Differential protein expression in the estuarine copepod Eurytemora affinis after diuron and alkylphenol exposures

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Running head: Proteomics in the copepod after organic contaminant exposure

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DIFFERENTIAL PROTEIN EXPRESSION OF THE ESTUARINE COPEPOD *EURYTEMORA AFFINIS* AFTER DIURON AND ALKYLPHENOL EXPOSURES

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Abstract - Proteomics was used in the calanoid copepod *Eurytemora affinis* for screening of protein expression modifications induced by organic contaminants. The copepods were exposed in a continuous flow-through system for 86 h to environmentally relevant concentrations of contaminants representative of the Seine Estuary's pollution (Diuron: 500 $ng.L^{-1}$, Alkylphenol (AP) mixture: 1000 $ng.L^{-1}$). The proteome analysis of whole body copepod extracts by two-dimensional gel electrophoresis (2DE) revealed that the contaminants induced modifications in protein expression, with the highest quantitative variations occurring after diuron exposure. Specifically, a total of 88 and 41 proteins were differentially expressed after diuron and AP treatments respectively. After mass spectrometry analysis, 51 (diuron exposure) and 15 (AP exposure) proteins were identified. The identified proteins were potentially related to energy metabolism, cell growth, nervous signal conductivity, excitotoxicity, oxidative stress response and antioxidant defense. The data suggest a massive general disturbance of physiological functions of *E. affinis* after diuron exposure whereas APs induced an alteration of a few targeted physiological functions. The protein expression signatures identified after contaminant exposure deserve to further investigate the development of novel potential biomarkers for water quality assessment.

Keywords - 2D Electrophoresis, Biomarkers, Calanoid, Estuary, Organic contaminants

INTRODUCTION

Many human activities introduce organic micro-pollutants into aquatic environments, particularly in North-Atlantic estuaries which are generally under high anthropogenic pressure because of their strategic geographical position and their economic importance. Thus, estuaries receive large quantities of organic contaminants from various sources including industrial discharges, urban and agriculture runoff and atmospheric deposition [1]. These contaminants can have deleterious effects on aquatic biota and have been frequently associated with biochemical and physiological disorders in aquatic organisms [1], representing a risk for these populations and the local food web. Most ecotoxicological research is usually based on the study of biomarkers, which are defined as "biochemical, cellular, physiological or behavioral variations that can be measured in tissue or body fluid samples or at the level of whole organisms, that provide evidence of exposure to and/or effects of, one or more chemical pollutants" [2]. Biomarkers can thus be used to assess the health status of aquatic organisms and to detect early warning responses to environmental risks [3]. However, such approaches are frequently discussed because of the deficiency or the specificity of the selected biomarkers in relation to the diversity and the variability of the contaminants and the abiotic factors [4]. The use of a battery of biomarkers of contaminant exposure and effects are thus recommended in biomonitoring programs [5]. In addition, some of the actual biomarkers – in particular biomarkers developed in vertebrates – are not applicable to invertebrates because of their major physiological differences [6].

Proteomic approaches may represent a relevant strategy (a) to better understand the physiological and biochemical effects of organic contaminants on aquatic organisms but also (b) to enlarge the panel of classical biomarkers with potentially more sensitive and/or specific molecular tools [7]. Adaptation to environmental stress - such as chemical exposure - includes changes in protein expression resulting from transcriptional, posttranscriptional, translational and

posttranslational modifications [8]. The proteome represents the bulk of proteins of an organism, a cell, an organ, or even a body fluid, quantified at a given moment and under precise conditions. It can thus be considered as a stress signature. In that line, the proteome has been increasingly studied over the last decade to identify key biochemical actors involved in physiological *versus* pathological pathways. Proteomics has opened novel horizons in many research areas of life sciences such as medicine [9], ecology [10] and ecotoxicology [10].

Several studies have reported the strong contamination of the Seine Estuary by organic micro-pollutants especially by neurotoxic, carcinogenic and estrogen-like compounds [11, 12]. In spite of this high chemical stress context, the calanoid micro-crustacea *Eurytemora affinis* dominates the zooplankton community, representing 90 to 99 % of zooplanktonic species [13]. Over the years, *E. affinis* has become a relevant ecological and ecotoxicological bioindicator [14]. Indeed, this euryhaline copepod is widely distributed in North-Atlantic brackish waters where it represents an essential basal organism in the trophic web. The *E. affinis* copepods are appropriate test organisms because of their small size, sexual dimorphism, short generation time, and ease of culturing in the laboratory [15, 16, 17, 18].

Among hydrophobic organic contaminants, diuron and alkylphenols (APs) are of major concern because of their persistence and toxicity. Diuron, (3-(3',4'-dichlorophenyl)-1,1-dimethyl-urea; subclass of phenylurea) has been widely used both as a herbicide to control a wide variety of weeds and mosses, and as an anti-foulant, in replacing tributyltin [19]. Due to its high persistence (one month to one year), diuron can be detected in soil, sediment and water [19]. The current diuron concentration is around 1 to 1,000 $ng.L^{-1}$ [20]. APs belong to the group on non-ionic surfactants. They have been extensively used as detergents, dispersants or solubilizers [21]. Literature data report an AP concentration in worldwide river waters ranging from 0.6 $ng.L^{-1}$ to 15 $\mu g.L^{-1}$ when related to sewage treatment plant discharges [21].

In this context, we investigated the effects of diuron and APs – categorized as Priority Hazardous Substances by the European commission (Directive on Environmental Quality Standards 2008/105/EC, [22]) – on the estuarine *E. affinis* proteome after experimental exposure at environmentally realistic concentrations in order to identify Protein Expression Signatures (PES).

MATERIALS AND METHODS

Eurytemora affinis collection and stabulation

The copepods *E. affinis* were collected in autumn using subsurface tows of WP2 plankton net (200-µm mesh size) at ebb tide, in Tancarville Station in the oligo-mesohaline part of the Seine Estuary (longitude 0°15′52″E, latitude 49°29′19″N; Haute-Normandie, France). Immediately after sampling, the copepods were sorted using 500 µm sieves in order to eliminate predators (especially Mysidacea and Gammaridae), transferred into isotherm containers and brought back to the laboratory.

The copepods were maintained in the laboratory for an acclimatization period of three days in a 300-L hydrodynamic canal under optimally controlled conditions i.e. allowing high embryonic developmental rates (constant aeration; salinity, salinity 15; temperature, 10 °C; photoperiod, 12:12h) [23]. The hydrodynamic canal contained freshly filtered sea water (GF/C Whatman filter, 0.45 µm) sampled in the English Channel mixed with ultra-pure water in order to reach the selected salinity of 15. The same water preparation was used for exposure experiments. During the detoxification period, the copepods were fed twice a day with an algal mixture (*Rhodomonas marina* and *Isochrysis galbana*) receiving a total of 20,000 cells.mL^{-1}. Algae cultures were grown at 20 °C in 10-L tanks under 24-h fluorescent illumination and constant aeration in Conway medium.

Material design

The experimental system was set up as described by Cailleaud et al. [18]. Briefly, the experimental system was composed of three compartments: the water reservoirs, the exposure tanks containing the copepods, and a recycling tank to remove contaminants from the water using activated carbon. Contaminant concentrations in the water reservoirs and in the exposure tanks were identical. All the tanks were made of glass and were systematically disinfected and washed before use. Peristaltic pumps were used to (i) draw up contaminated water from the water reservoirs to the exposure tanks, and (ii) eliminate excess water from the exposure tanks into the salvaged tank. In order to avoid aspiration of copepods by the peristaltic pumps, the rubber tubing extremities was capped with 50-µm mesh.

Experimental setups

Selected contaminants (purity > 98 %) i.e. diuron and alkyl phenols (APs; 4-nonylphenol, 4-NP; nonylphenol-ethoxy-acetic-acid, NP1EC) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France; diuron, 4-NP) and LGC Standards (Molsheim, France; NP1EC). Preliminary tests were performed with the exposure system in order to determine the appropriate experimental conditions to saturate the tanks and the rubber tubing with the highly hydrophobic selected contaminants (APs and diuron) to ensure that the real exposure concentrations were included within an acceptable range from the selected nominal concentrations during all the experiments [18]. Briefly, before each exposure experiment, the system was saturated using the experimental conditions. The stock solutions of contaminants were prepared at high concentrations in acetone in order to introduce low volumes of solvent in the water (i.e. 15 µL of acetone/L of water). The contaminant solution was weighed in a glass vial and manually introduced into the water of both reservoirs and exposure tanks at the beginning of the saturation

period using a glass pipette in order to reach the appropriate experimental concentrations. The water was then gently stirred and the flow-through system was activated.

Two copepod groups were exposed simultaneously in separate microcosms to environmentally realistic concentrations of diuron (500 $ng.L^{-1}$) and AP mixture (4-NP, 480 ng.L-1; NP1EC, 520 $ng.L^{-1}$) for 86 h in the experimental continuous flow-through system [20, 21]. In parallel, a control group was maintained in a continuous flow-through system in clean water. During the exposure experiments, food was not provided to the copepods to avoid algal sorption. Copepod samples (exposed and non-exposed organisms) were collected and immediately frozen in liquid nitrogen at the end of the exposure experiments for further proteomic analysis. Six pools of copepods (350 mg wet weight i.e. approximatively 7,000 individuals each) were collected for each experimental condition.

Protein extraction

Copepod pools of whole-body copepods were homogenized on ice, in ice-cold tris HCl buffer 50 mM pH 7.5 (1/3, v/w) composed of EDTA (2 mM), glycerol (10 %), magnesium acetate (5 mM), dithiothreitol (DTT 1 mM) and aprotinin (0.1 %) using an Ultra-Turax homogenizer followed by ultrasonication. Cellular debris was then removed by centrifugation (9,000 *g* for 15 min at 4°C). Then, supernatants were collected and centrifuged at 105,000 *g* for 1 hour at 4 °C. Final supernatants were collected and precipitated with trichloroacetic acid (50%) for 45 min. The precipitated proteins were centrifuged at 9,000 *g* for 30 min at 4 °C, washed with acetone and then resuspended in a hydratation buffer composed of 9 M urea, 55 mM CHAPS and 110 mM DTT. All reagents were purchased from Sigma-Aldrich. The protein quantification was evaluated according to the Bradford method using BSA as a standard.

Two-dimensional gel electrophoresis (2DE)

Depending on the final staining techniques, 150 µg of total protein extracts (silver staining) or 750 µg of total protein extracts (colloidal blue staining) were suspended in an isoelectric focusing (IEF) buffer composed of 1 % IPG carrier ampholytes (pH 3.5–10) and a pinch of Orange G. The first dimension gel separation was carried out with nonlinear Immobiline Dry Strips (18 cm, pH 3–10, nonlinear; GE Healthcare Life Sciences, Velizy-Villacoublay, France) using a Multiphor II apparatus (GE Healthcare Life Sciences). IEF was performed using the following parameters: increase to 500 V in 1 minute, 500 V for 5 hours, increase to 3500 V in 5 hours and 3500 V for 9 h 30 (1 mA, 5 W constant) for a total of 53.3 kV. After isoelectric focusing, strips were soaked in 15 mM dithiothreitol in equilibration buffer (50 mM tris-HCl buffer, pH 6.8, containing 6 M urea, 4% SDS, 25 % glycerol) for 12 min and soaked in 120 mM iodoacetamide and bromophenol blue in the equilibration buffer for 5 min. The second dimension consisted of an SDS–PAGE using 12 % polyacrylamide resolving gels using a Protean Plus Dodeca Cell (width, 19 cm; length, 20 cm; thickness, 0.75 mm; BioRad, Marnes la Coquette, France). Electrophoresis was carried out at 50 mA/gel for 15 min at 15 °C, 83 mA/gel for 15 min, and 20 V/gel for 6h. After migration, the 150 µg of protein extracts were visualized by silver nitrate staining (developing time: 15 min) for gel analysis (Figure 1A) and the 750 µg of protein extracts were visualized by colloidal blue staining for trypsin digestion and mass spectrometry (preparative gel; Figure 1B).

Image analyses

Silver-stained gels were scanned using the ImageScanner (GE Healthcare Life Sciences) and analysed using the Melanie software (ImageMaster™ 2D Platinium version 5.0; GE Healthcare Life Sciences). For each condition, six control and six exposed copepod gels were simultaneously considered. Gel replicates were matched together to form a standard (synthetic) image consisting of spots present in at least three of the six replicate gels. Standard gels of the exposed copepods

were then compared to the one from the non-exposed copepods for differential expression analysis. The quantification of each spot was expressed as volume on a Gaussian image (area of each spot multiplied by its intensity). The differences of protein expression between exposed and non-exposed copepods were statistically analysed by the STATISTICA Software v6.0 (Statsoft. Inc., 2002). To compare two groups, the non-parametric Mann-Whitney U test was used. In all cases, the level of significance (p) was set to 0.01.

Trypsin digestion and mass spectrometry analysis

Coomassie-stained spots of interest were excised from the polyacrylamide gel. Gel plugs were washed twice with 100 µL of NH_4HCO_3 buffer (25 mM) and then dried with acetonitrile (ACN). After DTT reduction, these plugs were incubated in the dark with iodoacetamide (25 mM) for 45 min at room temperature and totally dried using a SpeedVac centrifuge for few minutes before the trypsin solution (10 µL of a 15 ng/µL sequencing-grade trypsin (Promega) in 25 mM NH_4HCO_3 buffer) was added. After rehydration with the enzyme solution, buffer solution was added to cover the gel pieces and digestion was allowed to proceed overnight at 37 °C. Peptides were extracted by adding twice 30 µL of trifluoroacetic acid (TFA, 0.1 %, v/v) and 30 µL of ACN. These fractions were concentrated in a vacuum centrifuge and then dissolved in 10 µL of a buffer containing 2 % of ACN (v/v) and 0.2 % of formic acid.

Sequence fragments were determined using automated nanoLC/MS/MS. Briefly, injections of 5 µL of the sample were carried out into an LC Packings Ultimate nanoLC (Dionex, LCPackings). The peptides were enriched and desalted on a reversed-phase analytical column (RP-C18) and the chromatographic separation was performed using a C18 Pep-Map column (75 µm i.d. × 15 cm). Peptides were separated using a mixture of ACN (2 %)/formic acid (0.1%) (A) and a mixture of ACN (95 %)/ formic acid (0.2 %) (B) used as the mobile phase. The solvent program started with an initial B concentration of 5 % linearly increased to 50% within 45 min

with a constant flow rate of 200 nL/min. The eluant was analysed on a QTrap mass spectrophotometer equipped with a nanospray source (Applied Biosystems). The peptide fragmentation patterns were matched using MASCOT software against the NCBInr databases with the parameters: one missed cleavage by trypsin, mass accuracy of 1.2 Da for the parent ions (MS), 0.6 Da for the fragment ions (MS/MS), one fixed modification (carbamidomethylation of cysteines), variable modifications (oxidations of methionine residues). No species restriction was considered for searching in the database because copepods and crustaceans present few annotations in databases.

RESULTS AND DISCUSSION

The adaptation to environmental pollution, in the same way as other biological stresses, involves changes in protein expression which can be contaminant-specific or dose-dependent. In recent years, comparative proteomics became a powerful tool, with the potential to reveal new and unexpected associations between proteins and toxicant exposure, without prior hypotheses. However, the number of publications reporting the effects of environmental pollutants on the invertebrate proteome is still limited, in particular in crustaceans [24, 25]. Kimmel and Bradley published a notable proteomic study on the impact of salinity and temperature on *E. affinis*, that led to establishing characteristic PES (protein expression signature), but without any protein identification [26]. The present paper reports results from, to our knowledge, the first study designed to examine the effects of diuron and APs on crustacean proteome. The data fill an essential need for better understanding of the effects of such compounds in non-targeted species – in particular in copepods – since nowadays available studies mainly focus on lethal toxicity [27, 28].

Global protein expression patterns after contaminant exposure

The proteome of *E. affinis* was investigated after exposure to environmentally relevant concentrations of organic contaminants i.e. diuron (500 ng.L^{-1}) and a mixture of APs (4-NP, 480 ng.L-1; NP1EC, 520 ng.L-1) in a continuous flow-through system for a period of 86 h [20, 21]. A typical 2DE protein pattern is presented in Figure 2. Proteomic analyses revealed that both tested pollutants induced modifications in protein expression. However, two distinct patterns were established according to the contaminant. The diuron exposure induced the highest quantitative variations in protein expression. Indeed, the number of differentially expressed proteins after diuron exposure (i.e. 88 out of 1,017 spots ; Table 1) – mostly characterized by over-expressions in comparison with AP exposure (95.5% for diuron vs 24.4% for APs) – was 2-fold higher than after AP exposure (i.e. 41 out of 934 spots ; Table 2). Among the differentially expressed proteins, 12 were detected in both exposure conditions (Figure 2; Tables 1 and 2). Three of them exhibited the same pattern i.e. over-expressed. On the contrary, 9 presented an opposite pattern including 8 exhibiting an over-expression for diuron and an under-expression for APs. Among differentially expressed spots, a total of 51 diuron exposure spots and 15 AP exposure spots were identified using nanoelectrospray MS/MS micro-sequencing and a MASCOT search by sequence similarity (Tables 1 and 2). The percentage of identification was slightly better than the expected yield since many proteomic studies with aquatic invertebrates have identified, on average, 15 proteins indicating the problem of sequence divergences across species [7]. The still poor knowledge of copepod acid nucleic sequences explains why 21% of the identifications rested only on one peptide and should be considered with caution. Moreover, shifts in molecular weight and pI between experimental coordinates on 2D-gels and theoretical calculations were noticed and may probably be explained by the presence of post-translational protein modifications, such as phosphorylation for pI changes or proteolytic cleavage for molecular weight changes for instances [29]. Most of the identifications were obtained from homology with arthropod species

(77.3%) including 54.9% from crustacean sequences. Among crustaceans, 71.4% of identifications came from copepod sequences including 40% of calanoid matches and only 15% of *E. affinis* matches.

Differentially expressed proteins were also more diversified after diuron exposure than after AP exposure (Figure 3). Indeed, even if they can be classified into the same KEGG (Kyoto Encyclopedia of Genes and Genomes) functional categories i.e. metabolism, genetic information processing, environmental information processing and cellular processes, their layout was quite different, with approximately 50% of identified proteins implicated in either metabolism after diuron exposure, or genetic information processing after AP exposure. Moreover, diuron exposure affected the expression of proteins implicated in more than 15 subset functions against only 7 after AP exposure. These data would suggest a general disruption of physiological functions induced by diuron.

Diuron effects

Diuron exposure led to a general over-expressed pattern compared to the control animals, characterized by the induction of 84 spots and only 4 under-expressed spots (Table 1). Morever, for almost half of the over-expressed spots, the increase was more than 2, suggesting that diuron led to a massive disturbance of the copepod physiology.

Diuron exposure appears to induce cellular stress and damage, as suggested by the increase in protein synthesis and activation of defense mechanisms. These processes increase the demand for cellular energy as highlighted by the significant induction of enzymes involved in carbohydrate metabolism i.e. glycolytic pathway, tricarboxylic acid cycle or interrelated aminoacid pathway. In addition, a direct increase in the cell capacity to provide ATP can be suggested by the over-expression of energy metabolism proteins, such as ATP synthase and arginine kinase. Arginine kinase – involved in the ATP buffer system – contributes to maintaining the cell ATP supply

when a rapid energy demand occurs, and before any activation of the glycolytic pathway. In the same way, the over-expression of nucleoside diphosphate kinases could contribute to maintaining the level of triphosphate nucleosides (GTP, UTP and CTP). Their induction could be related to processes of DNA translation, replication and repair and protein synthesis as suggested by the over-expression of several proteins involved in these processes (endonuclease RNase H, t-box transcription factor TBX21, histone H4, elongation factor 1-alpha and elongation factor-2).

In the same way, other identified proteins – such as the proliferation-associated protein 2G4, the cell division cycle protein 27, and the 14-3-3 zeta – suggest an impact of diuron on cell control and growth. The proliferation-associated protein 2G4 – involved in both ribosome assembly and regulation of intermediate and late steps of rRNA processing – can interact with the cytoplasmic domain of the ErbB3 receptor and may contribute to transducing growth regulatory signals [30]. This protein is also a transcriptional corepressor of androgen receptor-regulated genes and other cell cycle regulatory genes through its interactions with histone deacetylases. The 14-3-3 zeta is an adapter protein which plays an important role in the regulation of a large spectrum of both general and specialized signaling pathways implicated in ovarian maturation and antiviral response in crustaceans [31, 32]. The down-regulation of 14-3-3 may thus contribute to inhibiting oocyte development and/or modifying the immune response in accordance with literature data highlighting reproductive and immunotoxic effects of diuron in aquatic organisms [33, 34, 35].

In accordance with previous studies which have reported the induction of oxidative stress response and antioxidant defense in marine organisms following diuron exposure [36], we observed an over-expression of two components of the anti-oxidative and detoxification systems, a superoxide dismutase precursor and a glutathione S-transferase enzyme, suggesting an oxidative effect of diuron on the copepod. This hypothesis is supported by the increased

abundance of proteins belong to the folding, sorting and degradation pathways, namely the heat shock proteins HSP 70, GRP78, HSC5, DnaK, HSP 60, cyclophilin and components of the proteasome i.e. the 20S proteasome regulatory subunit alpha type PSMA5 and the proteasome subunit beta type-7. We also identified the induction of a potential leucyl aminopeptidase (hypothetical protein ZHAS_00009025) presumably involved in the processing and regular turnover of intracellular proteins. Furthermore, the over-expression of the aconitase protein may be attributed to a functional property independent of its catalytic activity. This protein is also involved in the mitochondrial DNA (mtDNA) maintenance and inheritance. Aconitase is able to switch between an enzymatic form to a RNA binding form depending on whether the assembly or disassembly of the 4Fe-4S cluster is occurring. Aconitase activity – known to be reduced by oxidants in vertebrates and invertebrates – is commonly used as a biomarker for oxidative stress and has been suggested for use as an intramitochondrial sensor of redox status [37]. Based on these results, oxidative stress generated by the diuron exposure might induce a rise of the level of mitochondrial aconitase to counterbalance the concomitant decrease in activity, and consequently there may be no increase in the capacities of energy production in mitochondria. This result constitutes an interesting perspective in the identification of new biomarkers of oxidative stress in *E. affinis* which could be easily applied to an environmental biomonitoring program.

AP effects

AP exposure led to a general pattern of under-expression with 31 spots exhibiting a lower level in exposed copepod compared to the control animals, and only 10 over-expressions (Table 2). Most of the identified proteins belonged to the “genetic information processing” functional category of the KEGG classification since they were actors of folding, sorting and degradation processes (Figure 3). More clearly, an induction of the HSC5 and DnaK chaperones and of the

ubiquitin-conjugating enzyme E2 was particularly highlighted. In contrast, an under-expression of the proteasome subunit alpha type-2 and the molecular chaperones cyclophilin and calreticulin was observed. These data suggest changes in the processes of protein sorting and degradation in order to cope with the effect of the contaminant. AP exposure may also induce an inhibition of protein synthesis since the elongation factor 1-alpha was also shown to be under-expressed. Moreover, the down-regulation of proteins of the "metabolism" functional category of the KEGG classification (i.e. the enolase, the glyceraldehyde-3-phosphate dehydrogenase, the ATP synthase and the alpha esterase) may be an obstacle to face the increase in the ATP demand required for defense processes e.g. xenobiotic detoxification. These data are in accordance with previous studies which demonstrated the AP capability of interfering with physiological functions such as immunity [38]. However, the copepod *E. affinis* was shown to be able to accumulate and eliminate nonylphenol-ethoxy-acetic-acid and 4-nonylphenol [18]. In addition, two cytoskeleton proteins i.e. a myosin subunit which exhibits an over-expression, and a tropomyosin which is, in contrast, under-expressed could be related to behavioural changes. The glutamate receptor – involved in nervous conduction and excitotoxicity and identified in the "environmental information processing" KEGG functional category – was also shown to be under-expressed, in accordance with the neurotoxic effects of APs [39]. These results are consistent with previous studies which demonstrated that nonylphenol-ethoxy-acetic-acid and 4-nonylphenol are able to alter the *E. affinis* swimming behaviour with possible disruption of chemosensory communication [15].

Finally, it should be noted that the expression of 12 differentially expressed proteins after AP exposure was also affected by diuron. Three of these proteins presented the same pattern. They were in particular implicated in a stress-mediated protein response, as assumed by the over-expression of the molecular chaperones, HSC5 and DnaK. The other common spots

(glyceraldehyde-3-phosphate dehydrogenase, enolase, ATP synthase subunit beta mitochondrial precursor, proteasome 25 kDa subunit, tropomyosin and elongation factor1-alpha as identified proteins) were characterized by an opposite pattern, although with a similar depict i.e. over-expression after diuron exposure and under-expression after AP exposure.

CONCLUSIONS

The present study demonstrates that exposure to organic contaminants in environmentally relevant concentrations can alter the protein expression of copepods. Distinct 2DE patterns were identified for diuron and AP exposures. The expression changes may suggest (i) a massive general disturbance of physiological functions induced by diuron and, in contrast, (ii) an alteration of a few targeted physiological functions induced by APs. The PES identified after contaminant exposure may constitute a useful framework for further development of novel potential biomarkers for water quality assessment.

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REFERENCES

1. Pal A, Gin KY, Lin AY, Reinhard M. 2010. Impacts of emerging organic contaminants on freshwater resources: review of recent occurrences, sources, fate and effects. *Sci Total Environ* 408(24):6062-9.

2. Depledge MH, Amaral-Mendes JJ, Daniel B, Halbrook RS, Loepper-Sams P, Moore MN, Peakall DB. 1993. The conceptual basis of the biomarker approach. In D.B. Peakall & L. R.

Shugart (Eds), *Biomarkers: Research and Application in the assessment of Environmental Health*, vol. 68. NATO ASI Series H: Cell Biology. Springer Verlag, Berlin, Germany, pp 15-29.

3. Amiard-Triquet C, Amiard JC, Rainbow PS. 2012. *Ecological Biomarkers: Indicators of Ecotoxicological Effects*. CRC Press, Boca Raton, USA.

4. Forbes VE, Palmovist A, Bach L. 2006. The use and misuse of biomarkers in ecotoxicology. *Environ Toxicol Chem* 25(1):272-280.

5. Jemec A, Drobne D, Tisler T, Sepcić K. 2010. Biochemical biomarkers in environmental studies-lessons learnt from enzymes catalase, glutathione S-transferase and cholinesterase in two crustacean species. *Environ Sci Pollut Res Int* 17(3):571-81.

6. Amiard-Triquet C, Amiard JC, Mouneyrac C. 2015. *Aquatic Ecotoxicology, 1st dition Advancing Tools for Dealing with Emerging Risks.* Academic Press, USA.

7. Sanchez BC, Ralston-Hooper K, Sepúlveda MS. 2011. Review of recent proteomic applications in aquatic toxicology. *Environ Toxicol Chem* 30(2):274-82.

8. Regoli F, Giuliani ME, Benedetti M, Arukwe A. 2011. Molecular and biochemical biomarkers in environmental monitoring: a comparison of biotransformation and antioxidant defense systems in multiple tissues. *Aquat Toxicol* 105(3-4 Suppl):56-66.

9. Zhang AH, Sun H, Yan GL, Han Y, Wang XJ. 2013. Serum proteomics in biomedical research: a systematic review. *Appl Biochem Biotechnol* 170(4):774-86.

10. Tomanek L. 2011. Environmental Proteomics: Changes in the Proteome of Marine Organisms in Response to Environmental Stress, Pollutants, Infection, Symbiosis, and Development. *Ann Rev Mar Sci* 3:373-399.

11. Amiard JC, Bodineau L, Bragigand V, Minier C, Budzinski H. 2009. In Amiard-Triquet C, Rainbow PS (eds), *Environmental Assessment of Estuarine Ecosystems: A Case Study*. CRC Press - Taylor & Francis Group, Boca Raton, USA, pp 31-57.

12. Teil MJ, Tlili K, Blanchard M, Labadie P, Alliot F, Chevreuil M. 2014. Polychlorinated Biphenyls, Polybrominated Diphenyl Ethers, and Phthalates in Roach from the Seine River Basin (France): Impact of Densely Urbanized Areas. *Arch Environ Contam Toxicol* 66(1):41-57.

13. Mouny P, Dauvin JC. 2002. Environmental control of mesozooplankton community structure in the Seine Estuary (English Channel). *Oceanol Acta* 25:13-22.

14. Forget-Leray J., Souissi S., Devreker D., Cailleaud K. &Budzinski H. 2009. Ecological Status and Health of *Eurytemora affinis* Planktonic Copepod in Seine Estuary. In "*A comprehensive methodology for the assessment of the health status of estuarine ecosystems. Environmental assessment of estuarine ecosystems: A case study*" eds Amiard-Triquet C. & Rainbow P.S. (Taylor & Francis), pp 199-226.

15. Cailleaud K, Michalec FG, Forget-Leray J, Budzinski H, Hwang JS, Schmitt FG, Souissi S. 2011. Changes in the swimming behaviour of *Eurytemora affinis* (Copepoda, Calanoida) in response to a sublethal exposure to nonylphenols. *Aquat Toxicol* 102(3–4):228-231.

16. Lesueur T, Boulangé-Lecomte C, Xuereb B, Budzinski H, Cachot J, Vicquelin L, Giusti-Petrucciani N, Marie S, Petit F, Forget-Leray J. 2013. Development of a larval bioassay using the calanoid copepod, *Eurytemora affinis* to assess the toxicity of sediment-bound pollutants. *Ecotoxicol Environ Saf* 94(1):60-66.

17. Lesueur T, Boulangé-Lecomte C, Restoux G, Deloffre J, Xuereb B, Le Menach K, Budzinski H, Petrucciani N, Marie S, Petit F, Forget-Leray J. 2015. Toxicity of sediment-bound pollutants in the Seine estuary, France, using a *Eurytemora affinis* larval bioassay. *Ecotoxicol Environ Saf* 113:169-75.

18. Cailleaud K, Budzinski H, Lardy S, Augagneur S, Barka S, Souissi S, Forget-Leray J. 2011. Uptake and elimination, and effect of estrogen-like contaminants in estuarine copepods: an experimental study. *Environ Sci Pollut Res Int* 18(2):226-36.

19. Giacomazzi S, Cochet N. 2004. Environmental impact of diuron transformation: a review. *Chemosphere* 56(11):1021-32.

20. Barranger A, Akcha F, Rouxel J, Brizard R, Maurouard E, Pallud M, Menard D, Tapie N, Budzinski H, Burgeot T, Benabdelmouna A. 2014. Study of genetic damage in the Japanese oyster induced by an environmentally-relevant exposure to diuron: evidence of vertical transmission of DNA damage. *Aquat Toxicol* 146:93-104.

21. Soares A, Guieysse B, Jefferson B, Cartmell E, Lester JN. 2008. Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environ Int* 34(7):1033-49.

22. European Union. 2008. Directive 2008/105/EC of the European Parliament and of Council of 16 December 2008 on environmental quality standards in the field of water policy. *Official Journal of the European Union*: 84-97.

23. Devreker D, Souissi S,Winkler G, Forget-Leray J, Leboulenger F. 2009. Effects of salinity, temperature and individual variability on the reproduction of *Eurytemora affinis* (Copepoda; Calanoida) from the Seine estuary: a laboratory study. *J Exp Mar Biol Ecol* 368(2):113-123.

24. Pasquevich MY, Dreon MS, Gutierrez Rivera JN, Vázquez Boucard C, Heras H. 2013. Effect of crude oil petroleum hydrocarbons on protein expression of the prawn *Macrobrachium borellii*. *Comp Biochem Physiol C Toxicol Pharmacol* 157:390-396.

25. Trapp J, Armengaud J, Pible O, Gaillard JC, Abbaci K, Habtoul Y, Chaumot A, Geffard O. 2015. Proteomic Investigation of Male *Gammarus fossarum*, a Freshwater Crustacean, in Response to Endocrine Disruptors. *J Proteome Res* 14(1):292-303.

26. Kimmel DG, Bradley DG. 2001. Specific protein responses in the calanoid copepod *Eurytemora affinis* (Poppe, 1880) to salinity and temperature variation. *J. Exp Mar Biol Ecol* 266:135-149.

27. DeLorenzo ME, Fulton MH. 2012. Comparative risk assessment of permethrin, chlorothalonil, and diuron to coastal aquatic species. *Marine Pollution Bulletin* 64:1291-1299.

28. Forget-Leray J, Landriau I, Minier C, Leboulenger F. 2005. Impact of endocrine toxicants on survival, development, and reproduction of the estuarine copepod *Eurytemora affinis* (Poppe). *Ecotoxicol Environ Saf* 60(3):288-294.

29. Rabilloud T, Lelong C. 2011. Two-dimensional gel electrophoresis in proteomics: A tutorial. *Journal of Proteomics* 74(10):1829–1841.

30. Figeac N, Serralbo O, Marcelle C, Zammit PS. 2014. ErbB3 binding protein-1 (Ebp1) controls proliferation and myogenic differentiation of muscle stem cells. *Dev Biol* 386(1):135-51.

31. Talakhun W, Phaonakrop N, Roytrakul S, Klinbunga S, Menasveta P, Khamnamtong B. 2014. Proteomic analysis of ovarian proteins and characterization of thymosin-β and RAC-GTPase activating protein 1 of the giant tiger shrimp *Penaeus monodon. Comp Biochem Physiol Part D Genomics Proteomics* 11:9-19.

32. Chongsatja PO, Bourchookarn A, Lo CF, Thongboonkerd V, Krittanai C. 2007. Proteomic analysis of differentially expressed proteins in *Penaeus vannamei* hemocytes upon Taura syndrome virus infection. *Proteomics* 7(19):3592-601.

33. Akcha F, Spagnol C, Rouxel J. 2012. Genotoxicity of diuron and glyphosate in oyster spermatozoa and embryos. *Aquat Toxicol* 106-107:104-13.

34. Manzo S, Buono S, Cremisini C. 2006. Toxic effects of irgarol and diuron on sea urchin *Paracentrotus lividus* early development, fertilization, and offspring quality. *Arch Environ Contam Toxicol* 51(1):61-8.

35. Bouilly K, Bonnard M, Gagnaire B, Renault T, Lapègue S. 2007. Impact of diuron on aneuploidy and hemocyte parameters in Pacific oyster, *Crassostrea gigas*. A*rch Environ Contam Toxicol* 52(1):58-63.

36. Slaninova A, Smutna M, Modra H, Svobodova Z. 2009. A review: oxidative stress in fish induced by pesticides. *Neuro Endocrinol Lett* 30 suppl 1:2-12.

37. Cherkasov AA, Overton RA Jr, Sokolov EP, Sokolova IM. 2007. Temperature-dependent effects of cadmium and purine nucleotides on mitochondrial aconitase from a marine ectotherm, *Crassostrea virginica*: a role of temperature in oxidative stress and allosteric enzyme regulation. *J Exp Biol* 210:46-55.

38. Hébert N, Gagné F, Cyr D, Pellerin J, Blaise C, Fournier M. 2009. Effects of 4- nonylphenol on the immune system of rainbow trout (*Oncorhynchus mykiss*). *Fresen Environ Bull* 18:757-761.

39. Jie X, Jianmei L, Zheng F, Lei G, Biao Z, Jie Y. 2013. Neurotoxic effects of nonylphenol: a review. *Wien Klin Wochenschr* 125(3-4):61-70.

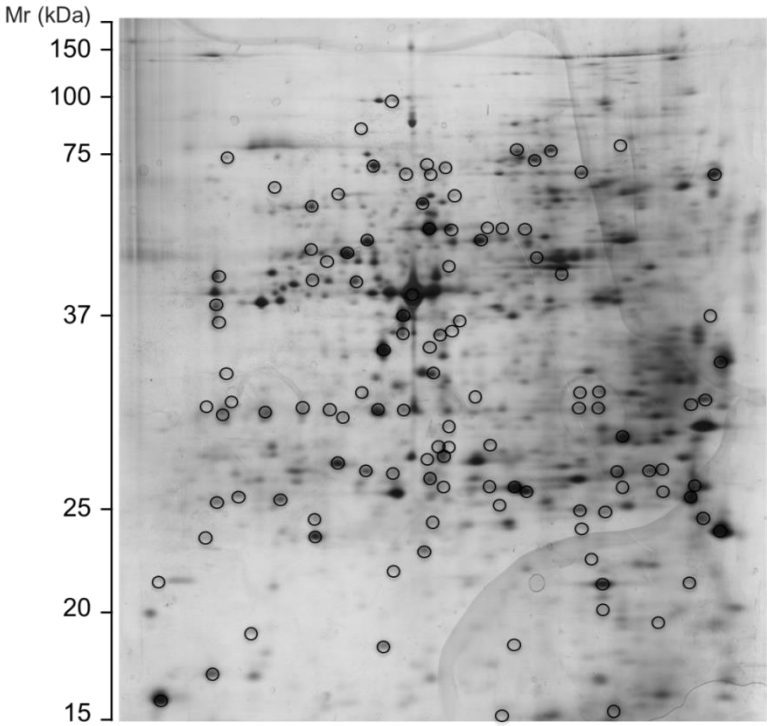
FIGURE LEGENDS

Figure 1: Representative 2DE control gels obtained from pools of *E. affinis* after silver nitrate staining (A, analysis gel) and colloidal blue staining (B, preparative gel). Differentially expressed proteins obtained after matching (circled spots) were excised from the analysis gel for mass spectrometry analysis. Mr, relative molecular mass.

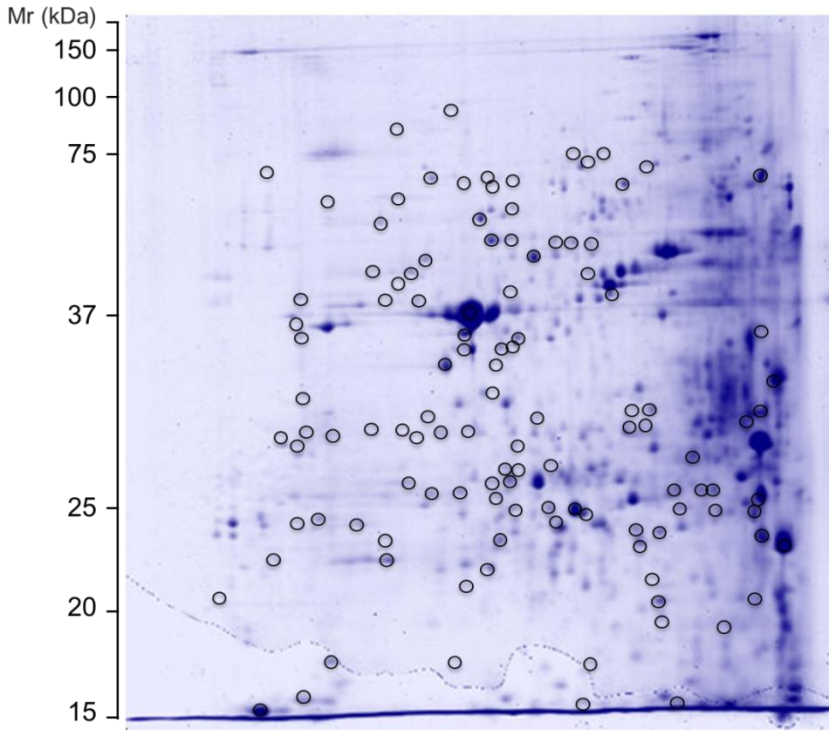
Figure 2: Differentially expressed proteins detected after diuron (A) or AP (B) exposures of *E. affinis* in a flow-through system. Under- and over-expressed spots – identified on a typical 2DE protein expression profile – are underlined in black by squares and circles respectively ($p<0.01$)Common spots are surrounded by white circles. Spot numbers refer to those reported in tables 1 and 2. Mr, relative molecular mass.

Figure 3: Pie diagrams illustrating the distribution of differentially expressed proteins after diuron (A) and AP (B) exposures according to their KEGG pathways. The data are derived from Tables 1 and 2.

A



B



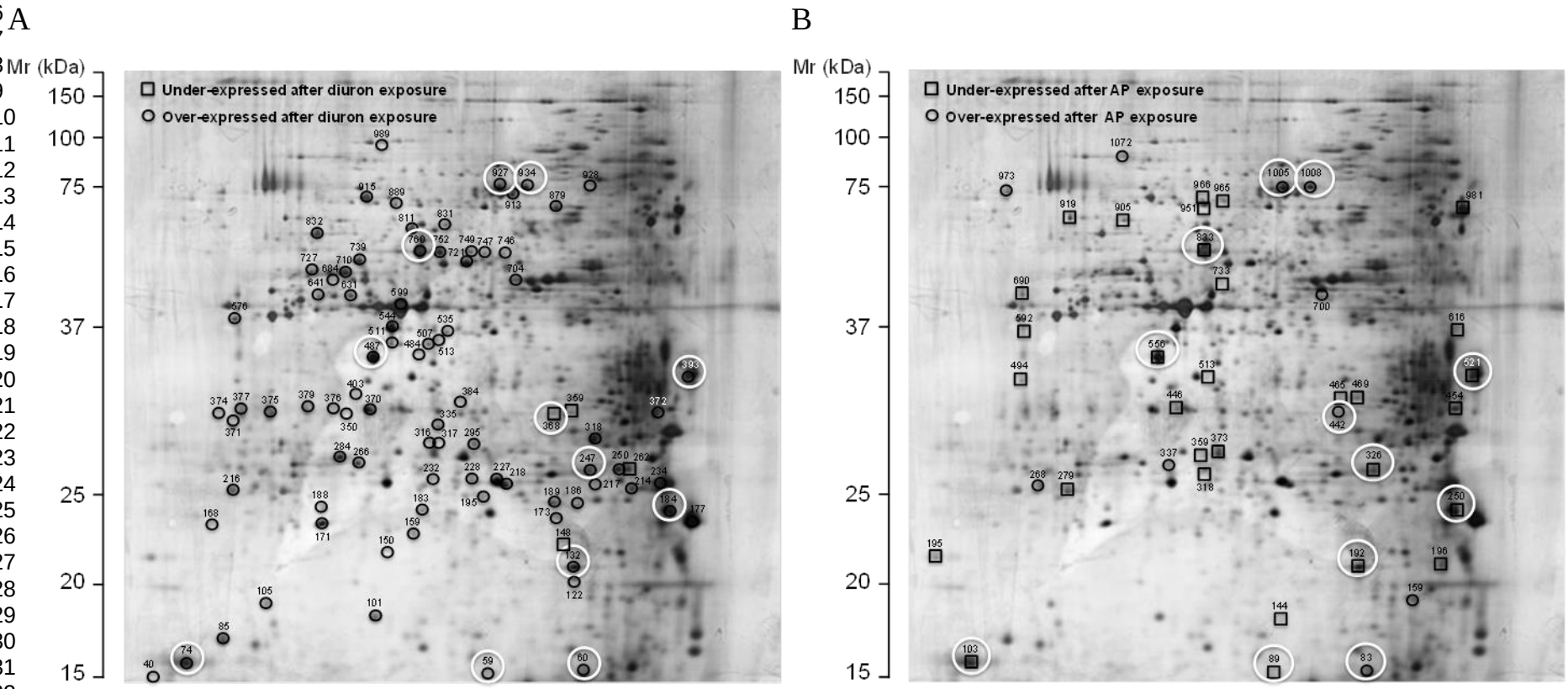

A
Mr (kDa)
150
100
75
37
25
20
15
Under-expressed after diuron exposure
Over-expressed after diuron exposure
B
Mr (kDa)
150
100
75
37
25
20
15
Under-expressed after AP exposure
Over-expressed after AP exposure

Figure 3

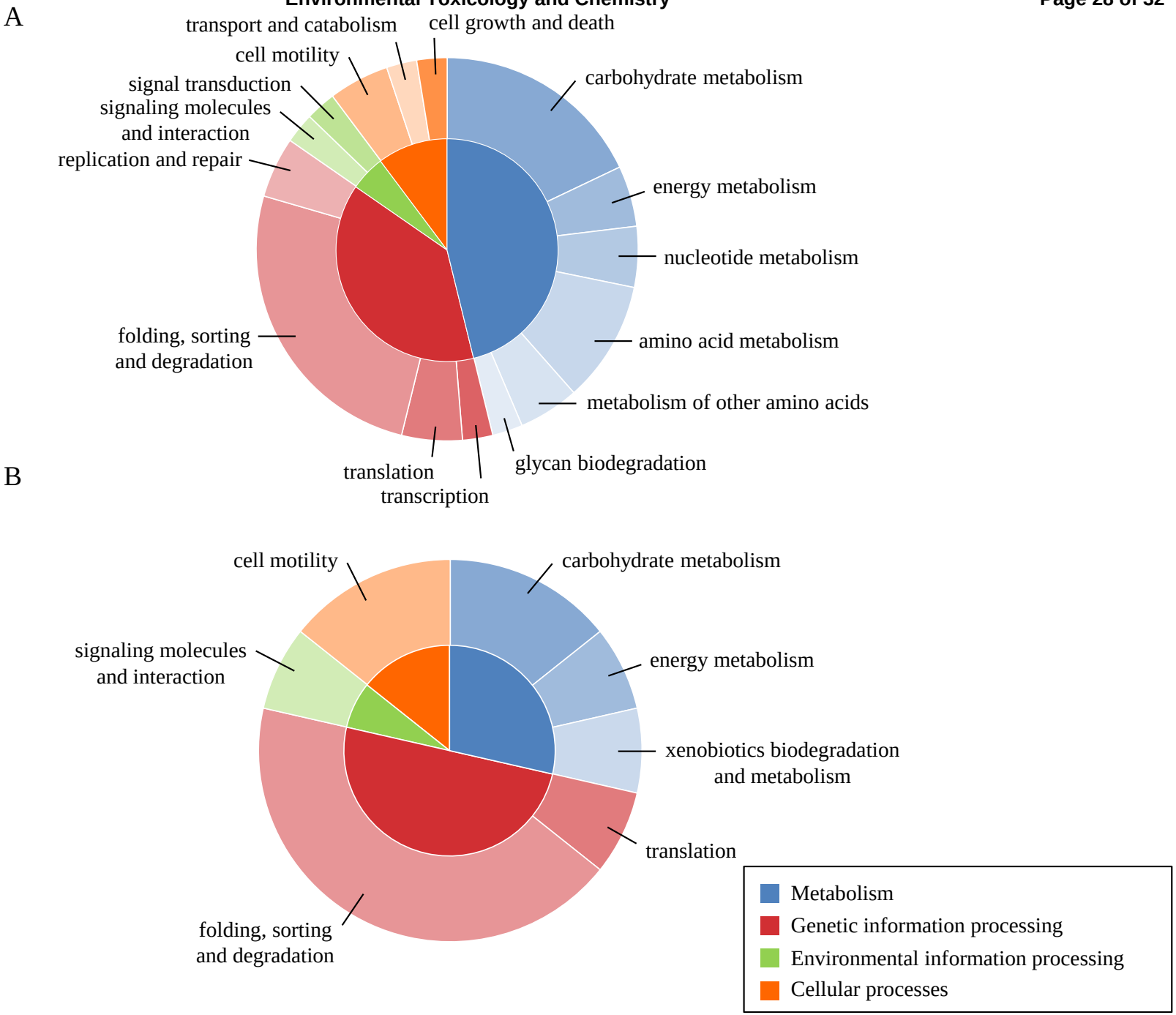

A
transport and catabolism
cell growth and death
cell motility
carbohydrate metabolism
signal transduction
signaling molecules
and interaction
energy metabolism
replication and repair
nucleotide metabolism
folding, sorting
and degradation
amino acid metabolism
metabolism of other amino acids
translation
transcription
glycan biodegradation
B
cell motility
carbohydrate metabolism
signaling molecules
and interaction
energy metabolism
xenobiotics biodegradation
and metabolism
translation
folding, sorting
and degradation
Metabolism
Genetic information processing
Environmental information processing
Cellular processes

Table 1. Protein spots differentially expressed after copepod exposure to diuron

Code number[a]	Variation[b]	Mascot identification[c]								
		Protein name	Protein function	Species	NCBI entry	Mr (Da)	pI	Score	Seq	Cov.
	under expressed									
148	0.62	proliferation-associated protein 2G4	cell proliferation - cell cycle protein	Lepeophtheirus salmonis	gi|225712832	44661	6.87	60	1	2
262	0.73	proteasome subunit alpha type-5, partial	protein degradation - direct interaction with the PSMG1-PSMG2 heterodimer which promotes 20S proteasome assembly	Calanus helgolandicus	gi|566637195	13338	4.41	65	1	10
359	0.83	14-3-3 zeta	cell proliferation - signal transduction by binding to phosphoserine-containing proteins	Artemia franciscana	gi|161898814	28049	4.79	82	1	5
368*	0.48									
	over expressed									
40	1.27	hypothetical protein DAPPUDRAFT_269172	undetermined	Daphnia pulex	gi|321451372	74668	6.34	60	1	1
59*	2.05	elongation factor 1 alpha	translation - delivery of aminoacylated tRNAs to the elongating ribosome	Ips typographus	gi|161722798	26711	8.42	62	2	7
60*	2.70									
74*	1.46	cyclophilin	protein folding - molecular chaperone	Aiptasia pallida	gi|140427776	17759	9.10	195	7	31
85	1.60	nucleoside diphosphate kinase B-like, predicted protein	nucleoside triphosphate synthesis - exchange of phosphate groups between nucleoside diphosphates	Nematostella vectensis	gi|156375296	18548	7.86	137	4	15
101	1.49	RNase H, hypothetical protein LOC100635540	DNA replication and repair - primer removal	Amphimedon queenslandica	gi|340383518	135435	9.07	50	2	1
105	1.65	nucleoside diphosphate kinase, putative	nucleoside triphosphate synthesis - exchange of phosphate groups between nucleoside diphosphates	Ixodes scapularis	gi|241859205	17230	6.74	79	2	13
122	2.78	nonmuscle myosin II heavy chain	cellularization - morphogenesis and cytokinesis	Hymenolepis microstoma	gi|674591345	126902	5.49	60	2	2
132*	1.52	ATP synthase subunit beta, mitochondrial precursor	energy metabolism - ATP synthesis from transfer of protons	Lepeophtheirus salmonis	gi|225712758	54281	5.04	64	1	3
150	2.20									
159	4.02									
168	2.56	histone H4	DNA replication and transcription - chromatin structure	Diprion pini	gi|1883026	11290	11.36	60	1	11
171	1.91	heat shock protein 70	protein folding - molecular chaperone	Pseudodiaptomus annandalei	gi|530407844	71376	5.27	174	4	8
173	2.04	ATP synthase beta subunit, partial	energy metabolism - ATP synthesis from transfer of protons	Modiolus americanus	gi|46909259	46240	5,00	407	7	20
177	1.39	Protein CBG25918, t-box transcription factor TBX21-like	transcription regulation	Caenorhabditis briggsae	gi|309361712	28953	9.5	55	1	3
183	2.54									
184*	2.10									
186	4.15	ATP synthase beta subunit, partial	energy metabolism - ATP synthesis from transfer of protons	Modiolus americanus	gi|46909259	46240	5,00	385	7	20
188	2.03	superoxide dismutase 1, mitochondrial precursor	anti-oxidative and detoxification systems - conversion of superoxide radicals into hydrogen peroxide and oxygen	Caligus clemensi	gi|225718514	24541	7.68	71	2	7
189	2.11	ATP synthase beta subunit, partial	energy metabolism - ATP synthesis from transfer of protons	Modiolus americanus	gi|46909259	46240	5,00	297	6	17
195	2.27									
214	3.22									
216	1.83	proteasome subunit beta type-7-like, predicted	protein degradation - member of the 20S proteasome core beta subunit	Amphimedon queenslandica	gi|340381844	29607	8.13	60	2	5
217	1.51									
218	1.47	glutathione S-transferase 1	anti-oxidative and detoxification systems - conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles	Ascaris suum	gi|1170109	23627	6.92	81	3	9
227	1.22	triosephosphate isomerase	energy metabolism - conversion of D-glyceraldehyde 3-phosphate into glycerone phosphate	Eurytemora affinis	gi|262305833	16441	5.09	79	2	12
228	1.74									

232	1.54									
234	1.25	cell division cycle protein 27 homologous partial	cell proliferation - cell cycle protein	Anopheles darlingi	gi|312380239	125404	6.2	51	3	2
240	1.32	leucine-rich repeat-containing protein 20, predicted	undetermined	Anopheles gambiae	gi|118782571	27619	5.03	52	1	3
247*	1.98	tropomyosin	muscle contraction - calcium dependent regulation of contraction	Lepeophtheirus salmonis	gi|225711890	33265	4.83	177	4	13
250	1.56	20S proteasome regulatory subunit alpha type PSMA5	protein degradation - direct interaction with the PSMG1-PSMG2 heterodimer which promotes 20S proteasome assembly	Glossina morsitans morsitans	gi|289740945	26810	4.75	130	2	10
266	2.11									
284	2.34	elongation factor-2	translation - GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome	Eurytemora affinis	gi|37703935	79396	5.78	107	3	4
295	3.75									
316	2.83									
317	2.19									
318	1.17	ATP synthase beta subunit, partial	energy metabolism - ATP synthesis from transfer of protons	Dendraster excentricus	gi|46909239	45827	4.95	502	9	26
335	3.33									
350	3.69									
371	4.64									
372	1.30	glucose-regulated protein 78kDa	protein folding - molecular chaperone	Eurytemora affinis	gi|359372671	72050	4.89	239	7	10
374	2.95									
375	1.97									
376	1.97									
377	2.52									
379	2.69									
384	3.12	omega-amidase NIT2-B		Crassostrea gigas	gi|405952265	30842	6.10	64	2	4
393*	1.77	glyceraldehyde-3-phosphate dehydrogenase	glycolytic pathway - conversion of D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate	Acartia pacifica	gi|509391698	36007	6.92	170	5	18
403	3.81									
484	1.81	elongation factor-2	translation - GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome	Streptocephalus seali	gi|37703993	82331	6.31	110	4	6
487*	1.47									
507	1.35	potassium channel subfamily T member 2 isoform X1, predicted	ion transport - Produces rapidly activating outward rectifier K+ currents	Acyrthosiphon pisum	gi|641660767	157748	6.98	50	2	1
511	1.84	glyceraldehyde 3-phosphate dehydrogenase	glycolytic pathway - conversion of D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate	Limenitis arthemis astyanax	gi|348167570	22037	6.47	153	4	10
513	2.90									
535	4.64									
544	1.30	glyceraldehyde-3-phosphate dehydrogenase	glycolytic pathway - conversion of D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate	Cherax quadricarinatus	gi|40887061	35830	6.54	191	5	14
576	1.55	elongation factor 1-alpha	translation - delivery of aminoacylated tRNAs to the elongating ribosome	Limnadia lenticularis	gi|4530094	40077	7.21	179	4	11
599	1.19	arginine kinase 3	energy metabolism - transfer of a phosphoryl group between ATP and arginine	Neocaridina denticulata	gi|226693235	39823	6.02	356	12	23
631	2.89									
641	2.02									
684	2.15									
704	2.58	enolase = 2-phospho-D-glycerate hydrolase	glycolytic pathway - conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate	Lepeophtheirus salmonis	gi|155966226	32045	5.27	125	2	7
710	2.01	citrate synthase 1, predicted mitochondrial precursor	tricarboxylic acid cycle - condensation reaction of acetyl coenzyme A and oxaloacetate into citrate	Lepeophtheirus salmonis	gi|225713236	52614	7.25	207	4	10
721	1.33	phosphoglycerate kinase, putative	glycolytic pathway - conversion of 1,3-diphosphoglycerate into 3-phosphoglycerate	Danaus plexippus	gi|357610086	44368	6.22	246	7	18

727	2.28									
739	1.62	fumarate hydratase, hypothetical	tricarboxylic acid cycle - conversion of fumarate to malate	Anopheles darlingi	gi|312380127	54822	8.92	147	5	12
746	1.57	enolase = 2-phospho-D-glycerate hydrolase	glycolytic pathway - conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate	Dendroctonus ponderosae	gi|332375414	47222	6.05	97	2	4
747	1.54	enolase = 2-phospho-D-glycerate hydrolase	glycolytic pathway - conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate	Hypocryphalus mangiferae	gi|14161523	40288	5.28	58	1	3
749	1.96									
752	1.34	adenosylhomocysteinase	methylation homeostasis - conversion of S-adenosylhomocysteine to homocysteine and adenosine	Lepeophtheirus salmonis	gi|225712068	47915	5.77	121	3	7
760*	1.39	enolase = 2-phospho-D-glycerate hydrolase	glycolytic pathway - conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate	Artemia sp. SBH266677	gi|41394395	39739	5.06	85	2	8
811	1.36	methylmalonate-semialdehyde dehydrogenase, predicted	aminoacid pathway - decarboxylation of methylmalonate semialdehyde to propionyl-CoA	Nematostella vectensis	gi|156407980	58005	7.94	152	4	8
831	1.41									
832	2.04	leucyl aminopeptidase provisional, hypothetical protein ZHAS_00009025	protein degradation - hydrolysis of leucine residues at the N-terminus of proteins	Anopheles sinensis	gi|668452503	57788	6.95	77	2	5
846	3.39									
879	1.71	mitochondrial heat shock protein 60	protein folding - molecular chaperone	Calanus glacialis	gi|166798221	61744	5.19	323	7	13
889	2.04									
913	2.70	aconitase (aconitate hydratase), predicted mitochondrial-like	tricarboxylic acid cycle - isomerization of citrate to isocitrate	Bombus terrestris	gi|340724974	86662	8.59	108	3	3
915	1.63	delta-1-pyrroline-5-carboxylate dehydrogenase, putative	aminoacid pathway (conversion of delta-1-pyrroline-5-carboxylate to glutamate) interconnecting urea and tricarboxylic acid cycles	Pediculus humanus corporis	gi|242004871	59457	6.25	104	2	4
927*	1.89	heat shock protein cognate 5	protein folding - molecular chaperone	Anopheles darlingi	gi|568255094	79593	7.96	172	5	9
928	3.12									
934*	1.79	molecular chaperone DnaK	protein folding - molecular chaperone	Culex quinquefasciatus	gi|170036376	72845	5.91	206	4	8
989	3.94									

[a]Spot numbers refer to those used in figure 2A

[b]Variation corresponds to fold change between exposure and control samples

[c]Proteins were identified after tandem mass spectrometry, Mascot request and named according to NCBI entries. Protein functional information was provided by UniProt (http://www.uniprot.org/)

Mr: molecular weight; pI: isoelectric point; seq: number matched sequences; cov: sequence coverage in %

*: spots differentially modified in both diuron and AP exposures

Table 2. Protein spots differentially expressed after copepod exposure to APs

Code number[a]	Variation[b]	Mascot identification[c]								
		Protein name	Protein function	Species	NCBI entry	Mr (Da)	pI	Score	Seq	Cov.
	under expressed									
89*	0.63	elongation factor 1 alpha	translation - GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes	Ips typographus	gi|161722798	26711	8.42	62	2	7
103*	0.50	cyclophilin	protein folding - molecular chaperone	Aiptasia pallida	gi|140427776	17759	9.10	195	7	31
144	0.62									
192*	0.80	ATP synthase subunit beta, mitochondrial precursor	energy metabolism - ATP synthesis from transfer of protons	Lepeophtheirus salmonis	gi|225712758	54281	5.04	64	1	3
195	0.77									
196	0.86									
250*	0.79									
279	0.78	proteasome subunit alpha type-2	protein degradation - member of the 20S proteasome core beta subunit	Danaus plexippus	gi|357622706	25937	6,07	102	2	7
318	0.51	ATP synthase subunit beta, mitochondrial precursor	energy metabolism - ATP synthesis from transfer of protons	Lepeophtheirus salmonis	gi|225712758	54281	5.04	64	1	3
326*	0.68	tropomyosin	muscle contraction - calcium dependent regulation of contraction	Lepeophtheirus salmonis	gi|225711890	33265	4.83	177	4	13
359	0.69									
373	0.73									
446	0.80									
454	0.68									
465	0.79	glutamate receptor	nervous conduction - glutamatergic synaptic transmission	Lymnaea stagnalis	gi|239628	103530	7.69	27	2	2
469	0.75									
494	0.67									
513	0.37									
521*	0.72	glyceraldehyde-3-phosphate dehydrogenase	glycolytic pathway - conversion of D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate	Acartia pacifica	gi|509391698	36007	6.92	170	5	18
556*	0.82									
592	0.63									
616	0.42									
690	0.69									
733	0.76									
833*	0.80	enolase = 2-phospho-D-glycerate hydrolase	glycolytic pathway - conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate	Artemia sp. SBH266677	gi|41394395	39739	5.06	85	2	8
905	0.60	alpha esterase	xenobiotic degradation - carboxylic ester hydrolase activity	Aedes aegypti	gi|157130962	64332	5.96	44	2	3
919	0.75									
951	0.70									
965	0.65									
966	0.55									
981	0.63	calreticulin	protein folding - molecular chaperone	Lepeophtheirus salmonis	gi|290462503	45846	4.46	63	1	3
	over expressed									
83*	1.55									
159	1.28	myosin light chain alkali	muscle contraction - Catalysis of movement along a microfilament, coupled to the hydrolysis of ATP	Caligus rogercresseyi	gi|225711070	18820	4.59	72	1	6

268	1.34									
337	1.59									
442*	1.23									
700	1.71	ubiquitin-conjugating enzyme E2, hypothetical protein AND_11650	protein degradation - catalyzis of ubiquitin attachment to other proteins	Anopheles darlingi	gi|312377149	143891	5.02	49	1	0
973	1.30									
1005*	1.93	heat shock protein cognate 5	protein folding - molecular chaperone	Anopheles darlingi	gi|568255094	79593	7.96	172	5	9
1008*	1.28	molecular chaperone DnaK	protein folding - molecular chaperone	Culex quinquefasciatus	gi|170036376	72845	5.91	206	4	8
1072	1.61									

[a]Spot numbers refer to those used in figure 2B

[b]Variation corresponds to fold change between exposure and control samples

[c]Proteins were identified after tandem mass spectrometry, Mascot request and named according to NCBI entries. Protein functional information was provided by UniProt (http://www.uniprot.org/)

Mr: molecular weight; pI: isoelectric point; seq: number matched sequences; cov: sequence coverage in %

*: spots differentialy modified in both diuron and AP exposures