques, the mixture mRNA expression fingerprints (Ni^{2+} + Cd^{2+}, Ni^{2+} + Pb^{2+}) were compared to those of the single metal treatments (Ni^{2+}, Cd^{2+}, Pb^{2+}). It was hypothesized that the molecular fingerprints of the mixtures would be additive combinations of the gene transcription profiles of the individual compounds present in the mixture. However, our results clearly showed additionally affected pathways after mixture treatment (e.g. additional affected genes involved in carbohydrate catabolic processes and proteolysis), indicating interactive molecular responses which are not merely the additive sum of the individual metals. These findings, although indicative of the complex nature of mixture toxicity evaluation, underline the potential of a toxicogenomics approach in gaining more mechanistic information on the effects of single compounds and mixtures.

1. Introduction

Although nickel is ubiquitous in the environment as a naturally occurring element, anthropogenic activities such as mining, smelting, refining and waste incineration activities contribute to the nickel loading in aquatic and terrestrial ecosystems. Nickel levels in estuaries and streams generally tend to range between 1 and 75 μg/L (Eisler, 1998). At highly nickel-contaminated sites groundwater levels can even reach concentrations of 2500 μg/L. Although most reported environmental exposure concentrations appear to be quite low, nickel toxicity is gaining interest with one of the key reasons lying within the European Union's (EU) Existing Substances Risk Assessment process categorizing Ni as a priority substance (EC 763/93). Moreover, the implementation of the Water Framework Directive guideline in 2000 (2000/60/EC) designed for development of European environmental quality guidelines also assigned a priority label to this metal.

In humans, nickel is known to cause a wide range of toxic effects, from skin dermatitis (Lidén and Norberg, 2005) over immunotoxic effects (Salsano et al., 2004) to several types of cancers (Hayes, 1997; Lu et al., 2005). Despite the reasonably well studied effects of this heavy metal in human and mammalian systems, there is still very little information present concerning modes of toxic action in aquatic ecosystems. In the rainbow trout mainly acute respiratory effects were detected, which were further evidenced by increases in hematocrit and plasma lactate values and a decrease in spleen haemoglobin (Pane et al., 2003a). However, in a study by Ptashynski et al. (2002) no differences regarding hematological endpoints were noted in lake whitefish after a dietary treatment. Ptashynski et al. (2002) reported histopathological alterations in liver and kidney

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to be the most sensitive endpoints. Waterborne exposures with *Daphnia magna* caused most significant effects on Mg^{2+} homeostasis, both acutely as chronically (Pane et al., 2003b). Additionally, a significant reduction in both oxygen consumption rate as well as whole-body haemoglobin concentration was seen in *D. magna* after chronic exposure.

Gaining relevant information about the modes of action of single compounds has already been proven a challenging task. However, in natural environments toxicants never occur as single contaminants, resulting in opportunities for interactions between compounds. Furthermore, from a toxicological point of view these interactions can highly influence the overall impact of chemical stressors on organisms. Although an increasing number of studies (Altenburger et al., 2004; Groten et al., 2004; Barata et al., 2006) invested large efforts for a better understanding of mixture toxicity, the complex interplay of different toxicants among several biological pathways still remains difficult to unravel. When mixture toxicity is discussed, effect data are usually fitted into either the concentration addition (CA) or the independent action (IA) model depending on the presumed (dis)similarity of modes of action of the compounds. However, a compound can elicit agonistic actions on one biological pathway, but show antagonistic responses on another. Therefore, contradictory results can easily be obtained depending on the endpoint studied (Cedergreen and Streibig, 2005; Barata et al., 2006). Nevertheless, single endpoint mixture studies generate considerable amounts of valuable information, specifically when compound-related biomarkers are used (e.g. loss of acetylcholinesterase activity in relation to organophosphate and carbamate pesticide exposures). So far, only the "top layer" of the toxic response has been revealed and it still remains impossible to elucidate actual modes of action or interactions between them. Acquiring in depth information regarding interactions of compounds requires multi-endpoint techniques which can provide an overview of several endpoints and mechanisms simultaneously, such as microarrays. Currently there are only a limited number of studies that have applied microarrays for evaluation of mixture toxicity responses (Bae et al., 2002; You and Bartolucci, 2004; Finne et al., 2007; Geoghegan et al., 2008). However, the potential assets of using cDNA microarray technology to help resolve mixture toxicity issues were highlighted in all of them.

The application of recent 'omics'-technologies in (eco)toxicology depends on the assumption that all toxicologically relevant effects are accompanied by alterations in gene expression patterns (Farr and Dun, 1999). However, to improve our understanding of complex exposure situations and develop adequate tools for effect assessment it is not only essential to study effects on multiple endpoints using microarrays but also to gain insight in effects on multiple levels of biological organization. The evaluation of additional endpoints such as the Cellular Energy Allocation (CEA) methodology (De Coen and Janssen, 2003; Smolders et al., 2004; Verslycke et al., 2004) for the assessment of – toxic stress induced – adverse effects on the energy budget of *D. magna* can provide valuable additional organismal information. Moreover, the relationship and usefulness of CEA measurements for prediction of long-term effects in *D. magna* was shown by De Coen and Janssen (2003).

In the present study, microarrays are applied with two major purposes. The first aim is to elucidate unknown toxic actions of waterborne nickel to the crustacean *D. magna* at both the gene transcription and (sub)organismal level (CEA and growth). The second objective is to investigate the potential biological/molecular interplay associated with binary mixtures of nickel and other metals (cadmium and lead) using DNA arrays as a multi-endpoint technique. Furthermore, it is questioned whether gene expression patterns of binary metal mixture stress are reducible to the sum of the effects of individual metals or whether additional pathways are affected.

2. Materials and methods

2.1. Experimental organisms

A single clone of *D. magna* was used to perform the toxicity tests. For the culturing, 1 L glass recipients containing aerated and biofiltered tap water were used to hold 20 organisms each. They were held at a constant temperature of $20 \pm 1\,^{\circ}C$ and at a photoperiod of 14 h light/10 h dark. The medium of the cultures was renewed three times weekly and the water fleas were fed a mixture of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio (4×10^5 cells/mL).

2.2. Chemicals and experimental setup

During all exposures, *Daphnia* were kept at a density of 10 organisms/100 mL OECD standard water ($CaCl_2 \cdot 2H_2O$, 2 mM; $MgSO_4 \cdot 7H_2O$, 500 μM; $NaHCO_3$, 771 μM; KCl, 77.1 μM; water hardness, 250 mg $CaCO_3$; pH 7.8; OECD guideline 203, annex 2) and were fed at 0 and 48 h with a mixture of *P. subcapitata* and *C. reinhardtii* in a 3:1 ratio (4×10^5 cells/mL). The volume of the test solution was adjusted according to the amount of organisms used in each test. Test concentrations of the used metals were confirmed by inductively coupled plasma mass spectrometry (ICP-MS) (Varian, Australia) and were all within 10% of the nominal values.

2.2.1. Single metal exposures

Daphnia were exposed for 96 h to either a control or four different concentrations of Ni^{2+} (0.125, 0.5, 1 and 2 mg/L) ($NiCl_2 \cdot 6H_2O$, INC Biomedicals, USA). These concentrations are equal 0, 0.02, 0.09, 0.18 and 0.36 Toxic Units (TU) when one TU is defined as the EC_{50} (immobility at 48 h) value of nickel when applied separately. After 96 h *Daphnia* were collected for cDNA microarray analysis and determinations of energy budgets. For the growth follow-up, organisms were exposed to the same concentrations, but were collected daily until day 4. All treatments were done in triplicate.

2.2.2. Binary mixture exposures

Daphnia were exposed to a Ni^{2+} (0.5 mg/L)–Pb^{2+} (0.5 mg/L) and a Ni^{2+} (0.5 mg/L)–Cd^{2+} (0.05 mg/L) mixture ($CdCl_2 \cdot H_2O$ and $Pb(NO_3)_2$ Merck, Germany). In the tested mixtures, each metal component contributes to the toxic loading of the mixture with 0.09 TU (calculations based on the individual compound EC_{50} (immobility at 48 h) values). Assuming that the toxicity of the metals remains unchanged in their mixture application, the total toxic dose of the mixture was assumed to be equal to 0.18 TU. Hereafter, conditions are indicated as 0.09 TU Ni^{2+} + 0.09 TU Pb^{2+} and 0.09 TU Ni^{2+} + 0.09 TU Cd^{2+}. The datasets of cadmium and lead as individual metals (equal to 0.09 and 0.18 TU of the individual metals) were presented previously (Soetaert et al., 2007b; Soetaert, 2007).

2.3. Microarray experiments

After 96 h of exposure, triplicate groups of approximately 45 *Daphnia*/replicate were collected. Samples were shock-frozen in liquid nitrogen and stored in RNAlater (Ambion, USA) at −80 °C until further analysis.

2.3.1. RNA extractions

These were performed using the TRIzol® method (Invitrogen, Belgium) followed by a DNAse treatment using 1 U RNAse-free DNAse and 1 U RNAse inhibitor (Fermentas, Germany) per 30 μL sample and subsequent phenol/chloroform extractions. The purity of the RNA samples was checked using the ND-1000 spectrophotometer (Nanodrop®, USA) through measurement of the

260 nm/280 nm and 260 nm/230 nm absorbance. For all used samples these ratios were above 1.90 and 2.10, respectively. To verify the intactness of the RNA samples, a denaturating formaldehyde agarose gel electrophoresis was performed to visualize the 18S and 28S ribosomal bands. Aliquots from the control samples – with equal RNA concentrations (μg) – were pooled for use as reference material.

2.3.2. The production of cDNA and subsequent amino-allyl labelling

These were performed according to the protocol described in detail by van der Ven et al. (2005) and Soetaert et al. (2006). In brief, 7 μg of total RNA was mixed with Lucidea control mRNA spike mix (Amersham, UK) to be reverse-transcribed using Superscript II, Random hexamer primers (both Invitrogen) in the presence of dNTPs with 2/3 aa-dTTP/dTTP (Sigma–Aldrich, Belgium). After an overnight incubation period (42 °C) the amino-allyl-incorporated cDNA was purified using a modified Qiagen PCR spin column protocol (van der Ven et al., 2005). In a next step, Cy3/Cy5 esters (Amersham) were used for covalent coupling to the amino-allyl labelled cDNA. The vacuum dried cDNA samples (dissolved in a 0.1 M carbonate buffer (pH 9.0)) were mixed with the Cy3/Cy5 esters (dissolved in 100% DMSO) and incubated for an hour in the dark at room temperature. Hereafter, a second cleanup reaction was performed to establish the removal of the remaining uncoupled dyes (QIAquick PCR purification kit, Qiagen, USA). The quality of eluted and labelled cDNA was analysed using the Nanodrop® spectrophotometer. Samples with an FOI (frequence of incorporated dye) between 20 and 50 were selected for hybridization and an amount of 150 pmol labelled target was vacuum dried.

2.3.3. Hybridization onto the D. magna microarray

The previously developed custom *D. magna* cDNA microarray (Soetaert et al., 2006, 2007a) containing 2445 life stage, moulting and energy metabolism related gene fragments was used. Triplicates of the cDNA fragments were spotted onto amino-coated glass slides (Asper biotech Ltd., Estonia) using the Qarray Mini (Genetix Ltd., UK). Sequencing of all fragments included in these three cDNA libraries was performed to get a more complete overview on the amount of unique fragments on the microarray. All bad quality sequences (too short or vector contaminated) were removed from the analysis and remaining gene fragments were aligned and assembled using CAP3. This resulted in 288 contigs and 901 singletons which provided us with an estimated 1189 unique gene fragments on the microarray.

Next to the *D. magna* cDNA fragments, 20 artificial quality control fragments from the Lucidea universal scorecard (Amersham) were spotted in 20-fold to ensure evaluation of the overall array quality. More specifically, 10 calibration controls were used to evaluate the dynamic range and sensitivity of the platform, 8 ratio controls represent low and high microarray expression levels and 2 negative controls allow evaluation of non-specific hybridization. The final step in the spotting protocol included rehydration of the spots over a hot water bath, snap-drying and cross-linking (UV Stratalinker 2400, Stratagene, USA). Prior to hybridization, arrays were incubated for 30–45 min at 42 °C in a prehybridization solution (50% formamide, 5× saline sodium citrate (SSC), 0.1% sodium dodecyl sulphate (SDS), 0.1 mg/mL bovine serum albumine (BSA)). The vacuum dried targets (both reference and treatment target) were resuspended in a hybridization solution (50% formamide, 5× SSC, 0.1% SDS, 0.1 mg/mL BSA and 0.1 mg/mL sheared salmon sperm) and denaturated at 95 °C. The cooled targets were subsequently applied onto the prehybridized slides and incubated overnight at 42 °C. After hybridization, arrays were washed in solutions with increasing stringency (decreasing concentrations of SSC and SDS) and dried with N_2.

2.3.4. Bioinformatics evaluation of the microarrays

Scanning and analysing of the slides was performed using the Genepix personal 4100 Scanner and Genepix pro Software (both Axon instruments, USA). Cy3 and Cy5 fluorescent signals were scanned at, respectively, 532 and 635 nm and the PMT (photomultiplier tube) values were adjusted to reach a ratio (Cy5/Cy3) around 1. Spots were identified and ratios quantified by means of the Genepix software 5.0 (Axon Instruments). The BioArray Software Environment Database (BASE 1.2.12, http://www.islab.ua.ac.be/base/) was used for storage, further evaluation and succeeding statistical analysis of the microarray datasets. In addition, the MIAME compliant data was submitted to the Gene Expression Omnibus (GEO) at the NCBI website (platform: GPL7742; series: GSE13866). After a local background correction, spots with a foreground larger than the mean local background +2 standard deviations for one or both colours were selected for further analysis. The $\log_2$ transformed Cy3/Cy5 ratio data were normalized by implementation of a Locally Weighed Scatterplot Smoothing (Lowess) (Yang et al., 2002) step, which is an intensity-based method. Significant vertical cut-off values of log −0.85 and 0.85 were determined through multiple self–self hybridizations. These were performed by labelling the same biological material with both Cy3 and Cy5 dyes and hybridizing them simultaneously on a microarray slide. To statistically evaluate the microarray results, Significance Analysis of Microarrays (SAM) described by Tusher et al. (2001) was applied on the Lowess normalized datasets. With this SAM approach a false discovery rate (FDR) below 5% was applied. As a result genes with $\log_2$ ratios outside the confidence interval of −0.85 and 0.85 were considered as differentially expressed gene fragments when FDR was below 5%.

2.4. Real-Time PCR confirmation

Real-Time quantitative PCR was used in order to validate relative mRNA expression changes of six selected gene fragments after nickel and metal mixture treatment (Table 1). In order to produce first strand cDNA of the different targets, the Superscript™ first strand synthesis system for Real-Time PCR kit (Invitrogen) was used according to manufacturer's instructions. The Lightcycler Fast start DNA master SYBR green I kit (Roche Diagnostics, Germany) enabled quantitative PCR confirmation on the LightCycler 3.5 (Roche Diagnostics, Germany). The used experimental protocol consisted of a denaturation phase (5′ at 95 °C), an amplification phase of 40 cycles (10″ at 95 °C, 10″ at 58 °C and 12″ at 72 °C) and a final melting phase. Primer sequences were designed using Roche Molecular LightCycler Probe Design software 1.0 (Table 1). Four potential housekeeping genes (beta-tubulin, actin, G3PDH, prohibitin 2) were compared to evaluate the variability of mRNA expression between exposure conditions. When comparing standard deviation on their respective Ct values the following order could be noted: Beta-tubulin (0.66) < actin (0.73) < G3PDH (1.06) < prohibitin 2 (1.21). As a result, beta-tubulin was selected as housekeeping gene for normalization and quantification of the Real-Time-PCR data. Relative mRNA expression levels were calculated and statistically evaluated according to the REST formula (Pfaffl et al., 2002).

2.5. (Sub)organismal parameters – Cellular Energy Allocation and growth

Whole-body homogenates of 30 pooled *Daphnia* taken after 0 and 96 h of exposure to the different nickel concentrations (0, 0.125, 0.5, 1 and 2 mg/L Ni^{2+}) were used to measure the available (proteins, lipids, sugars) and the consumed (Electron Transport System, ETS) energy according to the CEA protocol described in De Coen and Janssen (2003). All measurements of the energy reserve fractions were determined spectrophotometrically and transformed into

Table 1
Gene fragments and corresponding primers selected for Real-Time PCR.

Gene fragment – accession number	Forward primer	Reverse primer	Primer efficiency
a. Beta-tubulin – DV075795	5′-CCAGAACTCACTCAGC-3′	5′-AGCCTTACGACGGAAC-3′	1.94
b. Actin – AJ292554	5′-CCAAGGCTAATCGTGAGA-3′	5′-TGTAACCGCGTTCAGT-3′	1.87
c. G3PDH – AJ292555	5′-GGATTCGGTCGTATTGGC-3′	5′-TCAGCTCCAGCAGTTC-3′	1.92
d. Prohibitin 2 – DW724510	5′-AATTGTTCAAGCCGAGG-3′	5′-CGTCAAAGGAAACGTCAC-3′	1.70
b. δ-Aminolevilunate synthase (δ-ALAS) – DW724592	5′-GAAGGTAGACAACTGCG-3′	5′-CTCGGCACTGTCGGATA-3′	1.89
c. Haemoglobin – DW724693	5′-GGAAGCGGATTCACTG-3′	5′-TGTCACCCATAGCCGA-3′	1.87
d. Opsin – DW724560	5′-TCCTCGTGCTTGAAGAC-3′	5′-GCGCTTGTTTCGGATAC-3′	1.80
e. Vitellogenin – AB114859	5′-CCAGCGAATCCTACACC-3′	5′-CGCACAGACCACAGAG-3′	1.81
f. JH-esterase – DW724473	5′-TGGATACGCTTGCACTG-3′	5′-CGCTTCTACCTGTCATTG-3′	1.77
g. RNA terminal phosphate cyclase dom1 – DW724698	5′-CGGCAGTTTCACATGATAG-3′	5′-TTCGTCGCTGGCACCATA-3′	1.96

energetic equivalents using their respective energy of combustion (39.5 kJ/g lipid, 24 kJ/g protein, 17.5 kJ/g glycogen) and oxyenthalpic equivalents (480 kJ/mol O_2) (Gnaiger, 1983). Measurement of the protein content was performed by the Bradford method (Bradford, 1976), a standard curve of bovine serum albumine (Sigma, USA) was used and concentrations were determined at 592 nm. From the same body homogenate total carbohydrate content was extracted using trichloracetic acid (Across organics, Belgium) and visualized with 5% phenol and H_2SO_4 at 492 nm. A glucose (Sigma, USA) standard curve was used for quantification. Lipids were isolated through the chloroform–methanol extraction method and measured at 375 nm (Bligh and Dyer, 1959). Hereby, lipid concentrations were calculated by means of a standard curve of tripalmitin (Sigma, USA). The consumed energy, hence the consumed oxygen rate, was estimated through measurement of the electron transport activity (King and Packard, 1975) over a 96 h exposure period. Finally, the Cellular Energy parameter was calculated by integrating the available and consumed energy over the 96 h exposure period.

Besides energy parameters, growth was monitored by daily collection of 15 organisms per condition over the 4 days exposure period. *Daphnia* were stored in a sucrose–formaldehyde solution (4% formaldehyde; 12% sucrose) and lengths were determined by measuring the distance of the carapax from head till spine by means of a microprojector (Projectina, Switzerland).

On all (sub)organismal parameters measured, univariate statistics using the Statistica Software package (StatSoft, USA) was performed. After testing the data for normality and homogeneity of variance, a one-way analysis of variance (ANOVA) was done, followed by the post hoc Tukey HSD test if significant differences ($p < 0.05$) were found.

2.6. Multivariate and clustering analysis

Multivariate analysis techniques enable a reduction of the multidimensionality of complex datasets. In the present study, Principal Component Analysis (PCA) and Partial Least Squares (PLS) were applied. A major advantage of these projection methods is that they deal with microarray data associated problems such as missing values, collinearity and the presence of more variables than observations. All multivariate analyses were performed using the Simca-P 11.5 software package (UMETRICS, 1997). Clustering analyses were done using the MultiExperiment Viewer (MeV, TIGR).

3. Results

3.1. Single substance toxicity – Ni^{2+}

3.1.1. Microarray experiments

Microarray experiments were performed for all four Ni^{2+} treatments (0.125, 0.5, 1 and 2 mg/L Ni^{2+}). A reference design was used where the RNA from exposed (and control) *Daphnia* was compared to pooled RNA from control *Daphnia*. Using three biological microarray replicates, the biological relevance of the data was optimized.

Sequencing of all gene fragments present in the three selected cDNA libraries resulted in the identification of 1189 unique fragments on the used microarray platform. Evaluation of the four nickel treatments showed that 10.1% (120 genes) of all unique fragments on the microarray were affected in at least one of the treatments. Subsequently, the nucleotide sequences of these 120 significantly differentially expressed gene mRNAs were submitted to Blast2GO (http://www.blast2go.de) (Conesa et al., 2005), a Gene Ontology (GO) and annotation tool which enables blast-searching and GO annotation in one run. This Blast2GO analysis was able to identify 55.8% (Fig. 1, numbers 1–67) of all differentially transcribed fragments due to significant homology with other known protein/nucleotide sequences (Fig. 1, numbers 68–120). The genes lacking significant homology to other genes/proteins were appointed as "unknown" gene transcripts. Increasing nickel concentrations caused a higher number of differentially expressed genes to be influenced (7, 19, 55, 107 after respective 0.125, 0.5, 1 and 2 mg/L Ni^{2+}).

Using the Blast2GO tool, the biological processes and molecular functions were identified and evaluated for the "known" fragments. The biological processes primarily predicted to be affected are different metabolic processes (protein, chitin, carbohydrate metabolic processes), transport (ATP, lipid, oxygen) and signal transduction related processes such as visual and sensory perception. In the evaluation of the molecular functions, 'constituents of the cuticula' was the largest GO category predicted to be affected. Binding activity of different substrates (protein, ATP, chitin, zinc and calcium ion) was identified as the second largest. Fig. 2 – in which each gene is appointed to one single GO category – provides a summary of these results. Certain categories are composed of mainly repressed fragments due to nickel treatment, such as, transport of lipids, ATP and oxygen (Fig. 1). However, also some processes with significantly induced transcription – e.g. visual and sensory perception (within the signal transduction category) – could be identified. Examples of gene fragments involved in this process are homologues of opsin 1, opsin 2 and rhodopsin 4. Furthermore, the transcription of almost all gene fragments involved in the formation of the carapax and the chitin metabolism was induced in the organisms exposed to the three highest nickel concentrations.

3.1.2. (Sub)organismal effects – CEA and growth

Energy budgets were determined after 96 h of exposure to the four different Ni^{2+} treatments (Fig. 3A–C). Cellular Energy Allocation, which integrates both consumed as well as available energy over a 96 h time-interval, was significantly increased after exposure to the lowest selected nickel concentration (0.125 mg/L Ni^{2+}). Nonetheless, all other exposure conditions (0.5, 1 and 2 mg/L Ni^{2+}) caused a significant decrease of the CEA. In parallel with the energy budgets, the growth of the organisms was monitored during a consecutive period of 4 days (Fig. 3D). Already after an exposure period of 3 days a significant decrease in length was noticed in the two

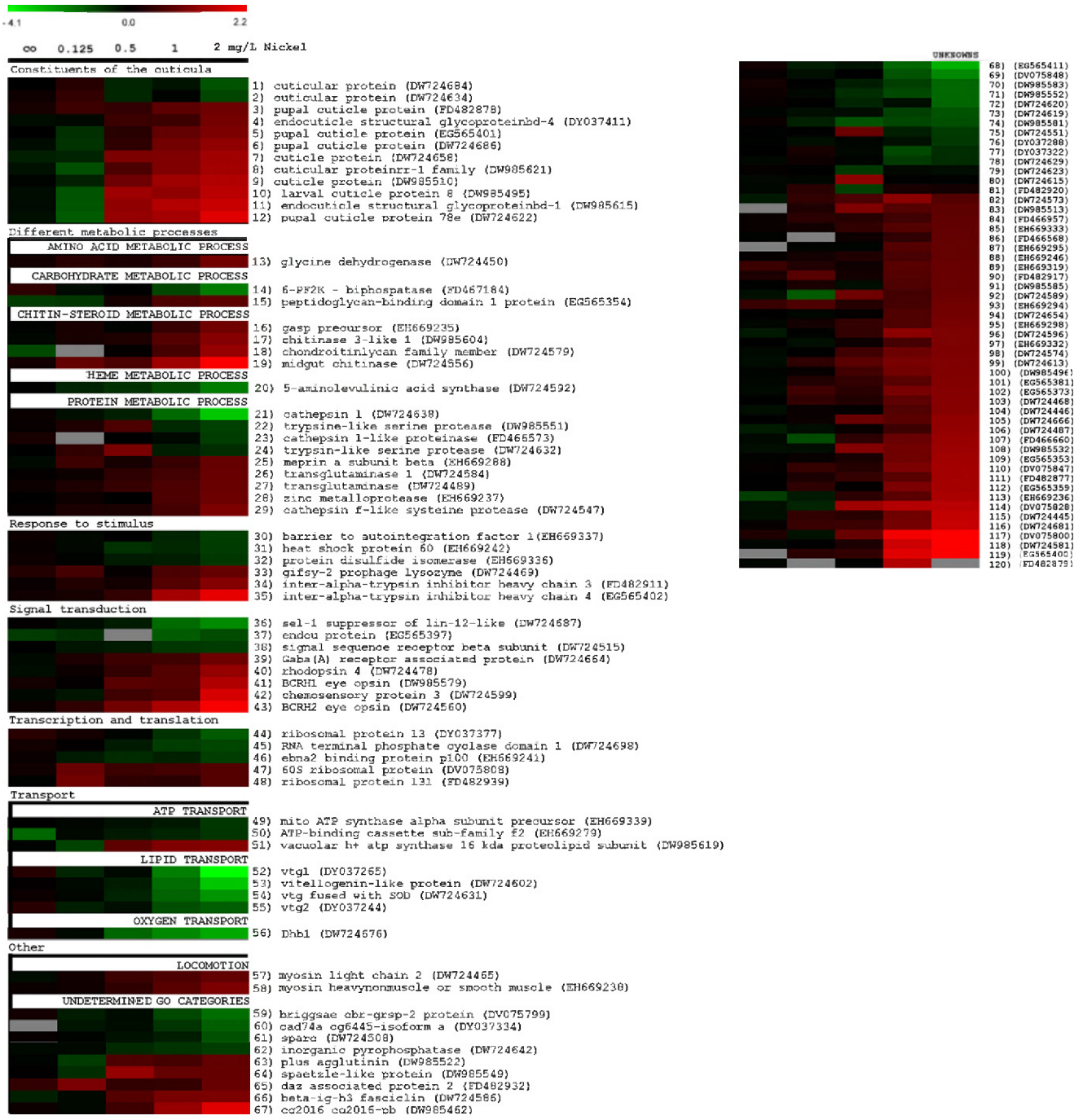

Fig. 1. A heat map of significantly differentially transcribed genes after 96 h of exposure to 0.125, 0.5, 1, 2 mg/L Ni^{2+}. Genes are divided into functional classes according to their GO annotation. mRNAs for down-regulated gene fragments are represented by a green horizontal bar and up-regulated ones by a red bar. The fragments with a darker shade of green/red ranging to black show less to no differential gene transcription.

highest nickel treatments (1 and 2 mg/L Ni^{2+}) ($p < 0.05$). From day 4, a decrease in length in the 0.5 mg/L Ni^{2+} exposure treatment was also seen ($p < 0.05$).

3.1.3. *Multivariate analysis: from genes to (sub)organismal effects*

A PLS analysis was performed in order to analyse the relationship between transcriptional and biochemical level responses. As CEA has been shown to be a valuable predictor for population effects (De Coen and Janssen, 2003) we tried to relate these biochemical responses with effects at the transcriptional level. The two-component PLS analysis led to a model with an $R^2 = 0.77$ and $Q^2 = 0.52$. These values represent the predictive capacities of the model, expressed as the predictive residual sum of squares, noted as Q^2 or the cross-validated R^2. Results are visualized in Fig. 4, which illustrates that the prediction of the overall energy budget of the organisms is best represented by the protein and lipid fraction. In a study by De Coen and Janssen (2003) – where a range of different chemicals was tested – they also identified the lipid fraction as most important. The genes which contributed the most to the model were identified by selecting those genes with a Variable Importance (VIP) score above one for both components. Among these genes two groups could be identified. A first group shows a direct link to

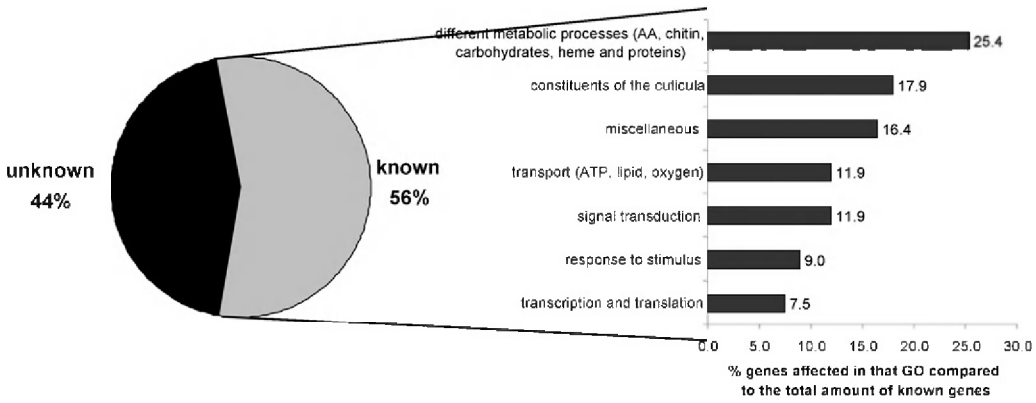

Fig. 2. All unique and "known" differentially transcribed fragments subdivided into functional classes according to their GO annotation.

the energy reserves and a second group has an inverse relationship with the biochemical endpoints. Genes which are part of this first group are genes coding for haemoglobin (fragment 56, numbers correspond to numbers in Fig. 1), two fragments involved in transcriptional and translational processes (a ribosomal fragment (fragment 44), and RNA terminal phosphate cyclase (fragment 45)), an unknown (fragment 77) and two cuticula proteins (fragments 1 and 2). Genes included in the second group of fragments are mainly fragments coding for processes involved in the cuticula metabolism. These results indicate that among the cuticula metabolism related fragments two different sets (probably indicating different regulatory control) were identified. mRNAs of cuticula related fragments 1 and 2 co-vary with the energy reserves while fragments 4–12 and 18 show an inverse relationship with the CEA parameters.

3.2. Mixture toxicity – microarray experiments

Exposing *Daphnia* for 96 h to binary metal mixtures of 0.09 TU Ni^{2} + 0.09 TU Pb^{2+} and 0.09 TU Ni^{2+} + 0.09 TU Cd^{2+} enabled the comparison of molecular responses with equitoxic treatments of the single metals (0.18 TU). The binary mixture and nickel molecular fingerprints were generated in this study, but the gene transcription datasets of single component exposures with cadmium and lead (both 0.09 TU and 0.18 TU of the individual metals) were previously published by Soetaert et al. (2007b) and Soetaert (2007). The latter datasets facilitated comparison between molecular fingerprints of all three metals at similar levels of toxicity (TU). Moreover, the same clone of *D. magna* and an identical microarray platform were used in both previous

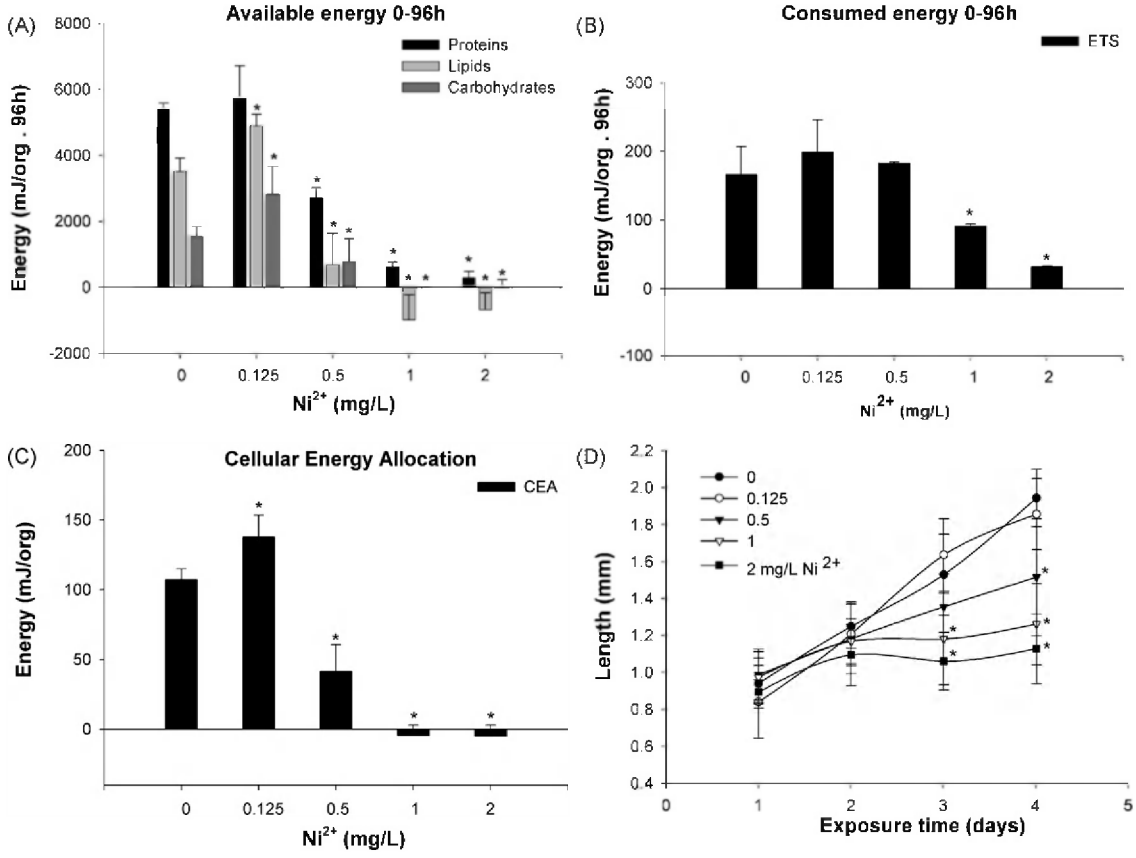

Fig. 3. (Sub)organismal effects after nickel exposure: (A) available energy, (B) consumed energy, (C) Cellular Energy Allocation and (D) length of the daphnids over a 4 days exposure period. Significant differences ($p < 0.05$) are indicated by "*".

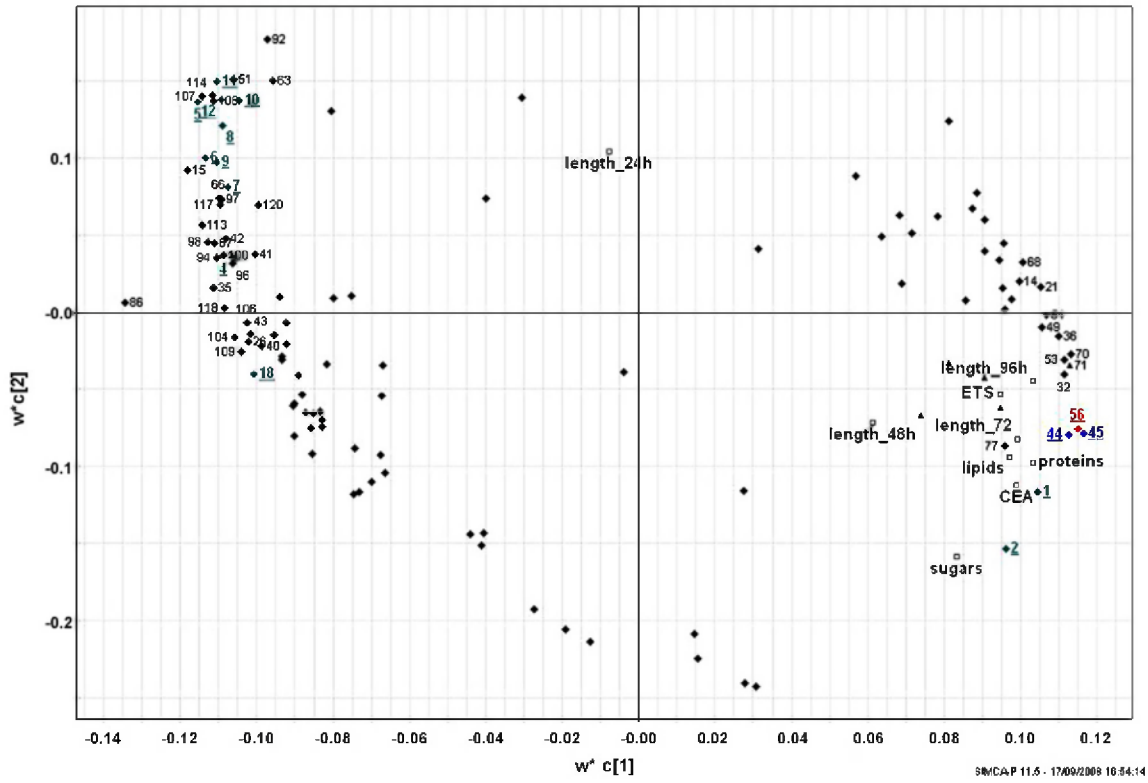

Fig. 4. PLS analysis of different nickel treatments, using differentially expressed mRNAs (●) as predictors and (sub)organismal effects (□) as response data. Genes are numbered from 1 to 120 concordant to numbering in Fig. 1. R^2 and Q^2 for this two-compartment model are, respectively, 0.77 and 0.52. Only the genes with a Variable Importance (VIP) score above 1 for both components in the model are labelled with their respective number.

as present studies, which greatly improves comparability of the datasets.

3.2.1. Similarity or dissimilarity of the single compound and mixture gene expression profiles

The differentially expressed gene transcripts, caused by the eight treatments (three individual metals tested at 0.09 TU and 0.18 TU and two mixtures), i.e. those of 258 unique gene fragments, were used for PCA analysis (Fig. 5). The score plot of the PCA shows a three-component model resulting in a R^2 value of 0.64 and a Q^2 of 0.44, with 64% of the total variance being explained. The first PC – explaining the majority of the variance (39%) – produces a first distinction between the profiles of the mixtures and the single compounds. The second and third PC accounted for 14 and 11% of the variance, respectively. Five major clusters were identified: (A) 0.09 TU Ni^{2+} + 0.09 TU Cd^{2+}, (B) 0.09 TU Ni^{2+} + 0.09 TU Pb^{2+}, (C) 0.18 TU Cd^{2+}, (D) 0.09 TU Cd^{2+} and (E) the biggest cluster containing all other treatments (0.09 TU Ni^{2+},0.09 TU Pb^{2+}, 0.18 TU Ni^{2+} and 0.18 TU Pb^{2+}).

We wanted to compare the molecular responses of the mixtures with those of the individual treatments, therefore the five equitoxic treatments were compared. We started to assess the compound-specificity of the molecular fingerprints by identifying the uniquely responding genesets (i.e. gene transcripts induced uniquely by one treatment) and the overlapping genesets (i.e. gene transcripts affected by more than one treatment) (Fig. 6). An increase in the number of differentially expressed gene transcripts could be observed in the following order: 0.18 TU Pb^{2+} (14) < 0.18 TU Ni^{2+} (55) < 0.18 TU Cd^{2+} (62) < 0.09 TU Ni^{2+} + 0.09 TU Cd^{2+} (127) < 0.09 TU Ni^{2+} + 0.09 TU Pb^{2+} (165). Among all five equally toxic treatments, seven common differentially expressed (repressed) gene transcripts were identified, i.e. those of genes coding for haemoglobin (Dhb1), cathepsin I, vtg1, vtg-like protein, RNA terminal phosphate

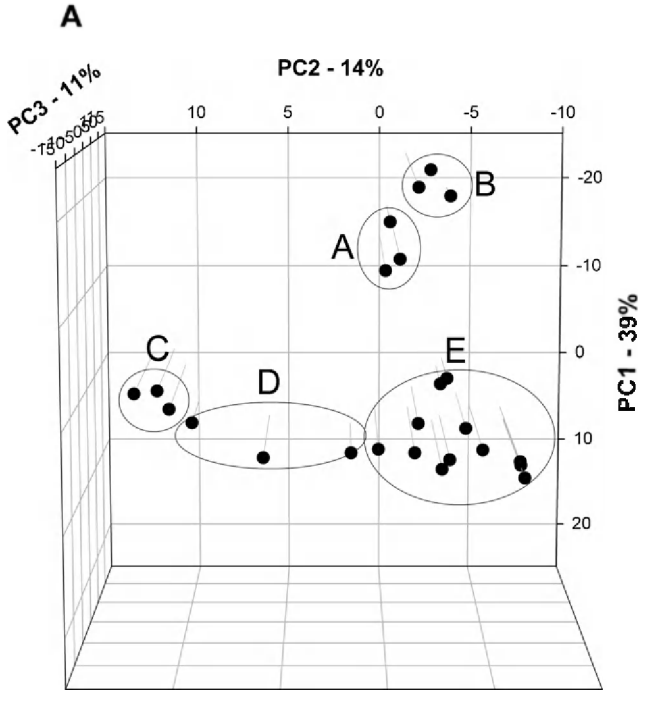

Fig. 5. PCA analysis of all differentially expressed genes of the mixtures (0.09 TU Ni^{2+} + 0.09 TU Pb^{2+}, 0.09 TU Ni^{2+} + 0.09 TU Cd^{2+}) and single metal treatments (0.09 TU Ni^{2+}, 0.09 TU Cd^{2+}, 0.09 TU Pb^{2+} and 0.18 TU Ni^{2+}, 0.18 TU Cd^{2+} and 0.18 TU Pb^{2+}). The total variance explained by the three components was 64%. The compositions of the different clusters are: (A) 0.09 TU Ni^{2+} + 0.09 TU Cd^{2+}, (B) 0.09 TU Ni^{2+} + 0.09 TU Pb^{2+}, (C) 0.18 TU Cd^{2+}, (D) 0.09 TU Cd^{2+} and (E) the biggest cluster containing all other treatments (0.09 TU Ni^{2+}, 0.09 TU Pb^{2+}, 0.18 TU Ni^{2+} and 0.18 TU Pb^{2+}).

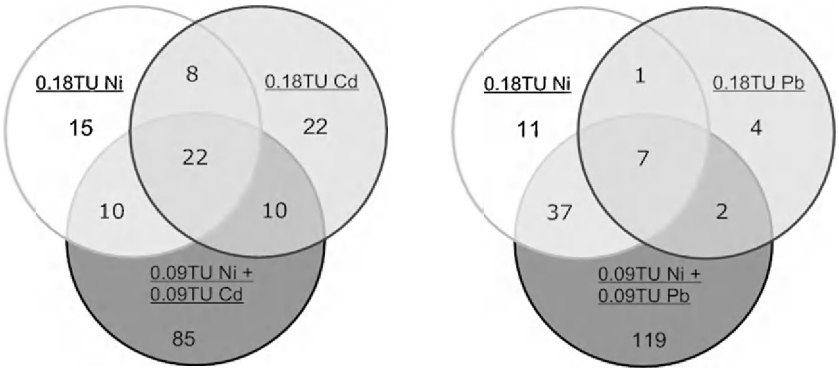

Fig. 6. A Venn diagram representation of the number of genes shared by the treatments equal to 0.18 TU for either Ni^{2+}, Cd^{2+} and Ni^{2+} + Cd^{2+} or for the Ni^{2+}, Pb^{2+} and Ni^{2+} + Pb^{2+} treatment.

cyclase domain 1, and 2 unknown fragments; respective GenBank accession nos. DW724678, DW724638, DY037265, DW724602, DW724698, DY037322 and EG565411).

3.2.2. *GO driven analysis of single compounds versus mixtures*

In order to further compare the mixtures and their interactions, a GO driven analysis was used to identify the genes and pathways underlying the differences in single compound compared to mixture exposure responses. Both binary mixtures and their respective components (at equitoxic concentrations) were evaluated using this approach (Fig. 7). The differentially expressed gene transcripts after exposure to one of the mixtures and its respective components were selected and subjected to a K-means clustering with the pre-definement of five types of responses (gene identities and their mRNA expression ratios are provided as supplementary information Tables 2 and 3). The rationale for selecting five types of responses was based on prior hierarchical clustering results (data not shown). The same set of genes (differentially expressed gene transcripts from one mixture and its individual components) was then submitted to Blast2GO for identification of the most important GO categories. To gain as much in depth GO information as possible – but still have a representation of all annotated fragments – we selected only those GO categories which were composed of at least five sequences (the GO categories presented in Fig. 7). In order to detect GO-related differences between the five clusters identified by K-means clustering, the GO terms associated within the individual clusters were plotted against the GO terms of the complete gene set.

The mRNA expression responses (or clusters) from which we gain most information regarding the mixture behaviour are the ones which behave differently in the mixture compared to the single compound treatments. Regarding the Ni–Cd mixture this refers to clusters 1, 2 and 4 (Fig. 7). Identification of the most prominent GO categories in the different clusters shows that cluster 1 is represented by different additionally induced processes of which the largest ones include polysaccharide catabolic processes, response to stimuli and proteolysis. Examples of genes involved in these processes are (among others) genes coding for a mannanase precursor, chymotrypsin-like serine proteinases, cellulases, carboxypeptidase, amylase. Cluster 2 is represented by genes coding for additionally repressed processes such as multicellular organismal processes and biopolymer metabolic processes. Clear interaction behaviour (with an up-regulation in the nickel treatment, a slight down-regulation in the cadmium treatment and a severe down-regulation in the mixture) is demonstrated by the fourth cluster which is mainly composed of genes coding for structural constituents of the cuticula. In the Ni–Pb mixture similar types of transcript expression responses – as identified in the Ni–Cd treatment – were seen (Fig. 7; clusters a–c). Cluster b which represents genes coding for additionally induced processes includes mainly response to stimuli and

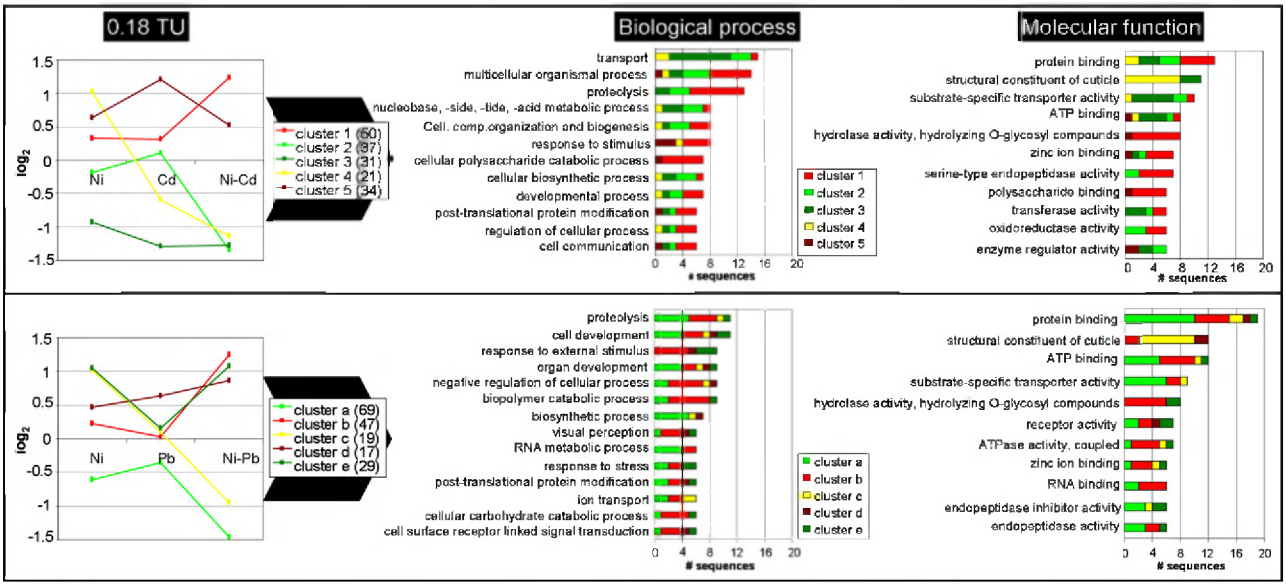

Fig. 7. The biological processes and molecular functions of the differentially transcribed genes due to Ni–Cd and Ni–Pb treatments and their respective individual components. The genes were subdivided into five different clusters using K-means clustering and the representation of these clusters within each GO category is represented as such.

Table 2
Real-Time PCR results for the selected gene fragments. Significantly differentially transcribed fragments are indicated by "*" (both for the microarray results as for the Real-Time PCR results).

	0.02 TU Ni		0.09 TU Ni		0.18 TU Ni		0.36 TU Ni		0.09 TU Ni + 0.09 TU Cd		0.09 TU Ni + 0.09 TU Pb	
	μA	RT-PCR	μA	RT-PCR	μA	RT-PCR	μA	RT-PCR	μA	RT-PCR	μA	RT-PCR
δ-ALAS	−0.59	0.43	−0.80	−0.53	−1.19*	−0.95	−1.99*	−1.91	−0.78	−0.64	−1.49*	−1.61
Dhb1	−0.15	0.70*	−1.64*	−1.30	−2.20*	−2.35*	−2.73*	−2.38*	−2.96*	−2.99*	−2.91*	−2.68*
Opsin	0.42	1.20*	0.99*	1.41*	1.49*	2.30*	2.14*	3.79*	2.17*	2.73	1.96*	2.97
Vtg1	−0.60	−0.89	−0.16	−0.02	−2.05*	−3.39*	−4.08*	−7.72	−3.11*	−6.46*	−3.53*	−5.60*
JH-esterase	−0.07	1.96*	0.29	0.32	0.20	1.13*	0.21	0.92	0.50	2.96	0.29	2.13
RNA terminal	0.04	0.25	−0.43	−0.40	−1.00*	−0.05	−1.11*	−0.04	−2.14*	−0.55	−2.44*	−1.45

catabolic processes (biopolymer, carbohydrate). Cluster a displays genes coding for additionally repressed processes and includes mostly biosynthetic and developmental processes. Cluster c which behaves in a similar manner as the fourth cluster in the Ni–Cd mixture is also composed of genes coding for structural constituents of the cuticula.

3.3. Real-Time PCR confirmation

A set of six genes was selected for Real-Time PCR confirmation (Table 1). For the majority of these selected genes the Real-Time PCR results confirmed the microarray responses (Table 2). In 92% of the cases, the trend (mRNA up- or down-regulation) was the same when comparing microarray results with Real-Time PCR results. All conditions for which the trend of transcription was not confirmed were also not significantly differentially transcribed according to the microarray analysis. Regarding actual levels of mRNA expression (and significance) we noted the most deviating response for the gene coding for RNA terminal phosphate cyclase fragment. The mRNA of this gene was – according to the microarray results – significantly differentially expressed in four out of six tested conditions and although the trend (down-regulation) was the same in the Real-Time PCR analysis, the level of repression was clearly less and not significant.

4. Discussion

The application of ecotoxicogenomics techniques opens possibilities for gaining insights into unknown modes of toxicity. In the present study this approach was used to test nickel toxicity in a standard test species, *D. magna*. Assuming that a large number of toxicologically relevant effects are accompanied by alterations in gene transcription, cDNA microarray analysis can produce insights into multiple endpoints and underlying mechanisms. Combined with specific (sub)organismal effects, a good image of the stress status of the organism is generated. Furthermore, since chemicals never occur solely in natural environments, our interest also focussed on molecular interactions of metals.

4.1. Single compound experiment – nickel exposure

Compared to the amount of mammalian toxicology data on nickel, the number of ecotoxicological studies is still very limited. Only a few studies have reported nickel effects in aquatic species (Dave and Xiu, 1991; Pane et al., 2003a; Ptashynski et al., 2002) and specifically crustaceans (Kszos et al., 1992; Pane et al., 2003b, 2004; Keithly et al., 2004). Relying on the specific respiratory effects observed in both *Daphnia* and rainbow trout (Pane et al., 2003a,b, 2004), respiratory effects were suggested as possibly important endpoints for nickel exposure.

In this study, a down-regulation of mRNA of a gene coding for haemoglobin (Dhb1) was detected (both by means of microarray analysis and quantitative RT-PCR) which is concordant with literature. Indeed, Pane et al. (2004) described a decrease in whole-body haemoglobin concentrations in daphnids after chronic waterborne nickel exposure. At the molecular level, it has already been extensively illustrated that haemoglobin levels can fluctuate in response to different types of stressors in daphnids (Gorr et al., 2004; Rider et al., 2005; Soetaert et al., 2007a,b; Soetaert, 2007). Soetaert et al. (2007a) reported besides an up-regulation of mRNA of a gene coding for haemoglobin as a result of fenarimol exposure, a mRNA down-regulation of the same fragment caused by cadmium and lead exposure (Soetaert et al., 2007b; Soetaert, 2007). The involvement of an endocrine regulated pathway for altered (elevated) haemoglobin expression due exposure to methyl farnesoate (and their synthetic analogues) was suggested by Rider et al. (2005). However, other studies (Gorr et al., 2004) – which demonstrated elevated haemoglobin concentrations in *D. magna* in response to low dissolved oxygen – suggest the activation of the hypoxia signalling pathway and subsequent hypoxia inducible factors (HIF). The results presented in the latter studies lead to the suggestion that dependent on the type of compound under investigation different underlying mechanisms result in differential haemoglobin expression levels. Therefore, it could be that although alterations in haemoglobin expression levels are clearly not compound specific, the underlying mechanisms could be compound specific or at least specific to a set of compounds, e.g. metals.

Transition metals such as nickel have been shown to be able to activate and stabilize the HIF-1α subunit in nickel-treated HIF-1-proficient cells (Salnikow et al., 1999, 2003). Therefore, based on our results, the involvement of this pathway in the regulation of oxygen-related gene fragments in *Daphnia* could be suggested. Another complementary finding to the observed haemoglobin effects was the identification of two additional differentially transcribed gene fragments which are involved in the heme biosynthesis. The first one, mRNA for a gene fragment homologue to δ-ALAS (i.e. a key enzyme in the heme biosynthesis) was found to be repressed. This enzyme catalyzes the formation of δ-aminolevilunic acid (δ-ALA) through carboxylation of succinyl-CoA and glycine. A decrease in δ-ALAS activity in the liver of rats after nickel injection was described (Maines and Kappas, 1977). Latunde-Dada et al. (2004) reported reduction of urinary δ-ALA in nickel-treated mice. The second gene encodes a glycine decarboxylating enzyme (GenBank accession no. DW724450) – a precursor enzyme in the heme biosynthesis pathway responsible for the breakdown of glycine – and its mRNA was found to be up-regulated after treatment. These last two gene fragments suggest that the haemoglobin effects might be due to a disturbance of the biosynthetic heme pathway rather than through HIF-related transcriptional regulation of the whole haemoglobin protein.

Among the down-regulated mRNAs of genes following nickel exposure, gene fragments encoding genes involved in lipid transport and embryo development were identified. The embryo development related group includes the *D. magna* vitellogenin (VTG) genes, the products of which serve as storage proteins providing nutrients to the developing embryo (Kato et al., 2004). Several studies (Poynton et al., 2006; Tokishita et al., 2006; Soetaert et al., 2007b; Soetaert, 2007) have indicated that besides nickel

other compounds are able to affect VTG transcription as well. For example, a repression of VTG mRNAs and proteins as a result of exposing neonate daphnids to juvenile hormone agonists was described by Tokishita et al. (2006). They related this differential expression of VTG mRNA to the presence of a homologue of the juvenile hormone response element in the VTG sequence and consequently proposed the utilization of VTG mRNAs as biomarkers for endocrine disrupting compounds. Our results, however, suggest that the down-regulation of VTG due to nickel exposure might be the result of a lack of sufficient energy reserves (Fig. 3A), rather than through a direct interaction with the gene encoding for VTG itself. This indicates that the down-regulation of VTG mRNA levels can be the result of either direct interaction (e.g. in the case of juvenile hormone agonist exposure) but it can also be a secondary response (e.g. in the case of lack of energy due to metal exposure). Therefore, whether this gene could reflect a general stress response to several types of toxicants in *D. magna* still remains to be further elucidated.

Another (repressed) gene which might give valuable insights into the nickel-induced toxic mechanism of action is coding for an inorganic pyrophosphatase (GenBank accession no. DW724642). As this enzyme hydrolyses pyrophosphate, it is involved in many different biochemical pathways (e.g. fatty acid synthesis, glycogen synthesis, etc.). However, for this enzyme to be active it requires the presence of magnesium ions (Braga and Avaeva, 1972). Pane et al. (2003b) described Mg^{2+} antagonism as one of the major mechanisms of acute nickel toxicity in *D. magna*. They noted a significant decrease in whole-body Mg^{2+} concentrations (both acute and chronic). Therefore, we believe that the effect of nickel on Mg^{2+} homeostasis might cause these direct effects on Mg^{2+}-dependent genes such as inorganic pyrophosphatase.

Furthermore, mRNAs of genes involved in photo-transduction (opsin 1, opsin 2 and rhodopsin 4) are induced due to nickel treatment (opsin 2 levels were confirmed by RT-PCR). Phototactic behaviour has been known to be influenced by metals (Michels et al., 2000) and previous studies at the mRNA level have also shown that the transcription of these genes responds to metal stress in *Daphnia* (Soetaert et al., 2007b; Shaw et al., 2007).

Generally, stressful exposure conditions will elicit toxic responses in organisms on many different biological levels. Integrating several biological levels fits into the so-called "systems toxicology" concept (Waters and Fostel, 2004). The potential and added value of such an approach has been shown in a small selection of studies (Moens et al., 2007; Heckmann et al., 2008). In the present study, a PLS analysis was used to connect the molecular (gene transcription) and (sub)organismal (energy parameters and growth) level in order to detect possible relationships between molecular and biochemical responses. This analysis (Fig. 4) pointed out that several gene fragments were co-varying with the CEA parameter, indicating a strong directly proportional relationship between both phenomena. These highly co-varying genes (coding for haemoglobin, RNA terminal phosphate cyclase, a ribosomal protein) could therefore be suggested to be linked to the energy budget parameters. As CEA was previously shown to be predictive for population level effects (De Coen and Janssen, 2003), we suggest that these genes could become molecular biomarkers with ecological relevance. However, their causal relationship still remains to be demonstrated. Beside these fragments, also a set of gene transcripts inversely related to the CEA parameter was identified, more specifically of genes related to the cuticula turnover. For crustaceans, a regular moulting behaviour is not only crucial for an adequate growing process, but also for further reproductive success. Several types of compounds have been shown to possess the ability to adversely affect this moulting process (Zou and Fingerman, 1997; Rodriguez et al., 2007; De Schamphelaere et al., 2008). At the mRNA level, an impairment of gene fragments involved in exoskeleton maintenance due to toxicant exposure was described (Poynton et al., 2006; Soetaert et al., 2007b; De Schamphelaere et al., 2008).

4.2. Binary mixture experiments

Investigating the potential interaction of nickel with two other metals (cadmium and lead) by means of a microarray platform enables the evaluation of these mixture toxicity responses on multiple endpoints/mechanisms of toxicity simultaneously. Since the gene expression fingerprints of the binary mixtures could provide insight into the nature of interaction of the single compounds two hypotheses were formulated. Firstly, in the case of similarly acting compounds (*A* and *B*) which produce similar gene transcription profiles ($A = B$), one would expect an equitoxic mixture ($C = \frac{1}{2}A + \frac{1}{2}B$) with an equal amount of TUs to result in an identical gene transcription profile ($A = B = C$). However, when deviations in the mixture gene transcription fingerprints are detected ($A = B \neq C$), this could indicate that molecular interactions between similarly acting compounds are occurring. Secondly, in the case of dissimilarly acting compounds (*A* and *D*), both compounds produce a compound-specific fingerprint ($A \neq D$). In this case, we expect an equitoxic mixture ($E = \frac{1}{2}A + \frac{1}{2}D$) to result in an intermediate molecular response profile being equal to the sum of a partial profile *A* and a partial profile *D* ($A \neq D \neq E = \frac{1}{2}A + \frac{1}{2}D$). Again, when unexpected deviations to the mixture expression pattern occur ($A \neq D \neq E \neq \frac{1}{2}A + \frac{1}{2}D$), it can be stated that interaction between dissimilar acting compounds has taken place.

To test this hypothesis, multivariate analyses were performed on all eight treatments (the 0.09 and 0.18 TU treatments of the single metals and the two mixtures). The three-component PCA (Fig. 5) enabled to classify treatments according to their (dis)similarities in mRNA expression profiles of genes. Overall, the largest proportion of variance (39%) was accounted for by the separation of the mixture and single metal profiles. Cadmium (both high and low doses) could be separated from the other metals by the second PC. Nickel and Lead were clearly most similar, regardless of the concentration. The main conclusion that could be drawn from this analysis was that the mRNA expression patterns of the tested binary metal mixtures were not the simple sum of their individual compounds' fingerprint, suggesting interaction among compounds. Additional molecular mechanisms were clearly affected when comparing the binary metal responses to the single compound fingerprints at equitoxic levels.

When comparing the differentially expressed mRNA fragments between all equitoxic treatments (Fig. 6), a set of compound-specific genes (uniquely altered due to, e.g. nickel, lead, cadmium treatment) could be detected for all metals. However, also a considerable overlap between treatments was present and among equitoxic treatments seven common (repressed) gene fragments were identified. Concordant to another microarray mixture study (Finne et al., 2007) a higher number of affected genes could be noted in the mixtures compared to the fingerprints of the individual compounds.

These higher numbers of genes affected in the mixtures again suggests the involvement of additional molecular mechanisms. Getting insights into the additionally altered pathways in the mixtures was accomplished by K-means clustering of the equitoxic responses (Fig. 7). The transcriptional responses which tell us the most about the mixture behaviour are the ones which behave dissimilarly in the mixture compared to the single compound treatments. Clusters with additionally altered (either induced or repressed) processes after mixture exposure compared to single component exposure could be identified for both binary mixtures. Also in both cases, a cluster indicative of clear interaction behaviour could be determined. The latter cluster was shown to be dominated by mRNA fragments coding for the 'structural constituents of the

cuticula'. It could be suggested that both metals in the mixtures are able to disrupt the normal moulting pattern by interference through different pathways. Cadmium has been shown to induce the gene coding for juvenile hormone esterase (JHE) (GenBank accession no. DW724473) (Soetaert et al., 2007b) in *D. magna*. This suggests an increased breakdown of juvenile hormone, a key regulator of the moulting process in daphnids. In the same experiment, mRNA fragments corresponding to cuticula proteins appeared to be slightly down-regulated by this metal. Other studies suggest impairment of the ecdysone secretion due to cadmium exposure as a possible mechanism of disrupting the moulting process in crustaceans (Bodar et al., 1988a,b; Moreno et al., 2003). Nickel on the other hand, caused a clear increase in cuticula mRNA levels, but the effects on the gene coding for juvenile hormone esterase are less clear (Table 2) since no differential mRNA expression was noted after microarray analysis, but quantitative RT-PCR showed significant up-regulation. The mixture which contained both metals is represented by an even more severely decreased mRNA expression of gene fragments coding for cuticula proteins compared to the cadmium treatment. Also here an increase in mRNA of the gene coding for juvenile hormone esterase could be noted. This indicates that although this JHE most probably influences the moulting process, other mechanisms also play an important role.

This GO driven analysis also demonstrated that for both mixtures catabolic processes (at the molecular level) are clearly additionally induced. The up-regulation of mRNAs – among others – of genes coding for a mannanase precursor, chymotrypsin-like serine proteinases, cellulases, carboxypeptidase, amylase clearly denote this. Metal-induced effects on the digestive system of daphnids have previously been shown both at the enzymatic (De Coen and Janssen, 1997) and at the mRNA level (Soetaert et al., 2007b; Poynton et al., 2008). However, these studies also indicate that depending on the concentration and duration of exposure the effects on the digestive enzymes due to metal exposure appear to differ. Reduced ingestion and feeding rates as a result of toxic stress (both metals as organic compounds) have been described previously (Bodar et al., 1988a,b; Villarroel et al., 2003; Zeman et al., 2008). In Bodar et al. (1988a,b) it was suggested that the decrease in assimilation efficiency is mainly due to the decreased filtering and food collection rather than malfunctioning of the digestive system. In this study we suggest that the up-regulation of the digestive metabolism related mRNA fragments could be the result of a reduced food uptake leading to an enhanced activation of digestive mechanisms.

The usefulness of identifying the different types of gene expression responses (using a GO driven analysis) for evaluation of gene transcription patterns of single compounds and mixtures is hereby illustrated. At the molecular level the metal mixtures clearly show additional mechanisms affected compared to the single compound responses. Whether these molecular effects show any relevance at a higher level of biological organization has to be further investigated. However, as molecular responses on important processes related to digestion and moulting illustrate: it is crucial that these additional effects are also studied at higher levels.

5. Conclusion

The usefulness of the custom cDNA *D. magna* microarray in detecting new pathways of nickel toxicity was demonstrated with the current study. A set of genes co- and inversely varying with the energy budgets were identified. This is indicative of the strength of integrating responses on multiple levels of biological organization for the selection of genes as potential biomarkers. Regarding the mixture toxicity, the hypothesis that gene transcription patterns of equitoxic binary mixtures would be the results of additive molecular combinations of both individual compounds was not proven in the present study. *D. magna* gene transcription patterns of binary metal mixtures are not the simple sum of their individual compounds' fingerprint. Moreover, when comparing equitoxic binary mixture fingerprints to single compound fingerprints, additional molecular mechanisms were clearly switched on. Multivariate and clustering techniques proved to be indispensable for the analysis of the microarray profiles. They helped in illustrating the (dis)similarity between treatments and the identification of additionally affected pathways after mixture treatment. To conclude, although analysing molecular mixture responses is clearly complex, the benefits of using a multi-endpoint technique such as microarrays for mixture toxicity evaluation are demonstrated with these findings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2008.12.012.

References

Altenburger, R., Walter, H., Grote, M., 2004. What contributes to the combined effect of a complex mixture? Environ. Sci. Technol. 38 (23), 6353–6362.

Bae, D.S., Hanneman, W.H., Yang, R.S.H., Campain, J.A., 2002. Characterization of gene expression changes associated with MNNG, arsenic or metal mixture treatment in human keratinocytes: application of cDNA microarray technology. Environ. Health Perspect. 110 (6), 931–941.

Barata, C., Baird, D.J., Noqueira, A.J., Soares, A.M., Riva, M.C., 2006. Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. Aquat. Toxicol. 78 (1), 1–14.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Bodar, C.W.M., van Der Sluis, I., Voogt, P.A., Zandee, D.I., 1988a. Effects of cadmium on consumption, assimilation and biochemical parameters on *Daphnia magna*: possible implications for reproduction. Comp. Biochem. Physiol. C Comp. Pharmacol. 90 (2), 341–346.

Bodar, C.W.M., Voogt, P.A., Zandee, D.I., 1988b. Ecdysteroids in *Daphnia magna*—their role in moulting and reproduction and their levels upon exposure to cadmium. Aquat. Toxicol. 17 (4), 339–350.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein, utilizing the principle of protein-dye landing. Anal. Biochem. 72, 248–254.

Braga, E.A., Avaeva, S.M., 1972. The interaction of yeast inorganic pyrophosphatase with bivalent cations and pyrophosphate. FEBS Lett. 27 (2), 251–255.

Cedergreen, N., Streibig, J.C., 2005. Can the choice of endpoint lead to contradictory results of mixture-toxicity experiments? Environ. Toxicol. Chem. 24 (7), 1676–1683.

Conesa, A., Götz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21 (182005), 3674–3676.

Dave, G., Xiu, R.Q., 1991. Toxicity of mercury, copper, nickel, lead and cobalt to embryos and larvae of zebrafish branch danio-rerio. Arch. Environ. Contam. Toxicol. 21 (1), 126–134.

De Coen, W.M., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing II. Digestive enzyme activity in *Daphnia magna* exposed to sublethal concentrations of cadmium, chromium and mercury. Chemosphere 35 (5), 1053–1067.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ. Toxicol. Chem. 22 (7), 1632–1641.

De Schamphelaere, K.A.C., Vandenbrouck, T., Muyssen, B.T.A., Soetaert, A., Blust, R., De Coen, W., Janssen, C.R., 2008. Integration of molecular with higher-level effects of dietary zinc exposure in *Daphnia magna*. Comp. Biochem. Physiol. Part D Genomics Proteomics 3 (4), 307–314.

Eisler, R., 1998. Nickel hazards to fish, wildlife, and invertebrates: a synoptic review. US Geological Survey, Biological Resources Division, Biological Science Report, 1998–2001.
Farr, S., Dun, R.T., 1999. Concise review: gene expression applied to toxicology. Toxicol. Sci. 50, 1–9.
Finne, E.F., Cooper, G.A., Koop, B.F., Hylland, K., Tollefsen, K.E., 2007. Toxicogenomics responses in rainbow trout hepatocytes exposed to model chemicals and a synthetic mixture. Aquat. Toxicol. 81, 293–303.
Geoghegan, F., Katsiadaki, I., Williams, T.D., Chipman, J.K., 2008. A cDNA microarray for the three-spined stickleback, *Gasterosteus aculeatus* L., and analysis of the interactive effects of oestradiol and dibenzantracene exposures. J. Fish Biol. 72, 2133–2153.
Gnaiger, E., 1983. Calculation of energetic and biochemical equivalents of respiratory oxygen consumption. In: Gnaiger, E., Forstner, H. (Eds.), Polarographic Oxygen Sensors. Aquatic and Physiological Applications. Springer Verlag, Berlin, pp. 337–345.
Gorr, T.A., Cahn, J.D., Yamagata, H., Bunn, H.F., 2004. Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna* is hypoxia-inducible factor dependent. J. Biol. Chem. 279, 36038–36047.
Groten, J.P., Heijne, W.H.M., Stierum, R.H., Freidig, A.P., Feron, V.J., 2004. Toxicology of chemical mixtures: a challenging quest along empirical sciences. Environ. Toxicol. Pharmacol. 18, 185–192.
Hayes, R.B., 1997. The carcinogenicity of metals in humans. Cancer Causes Control 8 (3), 371–385.
Heckmann, L.H., Sibly, R.M., Connon, R., Hooper, H.L., Hutchinson, T., Maund, S.J., Hill, C.J., Bouetard, A., Callaghan, A., 2008. Systems biology meets stress ecology: linking molecular and organismal stress responses in *Daphnia magna*. Genome Biol. 9, R40.
Kato, Y., Tokishita, S.I., Ohta, T., Yamagata, H., 2004. A vitellogenin chain containing a superoxide dismutase-like domain is the major component of yolk proteins in cladoceran crustacean *Daphnia magna*. Gene 334, 157–165.
Keithly, J., Brooker, J.A., DeForest, D.K., Wu, B.K., Brix, K.V., 2004. Acute and chronic toxicity of nickel to a cladoceran (*Ceriodaphnia dubia*) and an amphipod (*Hyalella azteca*). Environ. Toxicol. Chem. 23 (3), 691–696.
King, F.D., Packard, T.T., 1975. Respiration and the activity of the respiratory electron transport system in marine zooplankton. Limnol. Oceanogr. 20, 849–854.
Kszos, L.A., Stewart, A.J., Taylor, P.A., 1992. An evaluation of nickel toxicity to *Ceriodaphnia dubia* and *Daphnia magna* in a contaminated stream and in laboratory tests. Environ. Toxicol. Chem. 11, 1001–1012.
Latunde-Dada, G.O., Shirali, S., McKie, A.T., Simpson, R.J., Peters, T.J., 2004. Effect of transition metal ions (cobalt and nickel chlorides) on intestinal iron absorption. Eur. J. Clin. Invest. 34, 626–630.
Lidén, C., Norberg, K., 2005. Nickel on the Swedish market. Follow-up after implementation of the nickel directive. Contact Dermatitis 52, 29–35.
Lu, H., Shi, X., Costa, M., Huang, C., 2005. Carcinogenic effects of nickel compounds. Mol. Cell. Biochem. 279, 45–67.
Maines, M.D., Kappas, A., 1977. Regulation of heme pathway enzymes and cellular glutathione content by metals that do not chelate with tetrapyrroles: blockade of metal effects by thiols. Proc. Natl. Acad. Sci. U.S.A. 74 (5), 1875–1878.
Michels, E., Semsari, S., Bin, C., De Meester, L., 2000. Effect of sublethal doses of cadmium on the phototactic behaviour of *Daphnia magna*. Ecotoxicol. Environ. Saf. 47, 261–265.
Moens, L.N., Smolders, R., van der Ven, K., van Remortel, P., Del-Favero, J., De Coen, W.M., 2007. Effluent impact assessment using microarray-based analysis in common carp: a systems toxicology approach. Chemosphere 67, 2293–2304.
Moreno, R.P.A., Medesani, D.A., Rodriguez, E.M., 2003. Inhibition of moulting by cadmium in the crab *Chasmagnathus granulata* (Decapoda Brachyura). Aquat. Toxicol. 64, 155–164.
Pane, E.F., Richards, J.G., Wood, C.M., 2003a. Acute waterborne nickel toxicity in the rainbow trout (*Oncorhunchus mykiss*) occurs by a respiratory rather than ionoregulatory mechanism. Aquat. Toxicol. 63, 65–82.
Pane, E.F., Smith, C., McGeer, J.C., Wood, C.M., 2003b. Mechanisms of acute and chronic waterborne nickel toxicity in the freshwater cladoceran, *Daphnia magna*. Environ. Sci. Technol. 37, 4382–4389.
Pane, E.F., McGreer, J.C., Wood, C.M., 2004. Effects of chronic waterborne nickel exposure on two successive generations of *Daphnia magna*. Environ. Toxicol. Chem. 23 (4), 1051–1056.
Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, 36–45.
Poynton, H.C., Varshavsky, J.R., Chang, B., Cavigiolio, G., Chan, S., Holman, P.S., Loguinov, A.V., Bauer, D.J., Komachi, K., Theil, E.C., Perkins, E.J., Hughes, O., Vulpe, C.D., 2006. *Daphnia magna* ecotoxicogenomics provides mechanistic insights into metal toxicity. Environ. Sci. Technol. 41 (3), 1044–1050.
Poynton, H.C., Loguinov, A.V., Varshavsky, J.R., Chan, S., Perkins, E.J., Vulpe, C.D., 2008. Gene expression profiling in *Daphnia magna* part I: concentration-dependent provide support for the no observed transcriptional effect level. Environ. Sci. Technol. 42, 6250–6256.
Ptashynski, M.D., Pedlar, R.M., Evans, R.E., Baron, C.L., Klaverkamp, J.F., 2002. Toxicity of dietary nickel in lake whitefish (*Coregonus clupeaformis*). Aquat. Toxicol. 58, 229–247.
Rodriguez, E.M., Medesani, D.A., Fingerman, M., 2007. Endocrine disruption in crustaceans due to pollutants: a review. Comp. Biochem. Physiol. A 146, 661–671.
Rider, C.V., Gorr, T.A., Olmstead, A.W., Wasilak, B.A., Le Blanc, G., 2005. Stress signaling: coregulation of hemoglobin and male sex determination through a terpenoid signaling pathway in a crustacean. J. Exp. Biol. 208, 15–23.
Salnikow, K., An, W.G., Melillo, G., Blagosklonny, M.V., Costa, M., 1999. Nickel-induced transformation shifts the balance between HIF-1 and p53 transcription factors. Carcinogenesis 20 (9), 1819–1823.
Salnikow, K., Davidson, T., Zhang, Q., Chen, L.C., Su, W., Costa, M., 2003. The involvement of hypoxia-inducible transcription factor-1-dependent pathway in nickel carcinogenesis. Cancer Res. 63, 3524–3530.
Salsano, F., Francia, C., Roumpedaki, I., Proietti, M., Pisarri, S., Verna, N., Gabriele, E., Di Gioacchino, G., Di Gioacchino, M., 2004. Immune effects of nickel. Int. J. Immunopathol. Pharmacol. 17 (2), 63–69.
Shaw, J.R., Colbourne, J.K., Davey, J.C., Glaholt, S.P., Hampton, T.H., Chen, C.Y., Folt, C.L., Hamilton, J.W., 2007. Gene response profiles for *Daphnia pulex* exposed to the environmental stressor cadmium reveals novel crustacean metallothioneins. BMC Genomics 8, 477.
Smolders, R., Bervoets, L., De Coen, W., Blust, R., 2004. Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. Environ. Pollut. 129, 99–112.
Soetaert, A., Moens, L.N., van der Ven, K., Van Leemput, K., Naudts, B., Blust, R., De Coen, W.M., 2006. Molecular impact of propiconazole on *Daphnia magna* using a reproduction-related cDNA array. Comp. Biochem. Physiol. C 142, 66–76.
Soetaert, A., van der Ven, K., Moens, L.N., Vandenbrouck, T., van Remortel, P., De Coen, W.M., 2007a. *Daphnia magna* and ecotoxicogenomics: gene expression profiles of the anti-ecdysteroidal fungicide fenarimol using energy-, moulting- and life stage-related libraries. Chemosphere 67, 60–71.
Soetaert, A., Vandenbrouck, T., van der Ven, K., Maras, M., van Remortel, P., Blust, R., De Coen, W., 2007b. Molecular responses during cadmium-induced stress in *Daphnia magna*: integration of differential gene expression with higher level effects. Aquat. Toxicol. 83, 212–222.
Soetaert, A., 2007. Development of cDNA microarrays in the freshwater flea *Daphnia magna* for toxicity characterization of chemicals. PhD thesis. University of Antwerp, Antwerp, Belgium.
Tokishita, S., Kato, Y., Kobayashi, T., Nakamura, S., Ohta, T., Yamagata, H., 2006. Organization and repression by juvenile hormone of vitellogenin gene cluster in the crustacean, *Daphnia magna*. Biochem. Biophys. Res. Commun. 345, 362–370.
Tusher, V.G., Ribshirani, R., Chu, G., 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U.S.A. 98, 5116–5121.
van der Ven, K., De Wit, M., Keil, D., Moens, L., Van Leemput, K., Naudts, B., De Coen, W., 2005. Development and application of a brain-specific cDNA microarray for effect evaluation of neuro-active pharmaceuticals in zebrafish (*Danio rerio*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 141 (4), 408–417.
Verslycke, T., Roast, S.D., Widdows, J., Jones, M.B., Janssen, C.R., 2004. Cellular energy allocation and scope for growth in the estuarine mysid *Neomisis integer* (Crustacea: Mysidacea) following chlorpyrifos exposure: a method comparison. J. Exp. Mar. Biol. Ecol. 306, 1–16.
Villarroel, M.J., Sancho, E., Ferrando, M.D., Andreu, E., 2003. Acute, chronic and sublethal effects of the herbicide on *Daphnia magna*. Chemosphere 53, 857–864.
Waters, M.D., Fostel, J.M., 2004. Toxicogenomics and systems toxicology: aims and prospects. Nat. Rev. Genet. 5, 936–948.
Yang, Y.H., Dudoit, S., Lu, P., Lin, D.M., Peng, V., Ngai, J., Speed, T.P., 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. 30, e15.
You, L., Bartolucci, E.J., 2004. Gene expression profiles in mammary gland of male rats treated with genistein and methoxychlor. Environ. Toxicol. Pharmacol. 18, 161–172.
Zeman, F.A., Gilbin, R., Alonzo, F., Lecompte-Pradines, C., Garnier-Laplace, J., Aliaume, C., 2008. Effects of waterborne uranium on survival, growth, reproduction and physiological processes of the freshwater cladoceran *Daphnia magna*. Aquat. Toxicol. 86, 370–378.
Zou, E., Fingerman, M., 1997. Effects of estrogenic agents on moulting of the water flea, *Daphnia magna*. Ecotoxicol. Environ. Saf. 38, 281–285.