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Polybrominated diphenyl ethers do not affect metamorphosis but alter the proteome of the invasive slipper limpet *Crepidula onyx*

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ABSTRACT

Man-made polybrominated diphenyl ethers (PBDEs) used as flame retardants in various consumer products may be harmful to marine organisms. Larvae of some marine invertebrates, especially invasive species, can develop resistance to PBDEs through altered protein expression patterns or proteome plasticity. This is the first report of a proteomics approach to study BDE-47 induced molecular changes in the invasive limpet *Crepidula onyx*. Larvae of *C. onyx* were cultured for 5 days (hatching to metamorphosis) in the presence of BDE-47 (1 μg L^{-1}). Using a 2-DE proteomics approach with triple quadrupole and high-resolution TOF-MS, we showed that BDE-47 altered the proteome structure but not the growth or metamorphosis of *C. onyx* larvae. We found eight significant differentially expressed proteins in response to BDE-47, deemed the protein expression signature, consisting of cytoskeletal, stress tolerance, metabolism and energy production related proteins. Our data suggest *C. onyx* larvae have adequate proteome plasticity to tolerate BDE-47 toxicity.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are man-made chemicals commonly used as fire retardants in many consumer products. They have been recently recognized as ubiquitous environmental contaminants (Besis and Samara, 2012). Although production of PBDEs has been banned in European Union and USA (Michigan and Stupak, 2008), its production and use is not restricted in Asia, including in China and Hong Kong (Wang et al., 2007). Annually, about 70,000 tons of PBDEs in the form of electronic waste are imported into China (Bao et al., 2012; Chen et al., 2012). The coastal areas of Hong Kong and China are among the largest dumping sites of PBDEs in the world. In Hong Kong, >0.1 μg of PBDEs was found per gram of coastal sediments (Liu et al., 2005). The PBDE congener 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) has been recognized as a serious environmental pollutant because it is persistent in the environment (Ross et al., 2009) and it is an endocrine disruptor and neurotoxin. BDE-47 is not only harmful to humans but also to marine organisms (Tanabe and Ramu, 2012). The effects of BDE-47 and the molecular mechanisms of toxicity in humans have been fairly well-documented, but their toxic effect in marine benthic invertebrates is poorly understood (Chiu et al., 2012a).

The majority of benthic marine organisms have a complex life cycle. During the pelagic phase, the swimming larvae must select a hard substrate to attach before metamorphosing into benthic adults (Thiyagarajan, 2010). This irreversible pelagic–benthic transition is an energetically expensive process. Not surprisingly, the transition is highly vulnerable to environmental contaminants such as BDE-47 (Lam et al., 2010). Consequently, marine animals at metamorphosing larval stages are more susceptible to BDE-47 than their adults. Therefore, we need to determine whether larval forms of marine animals have the ability to tolerate the presence of BDE-47 and metamorphose normally to maintain a healthy population. Failure to successfully attach and metamorphose on a suitable habitat could lead to catastrophic population collapse (Pechenik, 1999). The majority of larvae never reach the metamorphosing stage due to predation and energy costs associated with rapid metamorphosis (Hadfield, 2000; Rumrill, 1990). During the past two decades, biologists have examined larval development and metamorphosis under varying behavioural and physiological conditions (Thiyagarajan, 2010). However, how BDE-47 effects the initiation or mediation of larval metamorphosis has yet to be elucidated. In a series of studies, we have shown that the response of marine invertebrate larvae to BDE-47 is highly species-specific.

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For instance, larval metamorphosis is promoted by BDE-47 in the sediment polychaete worms, *Pseudoploydora vexillosa* and *Capitella* sp. I (Lam et al., 2010). On the other hand, larval metamorphosis is not affected by BDE-47 in the invasive limpet species, *Crepidula onyx* (Chiu et al., 2012a). Similarly, environmentally relevant concentrations of BDE-47 are not toxic to marine fish larval forms (Mhadhbi et al., 2012). It appears that filter feeding larval forms of marine invertebrates are relatively tolerant to BDE-47, which raises the question of how the larvae adjust their molecular machinery to cope with BDE-47 toxicity. The protein expression signatures (PESs) in response to pollutants could give us an insight as to how larvae might become resistant to BDE-47.

Competitive analysis of global protein expression patterns of cells, tissues, or the whole organism exposed to environmental pollutants, including PBDEs, has been successfully adapted to investigate mechanisms of toxicity (Alm et al., 2008; Campos et al., 2012; Silvestre et al., 2012; Tomanek, 2011; Wang and Li, 2010). This environmental proteomics approach is commonly used in variety of non-model marine organisms (Apraiz et al., 2009; Riva et al., 2011). Differential expressions of proteins or proteome changes are primary regulators of cellular homeostasis. Species' ability to tolerate an environmental pollutant depends on its ability to maintain cellular homeostasis and detoxification. Thus, understanding differentially expressed proteins at the global proteomic level in response to environmental pollutants, including BDE-47, may provide a valuable insight into processes that lead to adaptation or shifts in population (Campos et al., 2012). However, the degree to which environmental stressors and pollutants alter the larval proteome and how PBDEs affect the up- and down-regulation of the global proteome remain a matter of speculation.

Over the past few years, two-dimensional electrophoresis (2-DE) based proteomic techniques have emerged as powerful tools for studying the proteome response to PBDEs in non-model marine invertebrate species (Apraiz et al., 2006; Chiu et al., 2012a,b; Silvestre et al., 2012). Several studies suggest that plasticity at the proteome level (the up- and down-regulation of proteins) enable species to overcome PBDEs stress (Riva et al., 2011). For example, the marine mussel, *Mytilus edulis*, was found to overexpress cytoskeletal proteins in response to BDE-47 (Apraiz et al., 2006). A set of protein expression signatures for the marine mussel exposed to BDE-47 was successfully identified in the above study. Using a similar methodology, we used a proteomic approach to investigate the effects of BDE-47 on the larvae of invasive slipper limpet, *C. onyx*. To our knowledge, this study represents the first proteomic analysis of *C. onyx* larvae. The larvae of *C. onyx* are highly tolerant to BDE-47 (Chiu et al., 2012a), so we expect only minimal changes to the proteome structure in response to BDE-47. The physiological mechanisms underlying this species' response to BDE-47 will enable us to better understand how exposure to PBDEs impacts at the species level (Ghosh et al., 2013).

2. Materials and methods

2.1. Test animal

Adults *C. onyx* limpets were collected from the intertidal area in Victoria Harbour, Hong Kong (22°17′N, 114°10′E), and were acclimatized at a salinity of 34‰ and a temperature of 25 °C for several weeks. The salinity and temperature corresponded to the typical ambient seawater conditions at the time of collection. Relatively unpolluted seawater from a coastal area in Hong Kong adjacent to South China Sea was used in this study. During acclimatization, adults were fed the flagellate, *Isochrysis galbana*. Under these optimal conditions, egg capsules developed underneath the female foot and hatched into veliger larvae within 2 weeks (Chiu et al., 2007a). Larvae released from several adults were used in the following experiment.

2.2. Effect of BDE-47 on larval metamorphosis

Larval growth and metamorphosis was monitored in the control group and in the experimental group exposed to BDE-47 (1 μg L^{-1}). The BDE-47 concentration was selected based on our previous work that showed a concentration of 1 μg L^{-1} BDE-47 did not impact larval growth and metamorphosis of *C. onyx* (Chiu et al., 2012a), which should allow adequate samples for the subsequent proteomic study. The BDE treatment solution was prepared by dissolving 1 μg of BDE-47 in 100 μl *n*-hexane made up to the correct concentration in 0.22 μm filtered natural sea water (FSW) (Chiu et al., 2012a). Control and BDE-47 treatment culture tanks were each replicated in triplicate. Appropriate blank and solvent controls were included in the experiment. The culture tanks were silyated using dichlorodimethylsilane to avoid adsorption of BDE-47. The newly released larvae were divided equally into the six culture tanks. Each culture tank contained 1 L of FSW (with or without BDE-47) and a larval concentration of one per ml. Culture tanks were randomly placed in a water bath maintained at 25 °C. During the entire culture period, larvae were fed with *I. galbana* (2×10^5 cells L^{-1}) (Chiu et al., 2007a). The larval culture medium was changed daily to replenish the food source and BDE-47. Larval metamorphosis bioassay was carried out when the majority of the larvae became competent to attach and metamorphose in the presence of natural settlement cues, such as natural multispecies biofilms (Chiu et al., 2007b). Biofilms were developed onto petri dishes (Falcon No. 1006) by deploying the dishes in plastic frames at the intertidal zone in Victoria Harbour for 7 days before use in the bioassay. The percentage of metamorphosis was assessed by counting the number of attached and metamorphosed individuals out of the total number of larvae (10 larvae) added into the bioassay dish after 24 h at 25 °C (Chiu et al., 2007b). Three bioassays were carried out per culture tank. All the remaining competent larvae from each culture were washed with double-distilled water to remove adhered salt and then immediately frozen for subsequent proteomic analysis. Samples of the competent larvae were used to measure the body burden of BDE-47 as described in our previous study (Chiu et al., 2012a).

2.3. Two-dimensional electrophoresis (2-DE) and proteome analysis

Briefly, proteins were extracted from the competent larvae in 2-DE lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 2% Bio-Lyte 3/10 ampholyte) (Thiyagarajan and Qian, 2008) by sonication (four 20 s cycles with 40 s pause-interval using a Branson Sonifier 150), followed by centrifugation for 20 min at 14,000 rpm. The supernatant was collected and stored at −80 °C until use. Proteins in the supernatant were purified using the 2-D Clean Up kit (Bio-Rad, USA) and quantified using the 2-D Quant kit (GE Healthcare Life Sciences, Sweden).

For 2-DE analysis, 500 μg of protein was mixed with a buffer (7 M urea, 2 M thiourea, 2% CHAPS, 40 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, 1% Bromophenol blue) (Görg et al., 2004). The samples were applied onto 11 cm IPG strips, linear pH 3–10 (Bio-Rad) and actively rehydrated overnight. The rehydrated IPG strips were subjected to isoelectric focusing (IEF) in a Protean IEF Cell (Bio-Rad) under the following conditions: 20 min at 25 V, followed by 2.5 h at a linear gradient from 250 to 8000 V and finally at 8000 V for a total of 80,000 Vh. The IPG strips were then equilibrated in equilibration buffer 1 (6 M urea, 2% SDS, 0.05 M Tris–HCl (pH 8.8), 50% glycerol, 2% w/v DTT) and buffer 2 (6 M urea, 2% SDS, 0.05 M Tris–HCl (pH 8.8), 50% glycerol, 2.5% iodoacetamide). The 2-dimensional separation of proteins on the IPG was