Ecotoxicology and Environmental Safety 122 (2015) 205–213

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Gender differences in responses in *Gammarus pulex* exposed to BDE-47: A gel-free proteomic approach

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ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form
28 July 2015
Accepted 29 July 2015
Available online 6 August 2015

Keywords:
Gammarus pulex
Gel-free proteomic
Sex-biased responses
Polybrominated diphenyl ether (PBDE)
Confounding factor

ABSTRACT

Very few ecotoxicological studies have considered differences in toxic effects on male and female organisms. Here, we investigated protein expression differences in caeca of *Gammarus pulex* males and females under control conditions (unexposed) and after 96 h exposure to BDE-47. Using gel-free proteomic analysis, we have identified 45 proteins, of which 25 were significantly differently expressed according to sex and/or BDE-47 exposure. These proteins were involved in several biological processes such as energy metabolism, chaperone proteins, or transcription/translation. In unexposed amphipods, 11 proteins were significantly over-expressed in females, and 6 proteins were over-expressed in males. Under BDE-47 stress, 7 proteins were differently impacted according to sex. For example, catalase was over-expressed in exposed females and under-expressed in exposed males, as compared to respective controls. Conversely, proteins involved in energy metabolism were up-regulated in males and down-regulated in females.

Our proteomic study showed differences in responses of males and females to BDE-47 exposure, emphasizing that sex is a confounding factor in ecotoxicological assessment. However, due to the limited information existing in databases on Gammarids, it was difficult to define a BDE-47 mechanism of action. The gel-free proteomic seems to be a promising method to develop in future ecotoxicological studies and thus, to improve our understanding of the mechanism of action of xenobiotics.

1. Introduction

The extent of the anthropogenic contamination of ecosystems, especially aquatic environments, is still growing and incurs potential hazard for aquatic organisms on the long term that could lead to population declines of sensitive species. Over the past 10 years, a growing number of studies have been devoted to the distribution and risk related to emerging pollutants (e.g. flame retardants, pharmaceuticals, steroids and hormones) which are chemical compounds for which it is considered that information is needed to exclude the possibility that they are harmful for ecosystems (La Farré et al., 2008). To investigate potential toxic effects of chemicals on aquatic organisms, ecotoxicological studies mainly use antitoxic compounds as biomarkers (Peakall, 1994). However most effect biomarkers used to determine the impact of contaminants on organisms are very specific and only provide information on the biological processes or physiological pathways targeted by the biomarkers experimenters choose to test.

Over the last decades, proteomics use in the field of ecotoxicology has been growing as it provides, through the screening of the expression changes in the whole proteome, a global insight into the mechanism of action of pollutants without the need of hypothesis testing or any preconception on the biological processes likely impacted (Monsinjon and Knigge, 2007). Until now, most ecotoxicological studies involving proteome analysis were carried out using the 2D-Differential Gel Electrophoresis (2D-DIGE) method, which is based on a first electrophoresis according to the isoelectric point of proteins and a second one based on the molecular weight of proteins. For example, Kling et al. (2008) demonstrated that a brominated flame retardant mixture altered proteins related to cellular maintenance and stress in the liver proteome of zebrafish model. In the same way, Riva and Binelli (2014) investigated the effects of dioxin-like PCBs on the gill proteome of the bivalve *Dreissena polymorpha*, and observed that the main metabolic pathways impacted by these pollutants were glycolysis and calcium homeostasis. In a study conducted to elucidate the mechanism of action of endocrine disrupting chemicals,

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http://dx.doi.org/10.1016/j.ecoenv.2015.07.038
0147-6513/

Giusti et al. (2013) tested the effect of chlordecone, testosterone, cyproterone acetate and tributyltin on the proteome of the reproductive organs of the gastropod *Lymnaea stagnalis*. These authors showed that the expression of two proteins essential for the reproduction of *L. stagnalis* were altered in exposed individuals. Indeed, chlordecone exposure increased the abundance of ovipostatin (protein involved in the oviposition process), while tributyltin reduced the expression of the yolk ferritin, which is the vitellogenin equivalent in this species.

However, although the 2D-DIGE is commonly used, this methodology presents some limitations such as under-selection of certain protein categories, limited dynamic range, co-migration of proteins, and the necessity to run many replicates (Görg et al., 2004). In addition, 2D-DIGE is labor intensive, the numerous manipulations it implies being potential sources of errors like e.g. during protein excision before mass spectroscopy analysis. Over the past 10 years, a new proteomic approach called "shotgun" proteomic or gel-free proteomic has been developed. This method offers the advantage to reduce sample manipulations (e.g. no spot excision) because this approach involves liquid chromatography (LC) which can be coupled directly with tandem mass spectrometry (MS) (Lambert et al., 2005). Indeed, when proteins are digested, samples can be injected directly into LC system and analyzed with the MS detector. In addition, this approach allows to analyze all proteins present in samples compared to the gel-based approach where the protein analysis is subjected to the manipulator's choice (Braconi et al., 2011). However, this method presents the disadvantage of providing only partial protein sequences.

Invertebrates are relevant biological models for ecotoxicological studies in aquatic ecosystems because they are major constitutents of aquatic foodchains. Among freshwater species, Gammarids are known to be sensitive to pollutants and easy to use in laboratory as well as in field studies (Kunz et al., 2010); hence, they are commonly used to evaluate impacts of xenobiotics on aquatic organisms (Gismondi et al., 2013; Gismondi and Thomé, 2014; Lebrun et al., 2014; Vellinger et al., 2013). Until now, very few ecotoxicoproteomic studies have been carried out using gammarids. Recently, Leroy et al. (2010) have investigated the impact of PCBs exposure on the proteome of *Gammarus pulex* by using 2D-DIGE, and concluded that the glycolysis and the glutamate pathways were altered. Moreover, most ecotoxicological studies on *Gammarus* sp. do not take into account the sex of the test animals, although it has been demonstrated that sex can be a confounding factor. Indeed, in most studies either only animals of one sex are used (usually males) to avoid this source of variation, or individuals are used regardless of their sex. Mixing both genders in experimental trials is not consistent with observations from studies in which males and females have been tested separately that showed significant differences between the responses of both sexes to toxicants (Sornom et al., 2010; Gismondi et al., 2013).

In the present study, we examined protein expression differences between *G. pulex* males and females in control conditions (i.e. unstressed) and after exposure to 2,2′,4,4′-tetrabromodiphenyl ether 47 (BDE-47) at 0.1 $\mu g\,L^{-1}$ for 96 h. BDE-47 congener was chosen because it is an emerging pollutant bioaccumulated by biota (De Wit et al., 2010) such as *Gammarus* sp. (Tlili et al., 2012; Lebrun et al., 2014). In addition, in a previous study we observed a sex-biased bioaccumulation of BDE-47 in *G. pulex* (Gismondi and Thomé, 2014). Proteomic analysis was carried out using hepatopancreatic caeca which is the detoxification tissue in amphipods. The results provide insight into the differences in male and female metabolism with or without xenobiotic pressure, and thus improve our understanding of the sex-biased sensitivity observed in previous gammarid studies (McCahon and Pascoe, 1988a; Sornom et al., 2010; Gismondi and Thomé, 2014). In addition, proteomic results will provide information on the mechanism of action of PBDEs, thanks to the identification of the biological functions altered.

2. Material and methods

2.1. Gammarus pulex collection

Adult males and non-ovigerous females *G. pulex* (size average: 10 mm and 7 mm, respectively) were collected in the Blanc-Gravier (Liège, Belgium), a stream of good physicochemical quality, as defined by the European Directive. Individuals were sorted out on the spot by observing gnathopods (smaller in females than in males) and transferred to the laboratory in stream water. In the laboratory, animals were maintained at 15 °C in two large aerated aquaria with Volvic mineral water (one for each sex) and fed ad libitum with alder leaves, until PBDEs exposures.

2.2. BDE-47 congener exposure

BDE-47 congener was purchased from Sigma-Aldrich Co. A stock solution at 1 $mg\,L^{-1}$ was prepared in acetone and stored in an amber glass vial at −20 °C. Animals were exposed to BDE-47 at 0.1 $\mu g\,L^{-1}$, which was prepared in Volvic mineral water. Control individuals were exposed to Volvic water containing 0.01% acetone.

Contaminations were performed in glass Petri dishes (100 mm diameter and 20 mm high), previously saturated with the BDE-47 solution for 3 days, except for control conditions which were saturated with Volvic and acetone solutions. This process allows to avoid BDE-47 adsorption onto the Petri dishes surface during the exposure of test animals. For each condition (i.e. males/females, exposed/control), four replicates of five *G. pulex* were carried out at 15 °C for 96 h with a photoperiod of 14 h light and 10 h dark. Experimental media were renewed every day in order to guarantee a constant concentration of BDE-47 throughout exposures. Gammarids were not fed during exposures. At the end of the incubation time, the five gammarids of each condition were dissected to collect the caeca tissues (i.e. four caeca per *G. pulex*) which were pooled (i.e. twenty caeca tissues per pool). Proteomic analysis was performed on each pool (i.e. $n=4$ per condition) using a gel-free proteomic analysis, where proteins were identified by liquid chromatography–electrospray tandem mass spectrometry (LC–ESI-MS/MS).

2.3. Proteomic analysis

2.3.1. Experimental assay conditions

The samples (i.e. pool of caeca tissues) were solubilized in Tris HCl 10 mM, pH 7.4, SDS 4%, 0.5 μL of Protease Inhibitor Cocktail EDTA-free and DNAse. The samples were sonicated thrice for 30 s, and vortexed for 30 min at room temperature, before storage overnight at 4 °C. The protein concentration of each sample was quantified using a *RC DC*™ Protein Assay Kit (Biorad).

The samples were reduced, alkylated and reduced before applying the 2D Clean-Up kit (GE Healthcare Life Sciences) according to the manufacturer's recommendations, in order to eliminate impurities not compatible with mass spectrometry analysis. After the washing steps, protein pellets were solubilized in 50 mM bicarbonate ammonium.

Each sample was digested for 16 h at 37 °C in solution with trypsin (ratio trypsin/total protein (w:w) 1/50) and then, 3 h at 37 °C in another solution with trypsin (ratio trypsin/total protein (w:w) 1/100 in 80% acetonitrile).

After the digestion step, samples were resuspended in 0.1%