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Investigating the emerging role of comparative proteomics in the search for new biomarkers of metal contamination under varying abiotic conditions

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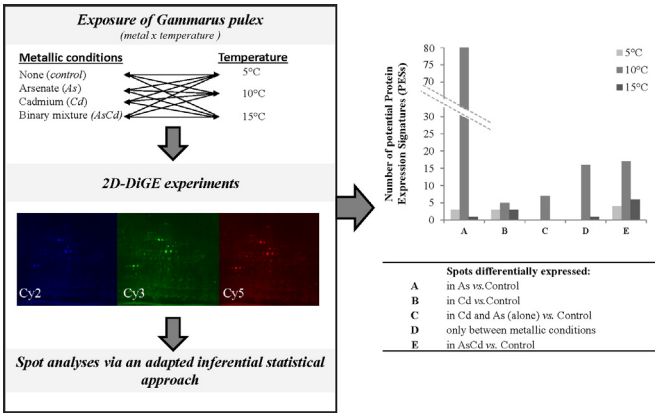
HIGHLIGHTS

- We tested comparative proteomics for identifying new biomarkers of chemical exposure.
- Both metal interactions and temperature confounding effects were taken into account.
- 129 spots have been highlighted as potential Protein Expression Signatures at 10 °C.
- Proteomics has high potential but a long way still to go to efficient multi-marker.
- Recommendations and precautions for future studies have been presented.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aims at investigating the potential use of comparative proteomics as a multi-marker approach of metal contamination, taking into account the potential confounding effect of water temperature. The major objective was to identify combinations of proteins specifically responding to a given metal, even if included in a metal mixture. The diagnostic approach was performed via the comparative analysis of protein expression on spot mapping provided by adult males of *Gammarus pulex* (Amphipoda, Crustacea) respectively exposed to arsenate (As), cadmium (Cd) or a binary mixture of these metals (AsCd) at three realistic temperatures (5, 10 and 15 °C). Proteomic expression analysis was performed by Differential in-Gel Electrophoresis (2D-DiGE), and completed by an adapted inferential statistical approach. Combinations of under/over-expressed protein spots discriminated the metal identity. However, none of these spots discriminated both the individual metal effect (As or Cd) and its effect in metal mixture (AsCd) whatever the tested temperature. Some limits of the two-dimensional analysis of protein spot maps in *G. pulex* have been highlighted: (i) the presence of contaminating peptides and/or abundant

Abbreviations: As, arsenate; Cd, cadmium; 2D-DiGE, two dimensional differential in gel electrophoresis; *Gammarus pulex*, *Gammarus pulex*; IEF, isoelectric focusing; IPG, immobilized pH gradient; MS, mass spectrometry; PESs, Protein Expression Signatures; PMT, photo multiplier tube; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MW, molecular weight.

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Ecotoxicological impact
Arsenate
Cadmium

"déja-vu" proteins which can mask the responses of other proteins of interest or (ii) the presence of post-translational modifications. An optimization of the experimental design (especially during the sample preparation) has been described for future investigations.
This study has also highlighted (i) the importance of precisely identifying the protein spots of interest to avoid erroneous interpretations in terms of action mechanisms of chemicals and (ii) the importance of working under controlled laboratory conditions with a temperature close to 10 °C. In such conditions, we have demonstrated a higher impact of As than Cd on the energetic metabolism of *Gammarus*. This As impact is reduced in AsCd mixture confirming the antagonistic interaction of this binary mixture previously observed on *G. pulex* mortality at 10 °C.

1. Introduction

Proteomics has recently taken a central place in the debate on the applicability of innovative technologies in environmental sciences (Sanchez et al., 2011). Indeed, the search for new biomarkers or a better understanding of the toxicant mechanisms of action have motivated the birth of the "ecotoxicoproteomics"; i.e. proteomics in ecotoxicological research (Bjørnstad et al., 2006; Gomiero et al., 2006). This discipline is primarily based on the "comparative proteomics", which compares the mapping of protein expression in tissues, cells or other body fluids of a model organism in "test" versus "control" conditions. Hence, comparative proteomics leads not only to an overview of the key proteins involved in normal physiological pathways, but also of those involved in diseases (Blackstock and Weir, 1999).

Comparative proteomics can be used without prior knowledge on the pollutant action mechanisms; an advantage on conventional biomarkers. Experimental studies of comparative proteomics, mainly based on marine organisms (e.g. Apraiz et al., 2006), have already shown that protein analysis could provide a specific "fingerprint" directly related (i) to exposure and/or (ii) to the effects of certain classes of chemicals, but also (iii) to tested chemical concentrations (Shepard and Bradley, 2000; Meiller and Bradley, 2002; Rodriguez-Ortega et al., 2003). Similar results were obtained when studying the effects of salinity and temperature on fish gills (Kultz and Somero, 1996). The combinations of proteins exhibiting a significantly altered expression (i.e. induction, repression, over- or under-expression) after a specific stress have been called Protein Expression Signatures (PESs) by Shepard and Bradley (2000). Because the alteration of protein expression is often indicative of the effects of an exposure to toxic stressors, the detection of PESs (i.e. quantitative proteomics) can be used to search for new biomarkers (Stagg, 1998; Shepard and Bradley, 2000), while their identification (i.e. qualitative proteomics) can be performed to better understand the action mechanisms of environmental stressors on exposed organisms (Silvestre et al., 2006; Leroy et al., 2010). Invertebrate-based proteomic approaches have provided promising results in risk assessment and monitoring programmes. For example, Bradley et al. (2002) showed that the PESs generated by three endocrine disruptors in rainbow trout (*Oncorhynchus mykiss*) could be detected in wastewaters. Romero-Ruiz et al. (2006) demonstrated that the burrowing bivalve *Scrobicularia plana* produces PESs varying both in number and intensity along a gradient of metal contamination. Amelina et al. (2007) discriminated polluted harbor areas from non-polluted marine areas, based on PESs detected in the mussel *Mytilus edulis*.

Even if the proteomic approach has already demonstrated its potential as diagnostic tool in environmental sciences (e.g. Liu and Wang, 2012), it has been so far mostly applied in studies focusing on the isolated effects of pollutants despite the frequent co-occurrence of pollutants – often dissimilarly acting – in many aquatic ecosystems. Indeed, the toxicity of pollutant mixtures can be additive, but also synergistic or antagonistic; i.e. significantly more or less important than the toxicity theoretically expected by the simple addition of the individual pollutant effects (Marking, 1985). Moreover, abiotic parameters (e.g. temperature) can be confounding factors, modifying the toxicity of a single substance or a mixture of chemicals. Nycthemeral, seasonal or larger scale (e.g. global warming related) temperature variations can increase the vulnerability of freshwater organisms by impairing physiological rates and biochemical reactions, especially ectotherms with environment-dependent body temperature (Willmer et al., 2000; Hochachka and Somero, 2002; Sokolova and Lannig, 2008).

Temperature can also modify chemical contaminant solubility and consequently its bioavailability, leading to change in toxicity in exposed organisms (e.g. Bat et al., 2000; Worms et al., 2006; Rainbow, 2007 for metals). In general, a temperature increase enhances metal toxicity (IPCC, 2007; Vellinger et al., 2012a, 2013, Vellinger, 2012d).

In this context, three main issues have been addressed:

(i) can comparative proteomics reliably highlight the presence and/or the effects of chemical contamination, while avoiding the confounding effect of water temperature?
(ii) can comparative proteomics specifically identify the nature of chemical contaminants and/or highlight the specific effects of each of these contaminants in chemical mixtures? Are PESs chemical contaminant-specific?
(iii) if chemical contaminant-specific PESs are identified, are they robust enough to still provide significant responses in binary mixtures?. We have considered as being robust: any protein response (i) statistically different (covering under- and over-expression) in a normalized volume of protein spot in "exposed" versus "control" organisms, but (ii) similarly observed for the single chemical contaminants and for the binary mixture exposures.

Experiments were conducted under laboratory controlled conditions using the amphipod *Gammarus pulex* considered as an excellent aquatic model organism (Kunz et al., 2010). We have chosen to analyse the effects of cadmium (Cd) and the pentavalent inorganic form of arsenic, i.e. arsenate (As), because these two trace metals have been frequently found together in aquatic ecosystems and have already caused severe and negative effects on aquatic communities. Moreover, several papers describing the physiological, biochemical, and behavioural effects of these two trace metals alone or in mixture, with or without the influence of temperature changes have already been published, with *Gammarus pulex* as model-species (Felten et al., 2008; Alonso et al., 2009; Vellinger et al., 2012a, 2012b, 2012c, 2012d, 2013).

Specimens of *G. pulex* were exposed to mineral water (control), arsenate alone (As), cadmium alone (Cd), and a binary mixture of the two metals (AsCd), in three realistic temperature conditions (5, 10 and 15 °C). Proteomic analyses were performed using "2D-Differential in-Gel Electrophoresis" (2D-DiGE) technology.

2. Material and methods

2.1. Collection, acclimation and metal exposure of organisms

Adult males of *G. pulex* collected in a reference site and acclimated to the tested temperature condition, were exposed in glass Petri dishes

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