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Biomarker-enhanced assessment of reproductive disorders in *Monoporeia affinis* exposed to contaminated sediment in the Baltic Sea

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ABSTRACT

Introducing biomarkers into monitoring programs requires understanding of their responses in relation to higher-level biological effects as well as modulating effects of confounding environmental factors. We evaluated relationships between the general toxicity biomarkers (acetylcholinesterase [AChE], lysosomal membrane stability [LMS], oxygen radical absorbance capacity [ORAC]) and reproductive performance (fecundity and embryo aberrations) in the amphipod *Monoporeia affinis* in the Baltic Sea. To further link biomarker response to contaminant (PCBs, PAHs and metals) levels in the surrounding sediments as well as environmental factors (salinity, bottom depth and total organic carbon in sediments [TOC]), correlation and partial least square regression (PLSR) analyses were applied. The observed contaminants levels were frequently elevated for heavy metals and PAHs, but not PCBs. In the amphipod populations, female ORAC values were positively related to the occurrence of females carrying malformed or membrane-damaged embryos and to the percentage of such embryos in their broods, but also to the fecundity. Female AChE activity was negatively related to the frequency of the membrane-damaged embryos, and positively to the frequency of embryos with arrested development in the broods. Moreover, higher AChE activity and ORAC values in the females occurred at elevated concentrations of metals and PAHs, while there was a negative correlation between embryo ORAC and some PCB congeners. The PLSR models explained over 80% of the variation in the female ORAC and AChE values by variation in contaminant concentrations in combination with environmental variables. Specifically, CB180 and PAM4,9 were identified as negative predictors for ORAC, whereas many PAHs and some metals were positive predictors. The AChE activity was positively related to some metals and negatively to PCBs. In the PLSR models, environmental factors had significant modulating effects, with positive effect of salinity on female ORAC and AChE, and negative effect of TOC on the AChE. The LMS data were less informative, with no apparent relation to any of the contaminants. Linking subcellular responses to the reproduction effects facilitates environmental stress assessment and understanding of the response mechanisms, but also calls for more experimental and field data providing a mechanistic understanding to these linkages.

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1. Introduction

To facilitate monitoring of environmental contaminants, various biomarkers are increasingly used. The main argument for the biomarker-based assessment of deleterious biological effects is a hierarchical response model, with lower-level (molecular, biochemical and physiological) responses preceding those at higher levels of biological organization (changes in body size, offspring production, population size, etc.; Depledge and Fossi, 1994). To reflect this linkage, adverse outcome pathway (AOP) concept has been developed to facilitate use and integration of biomarkers for forecasting chemical impacts on individuals and populations (Ankley et al., 2010). An ideal effect biomarker should respond to general or specific toxic stressors, with no or predictable effects of confounding factors. Therefore, understanding biomarker-contaminant relationships requires validation in situ, using organisms exposed to complex mixtures of commonly co-occurring contaminants, such as polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs) and metals, in various environmental settings. Therefore, to improve ecological relevance of contaminant risk assessment, biomarkers should be linked to

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ecologically relevant higher-order effects, such as growth and reproduction.

Various biomarkers are currently in use for assessment of contaminant effects (Martín-Díaz et al., 2004; Lam, 2009). Acetylcholinesterase (AChE) is an essential regulatory enzyme for acetylcholine turnover in neuronal synapses. Its inhibition indicates exposure to neurotoxic substances, such as organophosphate insecticides (Fulton and Key, 2001) but also metals (Gill et al., 1990). Another response to xenobiotics is decreased lysosomal membrane stability (LMS). This general toxicity biomarker has been linked to effects of organochlorines and PCBs (Broeg et al., 2002; Köhler et al., 2002), PAHs (Moore, 1990; Viarengo et al., 1992; Cajaraville et al., 2000) as well as heavy metals (Viarengo et al., 1981, 1985; Nolde et al., 2006) in fish and invertebrates. Also, overproduction of reactive oxygen species (ROS) and subsequent oxidative stress (Di Giulio, 1991; Livingstone, 2001, 2003) often accompany detoxification processes. Commonly used biomarkers of oxidative response include antioxidant enzymes, glutathione redox state and oxidative damage to cellular macromolecules (Martín-Díaz et al., 2004; Valavanidis et al., 2006; Lam, 2009). In amphipods exposed to contaminants and hypoxia, the overall antioxidant status measured as oxygen radical absorbance capacity (ORAC) has been found to correlate with various antioxidant enzymes (Gorokhova et al., 2013). Moreover, a coordinated response of ORAC levels and AChE activity to contaminant exposure has been observed in amphipods (Gorokhova et al., 2013) and daphnids (Wiklund et al., 2014).

Monoporeia affinis is a sentinel species used to assess biological effects of contaminants in the Baltic Sea within the Swedish National Monitoring Program (SNMP). In this semelparous soft-bottom amphipod, contaminant exposure can induce a variety of reproductive disorders (Reutgard et al., 2014). Moreover, several types of embryo aberrations were linked to specific contaminants in sediments, providing basis for indicator properties of these disorders in environmental assessment (Löf et al., 2016). When evaluating amphipod embryo aberrations as an indicator of biological effects of hazardous substances within EU Marine Strategy Framework Directive (MSFD) and the Baltic Sea Action Plan (BSAP) (HELCOM, 2013), it is relevant to ask – are there any biomarkers that can provide early warning for these reproductive disorders? Although responses of antioxidant enzymes, glutathione ratio, AChE and oxidative damage (DNA strand breaks and lipid peroxidation) to contaminants have been demonstrated in juvenile *M. affinis* (Gorokhova et al., 2010, 2013), the biomarkers and reproductive indicators have never been analyzed in concert and in relevant ecological settings.

Building on the previously identified biomarker responses to contaminated sediments in various invertebrates, including Baltic amphipods (Baršienė et al., 2006; Schiedek et al., 2006; Gorokhova et al., 2010, 2013), we studied biomarker profiles in *M. affinis* in relation to reproductive aberrations. We hypothesized that female AChE activity will decrease, whereas the total antioxidant capacity will increase with increasing embryo aberration frequencies. Moreover, based on the commonly observed variability in biomarker responses related to ontogeny and growth (Hoguet and Key, 2007; Xuereb et al., 2009), we hypothesized that biological and reproductive variables, such as fecundity and embryo developmental stage, will modify the biomarker responses. Using *M. affinis* collected in areas with varying contaminant loading and exhibiting a range of reproductive aberrations (Löf et al., 2016), we tested these hypotheses by evaluating relationships between the biomarkers and reproductive variables currently employed as indicators of biological effects of contaminants in SNMP. We also related variability in biomarker values to PAH, PCB and metal concentrations in sediments as well as environmental variables (salinity, depth and concentrations of sediment organic carbon) to evaluate biomarker applicability for screening contaminant effects in situ.

2. Material and methods

2.1. Embryo analysis

In *Monoporeia affinis*, mating takes place in early winter and developing embryos are carried in female brood pouch (marsupium) until early spring (Sundelin et al., 2008). According to the standard monitoring practice for embryo analysis (Sundelin and Eriksson, 1998; Sundelin et al., 2008), the embryos extracted from the marsupia were examined under stereomicroscope. For each female, fecundity (eggs per female), embryo developmental stage and embryo aberrations were recorded. The aberrations were classified as: (1) malformed embryos, (2) membrane-damaged embryos, (3) embryos with arrested development, and (4) partially dead broods (Table A.1 in Supplementary materials, Appendix A). These reproductive disorders were recently evaluated as indicators of biological effects of contaminants using same populations (Löf et al., 2016). Here, we focus on the linkages between the biomarkers and the observed aberrations.

2.2. Sampling sites and collections

The amphipods and sediment samples were collected in the Bothnian Bay (BB, 6 stations) and in the Bothnian Sea (BS, 4 stations) within a survey of embryo aberrations in relation to contaminant levels (Fig. 1; Löf et al., 2016). The amphipods were collected with Lundgren bottom sled (Blomqvist and Lundgren, 1996) and van Veen grab in December 2009 (BB) and January 2011 (BS) and transported in the ambient seawater to the laboratory within a few days. At each site, bottom depth (Depth; m) was recorded. Surface sediment samples for analysis of contaminants and total organic carbon (TOC, g kg^{-1} DW) were collected; these results are reported in detail elsewhere (Löf et al., 2016). Also, oxygen concentration (ml L^{-1}, measured by sodium thiosulfate titration) and salinity (Sal; psu) were measured ~2 m from the bottom. In no case bottom hypoxia was observed (>5 ml L^{-1} at all sites); thus oxygen level was not considered as environmental predictor of biological responses.

2.3. Biochemical assays

Biochemical assays were conducted using microplate reader FLUOstar Optima (BMG Lab Technologies, Germany) with absorbance (AChE, protein) and fluorescence (ORAC) configurations. All samples, standards and blanks were analyzed in duplicates.

2.3.1. Sample preparation

Females with the embryo brood removed and the extracted broods were analyzed separately. Each female was snap frozen in liquid nitrogen directly after the brood removal and the broods were treated in the same way directly after the embryo analysis. Samples were homogenized in a cold (4 °C) buffer (pH 7.2; 50 mmol L^{-1} tris(hydroxymethyl)aminomethane (Tris) buffer, 0.15 mol L^{-1} NaCl, 0.3 mol L^{-1} sucrose, 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 0.02 mol L^{-1} NaH_2PO_4, and 0.1% Triton X-100; Schreck et al., 2009) for 3× 20 s using FastPrep homogenizer; 500 μl and 200 μl were used for females and broods, respectively. The homogenate was centrifuged at 2300 × *g* for 5 min at 4 °C, aliquoted and frozen in −80 °C.

2.3.2. Protein assay

Protein concentration (mg ml^{-1}) in the homogenate was measured using the bicinchoninic acid assay (BCA, Pierce Ltd.) with bovine serum albumin as standard according to the manufacturer's instructions. For each assay, 10 μL of the homogenate $well^{-1}$ were

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