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Céline Boulange-Lecomte, Béatrice Rocher, Kevin Cailleaud, Pascal Cosette, Eléna Legrand, David Devreker, Hélène Budzinski, Sami Souissi, Joëlle Forget-Leray

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

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Mandatory Keywords:	biomarkers, estuarine toxicology, persistent organic pollutants (POPs)
Additional Keywords (Optional):	2D electrophoresis, calanoid
Abstract:	Proteomics was used in the calanoid copepod <i>Eurytemora affinis</i> 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 <sup>-1</sup> , Alkylphenol (AP) mixture: 1000 ng.L <sup>-1</sup> ). 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 <i>E. affinis</i> after diuron exposure whereas APs induced an alteration of a few targeted physiological functions. The protein expression signatures identified after

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	contaminant exposure deserve to further investigate the development of novel potential biomarkers for water quality assessment.

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3 **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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3 **Abstract** - Proteomics was used in the calanoid copepod *Eurytemora affinis* for screening of  
4 protein expression modifications induced by organic contaminants. The copepods were exposed  
5 in a continuous flow-through system for 86 h to environmentally relevant concentrations of  
6 contaminants representative of the Seine Estuary's pollution (Diuron: 500 ng.L<sup>-1</sup>, Alkylphenol  
7 (AP) mixture: 1000 ng.L<sup>-1</sup>). The proteome analysis of whole body copepod extracts by two-  
8 dimensional gel electrophoresis (2DE) revealed that the contaminants induced modifications in  
9 protein expression, with the highest quantitative variations occurring after diuron exposure.  
10 Specifically, a total of 88 and 41 proteins were differentially expressed after diuron and AP  
11 treatments respectively. After mass spectrometry analysis, 51 (diuron exposure) and 15 (AP  
12 exposure) proteins were identified. The identified proteins were potentially related to energy  
13 metabolism, cell growth, nervous signal conductivity, excitotoxicity, oxidative stress response  
14 and antioxidant defense. The data suggest a massive general disturbance of physiological  
15 functions of *E. affinis* after diuron exposure whereas APs induced an alteration of a few targeted  
16 physiological functions. The protein expression signatures identified after contaminant exposure  
17 deserve to further investigate the development of novel potential biomarkers for water quality  
18 assessment.

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43 **Keywords** - 2D Electrophoresis, Biomarkers, Calanoid, Estuary, Organic contaminants  
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## INTRODUCTION

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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

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3 25 posttranslational modifications [8]. The proteome represents the bulk of proteins of an organism,  
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5 26 a cell, an organ, or even a body fluid, quantified at a given moment and under precise conditions.  
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8 27 It can thus be considered as a stress signature. In that line, the proteome has been increasingly  
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10 28 studied over the last decade to identify key biochemical actors involved in physiological *versus*  
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12 29 pathological pathways. Proteomics has opened novel horizons in many research areas of life  
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15 30 sciences such as medicine [9], ecology [10] and ecotoxicology [10].  
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18 31 Several studies have reported the strong contamination of the Seine Estuary by organic micro-  
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20 32 pollutants especially by neurotoxic, carcinogenic and estrogen-like compounds [11, 12]. In spite  
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22 33 of this high chemical stress context, the calanoid micro-crustacea *Eurytemora affinis* dominates  
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24 34 the zooplankton community, representing 90 to 99 % of zooplanktonic species [13]. Over the  
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27 35 years, *E. affinis* has become a relevant ecological and ecotoxicological bioindicator [14]. Indeed,  
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29 36 this euryhaline copepod is widely distributed in North-Atlantic brackish waters where it  
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31 37 represents an essential basal organism in the trophic web. The *E. affinis* copepods are appropriate  
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33 38 test organisms because of their small size, sexual dimorphism, short generation time, and ease of  
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36 39 culturing in the laboratory [15, 16, 17, 18].  
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39 40 Among hydrophobic organic contaminants, diuron and alkylphenols (APs) are of major  
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41 41 concern because of their persistence and toxicity. Diuron, (3-(3',4'-dichlorophenyl)-1,1-  
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43 42 dimethyl-urea; subclass of phenylurea) has been widely used both as a herbicide to control a wide  
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45 43 variety of weeds and mosses, and as an anti-foulant, in replacing tributyltin [19]. Due to its high  
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47 44 persistence (one month to one year), diuron can be detected in soil, sediment and water [19]. The  
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49 45 current diuron concentration is around 1 to 1,000 ng.L<sup>-1</sup> [20]. APs belong to the group on non-  
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51 46 ionic surfactants. They have been extensively used as detergents, dispersants or solubilizers [21].  
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53 47 Literature data report an AP concentration in worldwide river waters ranging from 0.6 ng.L<sup>-1</sup> to  
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55 48 15 µg.L<sup>-1</sup> when related to sewage treatment plant discharges [21].  
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3 49 In this context, we investigated the effects of diuron and APs – categorized as Priority  
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5 50 Hazardous Substances by the European commission (Directive on Environmental Quality  
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8 51 Standards 2008/105/EC, [22]) – on the estuarine *E. affinis* proteome after experimental exposure  
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10 52 at environmentally realistic concentrations in order to identify Protein Expression Signatures  
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12 53 (PES).  
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## 17 55 MATERIALS AND METHODS

### 19 56 *Eurytemora affinis* collection and stabulation

20 57 The copepods *E. affinis* were collected in autumn using subsurface tows of WP2 plankton net  
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22 58 (200- $\mu\text{m}$  mesh size) at ebb tide, in Tancarville Station in the oligo-mesohaline part of the Seine  
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24 59 Estuary (longitude 0°15'52"E, latitude 49°29'19"N; Haute-Normandie, France). Immediately  
25  
26 60 after sampling, the copepods were sorted using 500  $\mu\text{m}$  sieves in order to eliminate predators  
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28 61 (especially Mysidacea and Gammaridae), transferred into isotherm containers and brought back  
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30 62 to the laboratory.  
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36 63 The copepods were maintained in the laboratory for an acclimatization period of three days in  
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38 64 a 300-L hydrodynamic canal under optimally controlled conditions i.e. allowing high embryonic  
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40 65 developmental rates (constant aeration; salinity, salinity 15; temperature, 10 °C; photoperiod,  
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42 66 12:12h) [23]. The hydrodynamic canal contained freshly filtered sea water (GF/C Whatman filter,  
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44 67 0.45  $\mu\text{m}$ ) sampled in the English Channel mixed with ultra-pure water in order to reach the  
45  
46 68 selected salinity of 15. The same water preparation was used for exposure experiments. During  
47  
48 69 the detoxification period, the copepods were fed twice a day with an algal mixture (*Rhodomonas*  
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50 70 *marina* and *Isochrysis galbana*) receiving a total of 20,000 cells.mL<sup>-1</sup>. Algae cultures were grown  
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52 71 at 20 °C in 10-L tanks under 24-h fluorescent illumination and constant aeration in Conway  
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54 72 medium.  
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3 73 *Material design*  
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5 74 The experimental system was set up as described by Cailleaud et al. [18]. Briefly, the  
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8 75 experimental system was composed of three compartments: the water reservoirs, the exposure  
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10 76 tanks containing the copepods, and a recycling tank to remove contaminants from the water using  
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12 77 activated carbon. Contaminant concentrations in the water reservoirs and in the exposure tanks  
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15 78 were identical. All the tanks were made of glass and were systematically disinfected and washed  
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17 79 before use. Peristaltic pumps were used to (i) draw up contaminated water from the water  
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20 80 reservoirs to the exposure tanks, and (ii) eliminate excess water from the exposure tanks into the  
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22 81 salvaged tank. In order to avoid aspiration of copepods by the peristaltic pumps, the rubber tubing  
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24 82 extremities was capped with 50- $\mu$ m mesh.  
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27 83 *Experimental setups*  
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29 84 Selected contaminants (purity > 98 %) i.e. diuron and alkyl phenols (APs; 4-nonylphenol, 4-  
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31 85 NP; nonylphenol-ethoxy-acetic-acid, NP1EC) were purchased from Sigma-Aldrich (Saint  
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33 86 Quentin Fallavier, France; diuron, 4-NP) and LGC Standards (Molsheim, France; NP1EC).  
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36 87 Preliminary tests were performed with the exposure system in order to determine the appropriate  
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38 88 experimental conditions to saturate the tanks and the rubber tubing with the highly hydrophobic  
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41 89 selected contaminants (APs and diuron) to ensure that the real exposure concentrations were  
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43 90 included within an acceptable range from the selected nominal concentrations during all the  
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46 91 experiments [18]. Briefly, before each exposure experiment, the system was saturated using the  
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48 92 experimental conditions. The stock solutions of contaminants were prepared at high  
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51 93 concentrations in acetone in order to introduce low volumes of solvent in the water (i.e. 15  $\mu$ L of  
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53 94 acetone/L of water). The contaminant solution was weighed in a glass vial and manually  
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55 95 introduced into the water of both reservoirs and exposure tanks at the beginning of the saturation  
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3 96 period using a glass pipette in order to reach the appropriate experimental concentrations. The  
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5 97 water was then gently stirred and the flow-through system was activated.  
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8 98 Two copepod groups were exposed simultaneously in separate microcosms to environmentally  
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10 99 realistic concentrations of diuron (500 ng.L<sup>-1</sup>) and AP mixture (4-NP, 480 ng.L<sup>-1</sup>; NP1EC, 520  
11  
12 100 ng.L<sup>-1</sup>) for 86 h in the experimental continuous flow-through system [20, 21]. In parallel, a  
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14 101 control group was maintained in a continuous flow-through system in clean water. During the  
15  
16 102 exposure experiments, food was not provided to the copepods to avoid algal sorption. Copepod  
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18 103 samples (exposed and non-exposed organisms) were collected and immediately frozen in liquid  
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20 104 nitrogen at the end of the exposure experiments for further proteomic analysis. Six pools of  
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22 105 copepods (350 mg wet weight i.e. approximately 7,000 individuals each) were collected for  
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24 106 each experimental condition.  
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#### 28 29 107 *Protein extraction*

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31 108 Copepod pools of whole-body copepods were homogenized on ice, in ice-cold tris HCl buffer  
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33 109 50 mM pH 7.5 (1/3, v/w) composed of EDTA (2 mM), glycerol (10 %), magnesium acetate (5  
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35 110 mM), dithiothreitol (DTT 1 mM) and aprotinin (0.1 %) using an Ultra-Turax homogenizer  
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37 111 followed by ultrasonication. Cellular debris was then removed by centrifugation (9,000 g for 15  
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39 112 min at 4°C). Then, supernatants were collected and centrifuged at 105,000 g for 1 hour at 4 °C.  
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41 113 Final supernatants were collected and precipitated with trichloroacetic acid (50%) for 45 min.  
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43 114 The precipitated proteins were centrifuged at 9,000 g for 30 min at 4 °C, washed with acetone  
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45 115 and then resuspended in a hydration buffer composed of 9 M urea, 55 mM CHAPS and 110  
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47 116 mM DTT. All reagents were purchased from Sigma-Aldrich. The protein quantification was  
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49 117 evaluated according to the Bradford method using BSA as a standard.  
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#### 53 118 *Two-dimensional gel electrophoresis (2DE)*

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3 119 Depending on the final staining techniques, 150  $\mu\text{g}$  of total protein extracts (silver staining) or  
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5 120 750  $\mu\text{g}$  of total protein extracts (colloidal blue staining) were suspended in an isoelectric focusing  
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8 121 (IEF) buffer composed of 1 % IPG carrier ampholytes (pH 3.5–10) and a pinch of Orange G. The  
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10 122 first dimension gel separation was carried out with nonlinear Immobiline Dry Strips (18 cm, pH  
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12 123 3–10, nonlinear; GE Healthcare Life Sciences, Velizy-Villacoublay, France) using a Multiphor II  
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15 124 apparatus (GE Healthcare Life Sciences). IEF was performed using the following parameters:  
16  
17 125 increase to 500 V in 1 minute, 500 V for 5 hours, increase to 3500 V in 5 hours and 3500 V for 9  
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19 126 h 30 (1 mA, 5 W constant) for a total of 53.3 kV. After isoelectric focusing, strips were soaked in  
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21 127 15 mM dithiothreitol in equilibration buffer (50 mM tris-HCl buffer, pH 6.8, containing 6 M  
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23 128 urea, 4% SDS, 25 % glycerol) for 12 min and soaked in 120 mM iodoacetamide and  
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25 129 bromophenol blue in the equilibration buffer for 5 min. The second dimension consisted of an  
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27 130 SDS–PAGE using 12 % polyacrylamide resolving gels using a Protean Plus Dodeca Cell (width,  
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29 131 19 cm; length, 20 cm; thickness, 0.75 mm; BioRad, Marnes la Coquette, France). Electrophoresis  
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31 132 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.  
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33 133 After migration, the 150  $\mu\text{g}$  of protein extracts were visualized by silver nitrate staining  
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35 134 (developing time: 15 min) for gel analysis (Figure 1A) and the 750  $\mu\text{g}$  of protein extracts were  
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37 135 visualized by colloidal blue staining for trypsin digestion and mass spectrometry (preparative gel;  
38  
39 136 Figure 1B).

#### 137 *Image analyses*

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

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3 143 were then compared to the one from the non-exposed copepods for differential expression  
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5 144 analysis. The quantification of each spot was expressed as volume on a Gaussian image (area of  
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8 145 each spot multiplied by its intensity). The differences of protein expression between exposed and  
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10 146 non-exposed copepods were statistically analysed by the STATISTICA Software v6.0 (Statsoft.  
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12 147 Inc., 2002). To compare two groups, the non-parametric Mann-Whitney U test was used. In all  
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15 148 cases, the level of significance ( $p$ ) was set to 0.01.

#### 17 149 *Trypsin digestion and mass spectrometry analysis*

20 150 Coomassie-stained spots of interest were excised from the polyacrylamide gel. Gel plugs were  
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22 151 washed twice with 100  $\mu$ L of  $\text{NH}_4\text{HCO}_3$  buffer (25 mM) and then dried with acetonitrile (ACN).  
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24 152 After DTT reduction, these plugs were incubated in the dark with iodoacetamide (25 mM) for 45  
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27 153 min at room temperature and totally dried using a SpeedVac centrifuge for few minutes before  
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29 154 the trypsin solution (10  $\mu$ L of a 15 ng/ $\mu$ L sequencing-grade trypsin (Promega) in 25 mM  
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32 155  $\text{NH}_4\text{HCO}_3$  buffer) was added. After rehydration with the enzyme solution, buffer solution was  
33  
34 156 added to cover the gel pieces and digestion was allowed to proceed overnight at 37 °C. Peptides  
35  
36 157 were extracted by adding twice 30  $\mu$ L of trifluoroacetic acid (TFA, 0.1 %, v/v) and 30  $\mu$ L of  
37  
38  
39 158 ACN. These fractions were concentrated in a vacuum centrifuge and then dissolved in 10  $\mu$ L of a  
40  
41 159 buffer containing 2 % of ACN (v/v) and 0.2 % of formic acid.

43 160 Sequence fragments were determined using automated nanoLC/MS/MS. Briefly, injections of  
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45  
46 161 5  $\mu$ L of the sample were carried out into an LC Packings Ultimate nanoLC (Dionex,  
47  
48 162 LCPackings). The peptides were enriched and desalted on a reversed-phase analytical column  
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50 163 (RP-C18) and the chromatographic separation was performed using a C18 Pep-Map column (75  
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52 164  $\mu$ m i.d.  $\times$  15 cm). Peptides were separated using a mixture of ACN (2 %)/formic acid (0.1%) (A)  
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55 165 and a mixture of ACN (95 %)/ formic acid (0.2 %) (B) used as the mobile phase. The solvent  
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58 166 program started with an initial B concentration of 5 % linearly increased to 50% within 45 min  
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3 167 with a constant flow rate of 200 nL/min. The eluant was analysed on a QTrap mass  
4  
5 168 spectrophotometer equipped with a nanospray source (Applied Biosystems). The peptide  
6  
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8 169 fragmentation patterns were matched using MASCOT software against the NCBIInr databases  
9  
10 170 with the parameters: one missed cleavage by trypsin, mass accuracy of 1.2 Da for the parent ions  
11  
12 171 (MS), 0.6 Da for the fragment ions (MS/MS), one fixed modification (carbamidomethylation of  
13  
14 172 cysteines), variable modifications (oxidations of methionine residues). No species restriction was  
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16 173 considered for searching in the database because copepods and crustaceans present few  
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18 174 annotations in databases.  
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## 26 **RESULTS AND DISCUSSION**

27 177 The adaptation to environmental pollution, in the same way as other biological stresses,  
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29 178 involves changes in protein expression which can be contaminant-specific or dose-dependent. In  
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31 179 recent years, comparative proteomics became a powerful tool, with the potential to reveal new  
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33 180 and unexpected associations between proteins and toxicant exposure, without prior hypotheses.  
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35 181 However, the number of publications reporting the effects of environmental pollutants on the  
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37 182 invertebrate proteome is still limited, in particular in crustaceans [24, 25]. Kimmel and Bradley  
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39 183 published a notable proteomic study on the impact of salinity and temperature on *E. affinis*, that  
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41 184 led to establishing characteristic PES (protein expression signature), but without any protein  
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43 185 identification [26]. The present paper reports results from, to our knowledge, the first study  
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45 186 designed to examine the effects of diuron and APs on crustacean proteome. The data fill an  
46  
47 187 essential need for better understanding of the effects of such compounds in non-targeted species –  
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49 188 in particular in copepods – since nowadays available studies mainly focus on lethal toxicity [27,  
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51 189 28].  
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53 190 *Global protein expression patterns after contaminant exposure*  
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3 191 The proteome of *E. affinis* was investigated after exposure to environmentally relevant  
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5 192 concentrations of organic contaminants i.e. diuron (500 ng.L<sup>-1</sup>) and a mixture of APs (4-NP, 480  
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7 193 ng.L<sup>-1</sup>; NP1EC, 520 ng.L<sup>-1</sup>) in a continuous flow-through system for a period of 86 h [20, 21]. A  
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9 194 typical 2DE protein pattern is presented in **Figure 2**. Proteomic analyses revealed that both tested  
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11 195 pollutants induced modifications in protein expression. However, two distinct patterns were  
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13 196 established according to the contaminant. The diuron exposure induced the highest quantitative  
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15 197 variations in protein expression. Indeed, the number of differentially expressed proteins after  
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17 198 diuron exposure (i.e. 88 out of 1,017 spots ; **Table 1**) – mostly characterized by over-expressions  
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19 199 in comparison with AP exposure (95.5% for diuron vs 24.4% for APs) – was 2-fold higher than  
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21 200 after AP exposure (i.e. 41 out of 934 spots ; **Table 2**). Among the differentially expressed  
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23 201 proteins, 12 were detected in both exposure conditions (Figure 2; Tables 1 and 2). Three of them  
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25 202 exhibited the same pattern i.e. over-expressed. On the contrary, 9 presented an opposite pattern  
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27 203 including 8 exhibiting an over-expression for diuron and an under-expression for APs. Among  
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29 204 differentially expressed spots, a total of 51 diuron exposure spots and 15 AP exposure spots were  
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31 205 identified using nanoelectrospray MS/MS micro-sequencing and a MASCOT search by sequence  
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33 206 similarity (Tables 1 and 2). The percentage of identification was slightly better than the expected  
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35 207 yield since many proteomic studies with aquatic invertebrates have identified, on average, 15  
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37 208 proteins indicating the problem of sequence divergences across species [7]. The still poor  
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39 209 knowledge of copepod acid nucleic sequences explains why 21% of the identifications rested  
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41 210 only on one peptide and should be considered with caution. Moreover, shifts in molecular weight  
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43 211 and pI between experimental coordinates on 2D-gels and theoretical calculations were noticed  
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45 212 and may probably be explained by the presence of post-translational protein modifications, such  
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47 213 as phosphorylation for pI changes or proteolytic cleavage for molecular weight changes for  
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49 214 instances [29]. Most of the identifications were obtained from homology with arthropod species  
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3 215 (77.3%) including 54.9% from crustacean sequences. Among crustaceans, 71.4% of  
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6 216 identifications came from copepod sequences including 40% of calanoid matches and only 15%  
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8 217 of *E. affinis* matches.  
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10 218 Differentially expressed proteins were also more diversified after diuron exposure than after  
11  
12 219 AP exposure (Figure 3). Indeed, even if they can be classified into the same KEGG (Kyoto  
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14 220 Encyclopedia of Genes and Genomes) functional categories i.e. metabolism, genetic information  
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16 221 processing, environmental information processing and cellular processes, their layout was quite  
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18 222 different, with approximately 50% of identified proteins implicated in either metabolism after  
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20 223 diuron exposure, or genetic information processing after AP exposure. Moreover, diuron  
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22 224 exposure affected the expression of proteins implicated in more than 15 subset functions against  
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24 225 only 7 after AP exposure. These data would suggest a general disruption of physiological  
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26 226 functions induced by diuron.  
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### 31 227 *Diuron effects*

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34 228 Diuron exposure led to a general over-expressed pattern compared to the control animals,  
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36 229 characterized by the induction of 84 spots and only 4 under-expressed spots (Table 1). Moreover,  
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38 230 for almost half of the over-expressed spots, the increase was more than 2, suggesting that diuron  
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40 231 led to a massive disturbance of the copepod physiology.  
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43 232 Diuron exposure appears to induce cellular stress and damage, as suggested by the increase in  
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45 233 protein synthesis and activation of defense mechanisms. These processes increase the demand for  
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47 234 cellular energy as highlighted by the significant induction of enzymes involved in carbohydrate  
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49 235 metabolism i.e. glycolytic pathway, tricarboxylic acid cycle or interrelated aminoacid pathway. In  
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51 236 addition, a direct increase in the cell capacity to provide ATP can be suggested by the over-  
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53 237 expression of energy metabolism proteins, such as ATP synthase and arginine kinase. Arginine  
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55 238 kinase – involved in the ATP buffer system – contributes to maintaining the cell ATP supply  
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3 239 when a rapid energy demand occurs, and before any activation of the glycolytic pathway. In the  
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6 240 same way, the over-expression of nucleoside diphosphate kinases could contribute to maintaining  
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8 241 the level of triphosphate nucleosides (GTP, UTP and CTP). Their induction could be related to  
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10 242 processes of DNA translation, replication and repair and protein synthesis as suggested by the  
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12 243 over-expression of several proteins involved in these processes (endonuclease RNase H, t-box  
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14 244 transcription factor TBX21, histone H4, elongation factor 1-alpha and elongation factor-2).

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17 245 In the same way, other identified proteins – such as the proliferation-associated protein 2G4,  
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19 246 the cell division cycle protein 27, and the 14-3-3 zeta – suggest an impact of diuron on cell  
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21 247 control and growth. The proliferation-associated protein 2G4 – involved in both ribosome  
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23 248 assembly and regulation of intermediate and late steps of rRNA processing – can interact with the  
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25 249 cytoplasmic domain of the ErbB3 receptor and may contribute to transducing growth regulatory  
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27 250 signals [30]. This protein is also a transcriptional corepressor of androgen receptor-regulated  
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29 251 genes and other cell cycle regulatory genes through its interactions with histone deacetylases. The  
30  
31 252 14-3-3 zeta is an adapter protein which plays an important role in the regulation of a large  
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33 253 spectrum of both general and specialized signaling pathways implicated in ovarian maturation  
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35 254 and antiviral response in crustaceans [31, 32]. The down-regulation of 14-3-3 may thus contribute  
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37 255 to inhibiting oocyte development and/or modifying the immune response in accordance with  
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39 256 literature data highlighting reproductive and immunotoxic effects of diuron in aquatic organisms  
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41 257 [33, 34, 35].

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44 258 In accordance with previous studies which have reported the induction of oxidative stress  
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46 259 response and antioxidant defense in marine organisms following diuron exposure [36], we  
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48 260 observed an over-expression of two components of the anti-oxidative and detoxification systems,  
49  
50 261 a superoxide dismutase precursor and a glutathione S-transferase enzyme, suggesting an  
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52 262 oxidative effect of diuron on the copepod. This hypothesis is supported by the increased  
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3 263 abundance of proteins belong to the folding, sorting and degradation pathways, namely the heat  
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5 264 shock proteins HSP 70, GRP78, HSC5, DnaK, HSP 60, cyclophilin and components of the  
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8 265 proteasome i.e. the 20S proteasome regulatory subunit alpha type PSMA5 and the proteasome  
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10 266 subunit beta type-7. We also identified the induction of a potential leucyl aminopeptidase  
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12 267 (hypothetical protein ZHAS\_00009025) presumably involved in the processing and regular  
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15 268 turnover of intracellular proteins. Furthermore, the over-expression of the aconitase protein may  
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17 269 be attributed to a functional property independent of its catalytic activity. This protein is also  
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20 270 involved in the mitochondrial DNA (mtDNA) maintenance and inheritance. Aconitase is able to  
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22 271 switch between an enzymatic form to a RNA binding form depending on whether the assembly or  
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24 272 disassembly of the 4Fe-4S cluster is occurring. Aconitase activity – known to be reduced by  
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27 273 oxidants in vertebrates and invertebrates – is commonly used as a biomarker for oxidative stress  
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29 274 and has been suggested for use as an intramitochondrial sensor of redox status [37]. Based on  
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31 275 these results, oxidative stress generated by the diuron exposure might induce a rise of the level of  
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33 276 mitochondrial aconitase to counterbalance the concomitant decrease in activity, and consequently  
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36 277 there may be no increase in the capacities of energy production in mitochondria. This result  
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39 278 constitutes an interesting perspective in the identification of new biomarkers of oxidative stress in  
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41 279 *E. affinis* which could be easily applied to an environmental biomonitoring program.  
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#### 45 281 *AP effects*

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48 282 AP exposure led to a general pattern of under-expression with 31 spots exhibiting a lower  
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50 283 level in exposed copepod compared to the control animals, and only 10 over-expressions (Table  
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52 284 2). Most of the identified proteins belonged to the “genetic information processing” functional  
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55 285 category of the KEGG classification since they were actors of folding, sorting and degradation  
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58 286 processes (Figure 3). More clearly, an induction of the HSC5 and DnaK chaperones and of the  
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3 287 ubiquitin-conjugating enzyme E2 was particularly highlighted. In contrast, an under-expression  
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5 288 of the proteasome subunit alpha type-2 and the molecular chaperones cyclophilin and calreticulin  
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8 289 was observed. These data suggest changes in the processes of protein sorting and degradation in  
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10 290 order to cope with the effect of the contaminant. AP exposure may also induce an inhibition of  
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12 291 protein synthesis since the elongation factor 1-alpha was also shown to be under-expressed.  
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14 292 Moreover, the down-regulation of proteins of the “metabolism” functional category of the KEGG  
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16 293 classification (i.e. the enolase, the glyceraldehyde-3-phosphate dehydrogenase, the ATP synthase  
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18 294 and the alpha esterase) may be an obstacle to face the increase in the ATP demand required for  
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20 295 defense processes e.g. xenobiotic detoxification. These data are in accordance with previous  
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22 296 studies which demonstrated the AP capability of interfering with physiological functions such as  
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24 297 immunity [38]. However, the copepod *E. affinis* was shown to be able to accumulate and  
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26 298 eliminate nonylphenol-ethoxy-acetic-acid and 4-nonylphenol [18]. In addition, two cytoskeleton  
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28 299 proteins i.e. a myosin subunit which exhibits an over-expression, and a tropomyosin which is, in  
29  
30 300 contrast, under-expressed could be related to behavioural changes. The glutamate receptor –  
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32 301 involved in nervous conduction and excitotoxicity and identified in the “environmental  
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34 302 information processing” KEGG functional category – was also shown to be under-expressed, in  
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36 303 accordance with the neurotoxic effects of APs [39]. These results are consistent with previous  
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38 304 studies which demonstrated that nonylphenol-ethoxy-acetic-acid and 4-nonylphenol are able to  
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40 305 alter the *E. affinis* swimming behaviour with possible disruption of chemosensory  
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42 306 communication [15].  
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50 307 Finally, it should be noted that the expression of 12 differentially expressed proteins after AP  
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52 308 exposure was also affected by diuron. Three of these proteins presented the same pattern. They  
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54 309 were in particular implicated in a stress-mediated protein response, as assumed by the over-  
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56 310 expression of the molecular chaperones, HSC5 and DnaK. The other common spots  
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3 311 (glyceraldehyde-3-phosphate dehydrogenase, enolase, ATP synthase subunit beta mitochondrial  
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5 312 precursor, proteasome 25 kDa subunit, tropomyosin and elongation factor1-alpha as identified  
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8 313 proteins) were characterized by an opposite pattern, although with a similar depict i.e. over-  
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10 314 expression after diuron exposure and under-expression after AP exposure.

### 315 CONCLUSIONS

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

323  
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326 conflict of interest. All authors declare that they have no conflict of interest.

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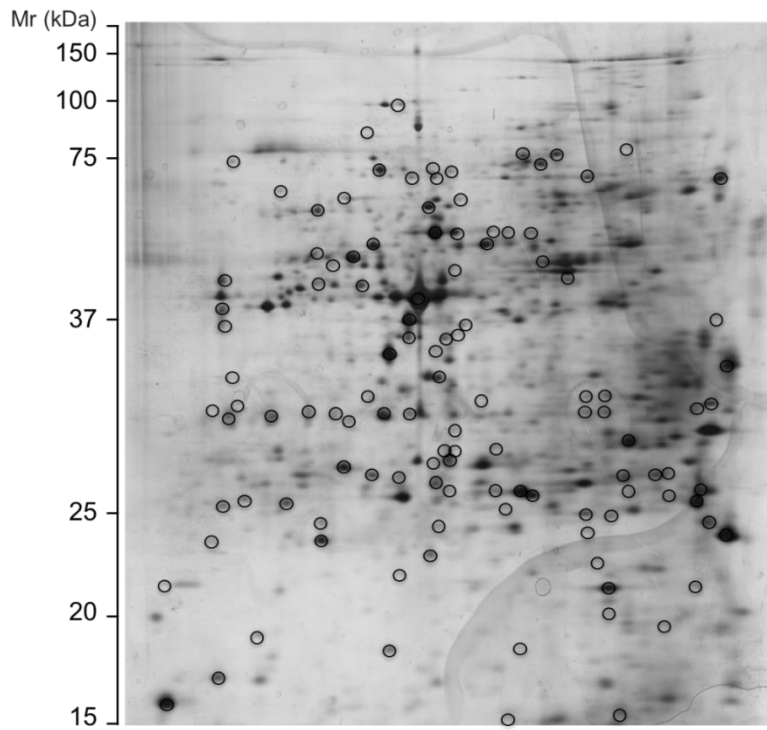
### 442 **FIGURE LEGENDS**

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32 443 Figure 1: Representative 2DE control gels obtained from pools of *E. affinis* after silver nitrate  
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34 444 staining (A, analysis gel) and colloidal blue staining (B, preparative gel). Differentially expressed  
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36 445 proteins obtained after matching (circled spots) were excised from the analysis gel for mass  
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38 446 spectrometry analysis. Mr, relative molecular mass.  
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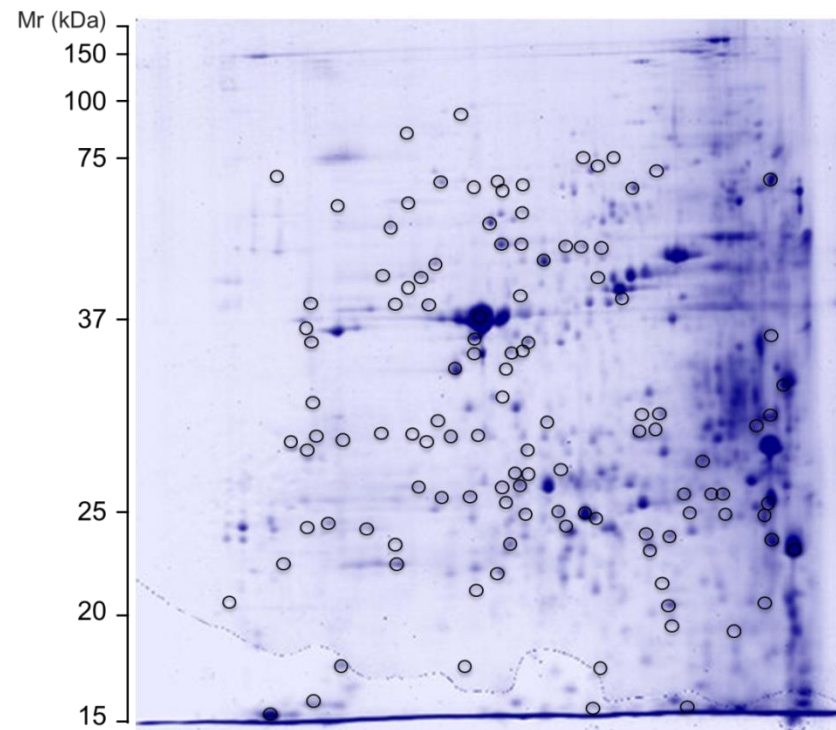
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44 448 Figure 2: Differentially expressed proteins detected after diuron (A) or AP (B) exposures of *E.*  
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46 449 *affinis* in a flow-through system. Under- and over-expressed spots – identified on a typical 2DE  
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48 450 protein expression profile – are underlined in black by squares and circles respectively  
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50 451 ( $p < 0.01$ ) Common spots are surrounded by white circles. Spot numbers refer to those reported in  
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53 452 tables 1 and 2. Mr, relative molecular mass.  
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3 454 Figure 3: Pie diagrams illustrating the distribution of differentially expressed proteins after diuron  
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5 455 (A) and AP (B) exposures according to their KEGG pathways. The data are derived from Tables  
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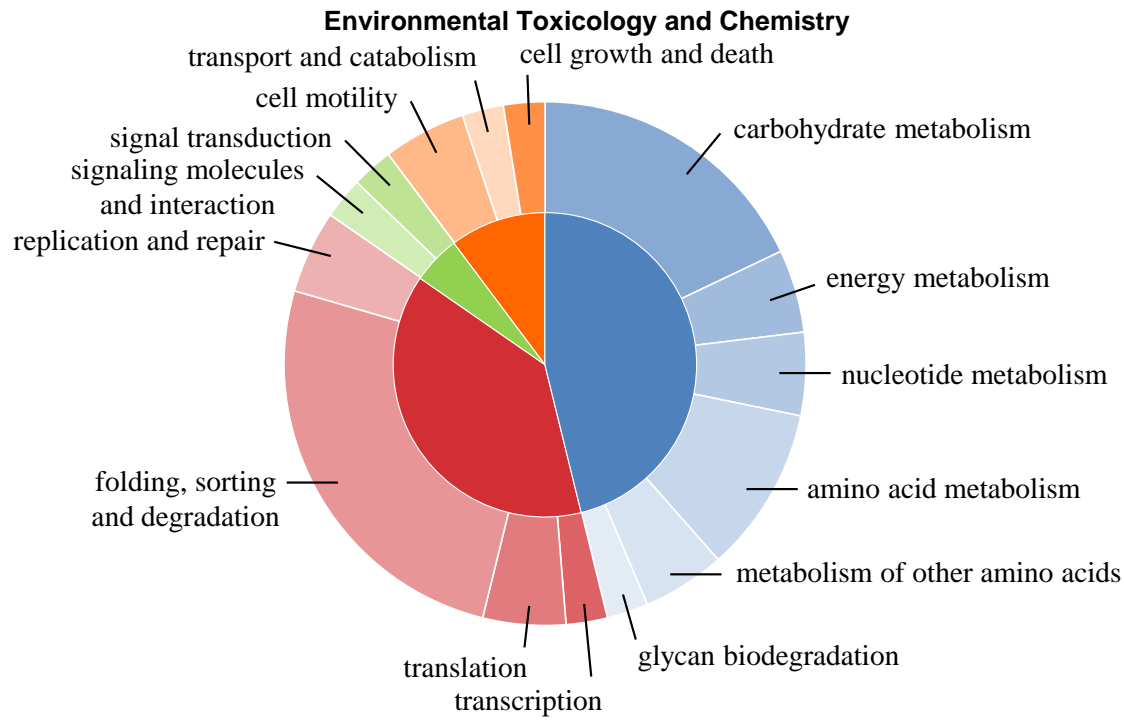


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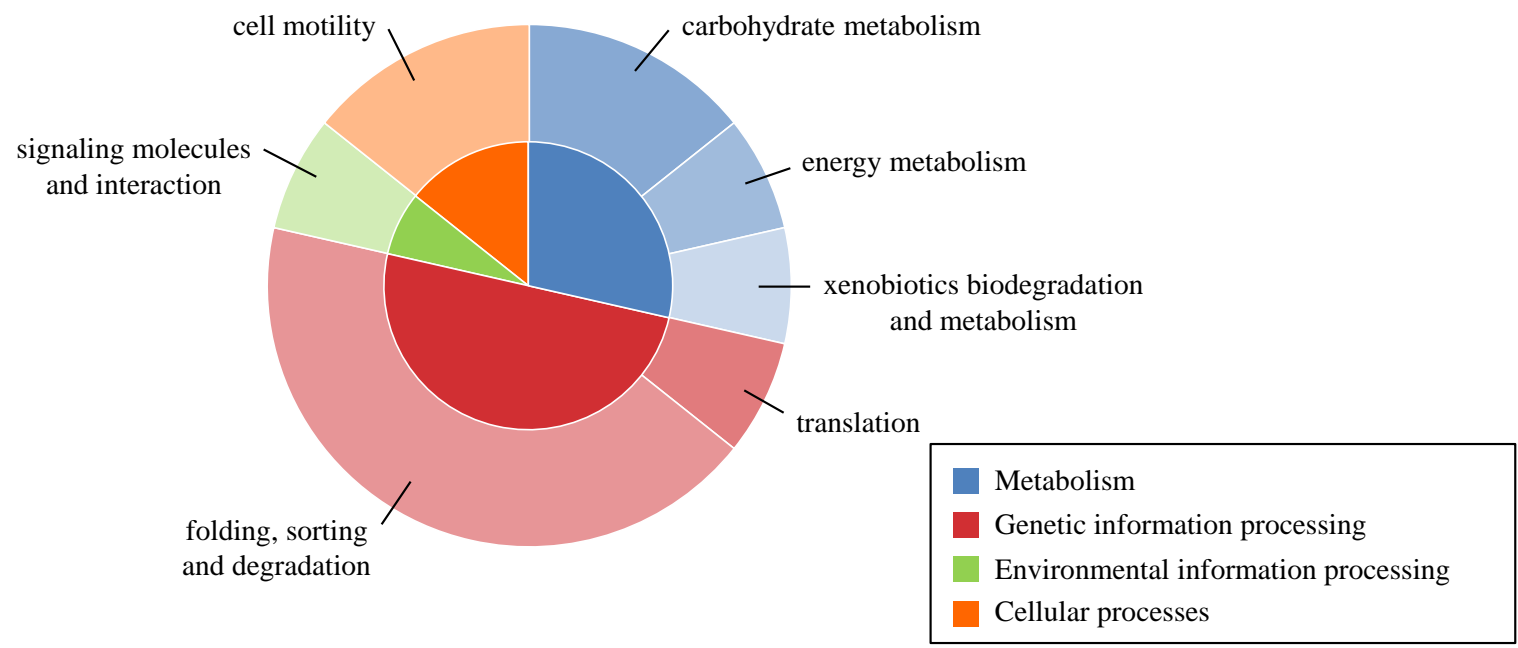




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232	1.54										
234	1.25	cell division cycle protein 27 homologous partial	cell proliferation - cell cycle protein	Anopheles darlingi	gi 312380239	125404	6.2	51	3	2	
240	1.32	leucine-rich repeat-containing protein 20, predicted	undetermined	Anopheles 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	Dendroaster 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 <sup>+</sup> currents	Acyrtosiphon 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	Limnitis 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 15596226	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										

<sup>a</sup>Spot numbers refer to those used in figure 2A

<sup>b</sup>Variation corresponds to fold change between exposure and control samples

<sup>c</sup>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 <sup>a</sup>	Variation <sup>b</sup>	Mascot identification <sup>c</sup>								
		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	<i>Ips typographus</i>	gi 161722798	26711	8.42	62	2	7
103*	0.50	cyclophilin	protein folding - molecular chaperone	<i>Aiptasia pallida</i>	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	<i>Lepeophtheirus salmonis</i>	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	<i>Danaus plexippus</i>	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	<i>Lepeophtheirus salmonis</i>	gi 225712758	54281	5.04	64	1	3
326*	0.68	tropomyosin	muscle contraction - calcium dependent regulation of contraction	<i>Lepeophtheirus salmonis</i>	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	<i>Lymnaea stagnalis</i>	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	<i>Acartia pacifica</i>	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	<i>Artemia sp. SBH266677</i>	gi 41394395	39739	5.06	85	2	8
905	0.60	alpha esterase	xenobiotic degradation - carboxylic ester hydrolase activity	<i>Aedes aegypti</i>	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	<i>Lepeophtheirus salmonis</i>	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	<i>Caligus rogercresseyi</i>	gi 225711070	18820	4.59	72	1	6

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268	1.34										
337	1.59										
442*	1.23										
700	1.71	ubiquitin-conjugating enzyme E2, hypothetical protein AND_11650	protein degradation - catalysis 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										

<sup>a</sup>Spot numbers refer to those used in figure 2B

<sup>b</sup>Variation corresponds to fold change between exposure and control samples

<sup>c</sup>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