Aquatic Toxicology 173 (2016) 36–42

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Linking sub-cellular biomarkers to embryo aberrations in the benthic amphipod *Monoporeia affinis*

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ARTICLE INFO

Article history:
Received 13 October 2015
Received in revised form 13 January 2016
Accepted 18 January 2016
Available online 21 January 2016

Keywords:
Monoporeia affinis
Embryo aberrations
Oxidative stress
Darwinian fitness
Acetylcholinesterase

ABSTRACT

To adequately assess and monitor environmental status in the aquatic environment a broad approach is needed that integrates physical variables, chemical analyses and biological effects at different levels of the biological organization. Embryo aberrations in the Baltic Sea key species *Monoporeia affinis* can be induced by both metals and organic substances as well as by hypoxia, increasing temperatures and malnutrition. This amphipod has therefore been used for more than three decades as a biological effect indicator in monitoring and assessment of chemical pollution and environmental stress. However, little is known about the sub-cellular mechanisms underlying embryo aberrations. An improved mechanistic understanding may open up the possibility of including sub-cellular alterations as sensitive warning signals of stress-induced embryo aberrations. In the present study, *M. affinis* was exposed in microcosms to 4 different sediments from the Baltic Sea. After 88–95 days of exposure, survival and fecundity were determined as well as the frequency and type of embryo aberrations. Moreover, oxygen radical absorption capacity (ORAC) was assayed as a proxy for antioxidant defense, thiobarbituric acid reactive substances (TBARS) level as a measure of lipid peroxidation and acetylcholinesterase (AChE) activity as an indicator of neurotoxicity. The results show that AChE and ORAC can be linked to the frequency of malformed embryos and arrested embryo development. The occurrence of dead broods was significantly associated with elevated TBARS levels. It can be concluded that these sub-cellular biomarkers are indicative of effects that could affect Darwinian fitness and that oxidative stress is a likely mechanism in the development of aberrant embryos in *M. affinis*.

1. Introduction

There is a general consensus that in order to adequately assess and monitor environmental stress in the aquatic environment, a broad approach that integrates physical variables, chemical characterization and biological effects is needed (Lehtonen et al., 2014; Lyons et al., 2010; Moore et al., 2004). Assessing embryo aberrations in *Monoporeia affinis*, a benthic key species of the Baltic Sea, is an established method that has been deployed for monitoring and assessing environmental status since the late 1970s (Elmgren et al., 1983). Embryo aberrations have been shown to be induced by hypoxia and increased temperatures (Wiklund and Sundelin, 2001), as well as by metals and organic substances in laboratory studies (Eriksson et al., 1996; Sundelin, 1983, 1988, 1989). In addition, field studies, together with a recent meta-analysis, verify a relationship between embryo aberrations and point sources of hazardous substances along the Baltic Sea coast (Reutgard et al., 2014; Sundelin and Eriksson, 1998; Sundelin et al., 2008). However, the biological part of an integrated monitoring and assessment approach should not only include variables that represent various compartments of the ecosystems and trophic levels, but also different levels of biological organization, from sub-cellular to community level. By this approach, initial changes at the sub-cellular level can be connected to effects at the individual level, similar to the concept of adverse outcome pathway (Ankley et al., 2010).

The use of biomarkers, here defined as sub-cellular biological responses, in environmental monitoring and assessment rests on the basic notion that any effect on organisms or higher levels of biological organization are preceded by specific alterations at lower levels of organization (Depledge, 1989; Depledge and Fossi, 1994). Biomarkers may therefore increase our mechanistic understanding of individual or community effects and provide us with warning signals of stress, before it manifests in harmful effects on higher biological organization levels (van der Oost et al., 2003). However, the relationship between biomarkers and effects on organismal fit-

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http://dx.doi.org/10.1016/j.aquatox.2016.01.005
0166-445X/

ness is for a number of reasons often uncertain (Jemec et al., 2010; Lam, 2009; Wu et al., 2005). For instance, responses at sub-cellular level can simply be signs of adaptation in order to maintain cellular homeostasis and functions. Sub-cellular effects can also be transient, as an initial induction or inhibition may return to normal levels within time. Moreover, several cellular processes have efficient repair systems, which can keep sub-cellular damages at low and harmless levels (Lam, 2009; van der Oost et al., 2003). For these reasons, it is important to establish whether biomarkers solely indicates stress or if they are associated with irreversible biochemical and cellular effects, that ultimately may lead to reduced Darwinian fitness and ecological integrity.

Biomarkers of oxidative stress are commonly used in ecology and ecotoxicology as indicators of stressful events (Livingstone, 2001; Lushchak, 2011; Valavanidis et al., 2006). Oxidative stress arises when there is an excess of reactive oxygen species (ROS) relative to the amount and the activity of antioxidative agents (enzymatic and non-enzymatic) (Halliwell and Gutteridge, 2007). Numerous xenobiotics, such as polyaromatic hydrocarbons, polychlorinated biphenyls, chlorophenols and transition metals, as well as factors like UV light, food quality and quantity, and hypoxia are well-known to interfere with the intracellular redox balance and ultimately cause oxidative damage to cellular components (e.g., membrane lipids, proteins and DNA) (Valavanidis et al., 2006). Oxidative stress has been linked to reduced health and fitness in humans as well as in terrestrial and aquatic organisms, where it also has been connected to embryo toxicity and teratogenicity (Paskova et al., 2011; Wells et al., 2009).

A commonly used biomarker in ecotoxicology is the activity of acetylcholinesterase (AChE) (Fulton and Key, 2001). AChE is an enzyme that catalyzes the breakdown of the neurotransmitter acetylcholine, and interference with AChE can thus lead to uncontrolled nerve signaling. Inhibition of AChE activity has primarily been applied as a biomarker of exposure to organophosphorous and carbonate pesticides, which directly bind to the enzymes' active site. However, other organic compounds, e.g., certain cyanotoxins, and metals have also been shown to alter the AChE activity (Fulton and Key, 2001; Nunes, 2011; Wiklund et al., 2014).

In a recent field study, Löf et al. (2016) examined the connection between embryo aberrations and biomarkers in gravid *M. affinis* from 10 sites in the northern Baltic Sea. A positive association was found between the AChE activity, the antioxidant defense and different types of embryo aberrations (malformed embryos and arrested development embryos). The aim of this study was to investigate the associations between sub-cellular changes and embryo aberrations in controlled laboratory settings to establish their suitability as field biomarkers. Toward this aim, *M. affinis* were exposed in the laboratory for 3 months to sediments collected from four sites in the northern Baltic Sea. At the organismal level survival, fecundity, and the occurrence of embryo aberrations were determined. Embryo aberrations were classified as malformed, arrested development embryos, single dead embryos or dead broods according to Sundelin and Eriksson (1998). Additionally, we assayed oxygen radical absorption capacity (ORAC) as a proxy for antioxidant defense. The use of the ORAC assay is novel within ecotoxicology (Furuhagen et al., 2014; Gorokhova et al., 2013; Wiklund et al., 2014), but the assay has previously been successfully applied to measure the antioxidant capacity in various food items and human tissue (Cao and Prior, 1998; Prior et al., 2005). Moreover, ORAC is positively correlated with the activity of catalase and superoxide dismutase and negatively correlated with GSH/GSSG ratio in *M. affinis* (Gorokhova et al., 2013). Lipid peroxidation and neurotoxicity were assessed using the well-established ecotoxicological biomarkers thiobarbituric reactive substances (TBARS) and AChE activity, respectively. It was hypothesized that embryo aberrations would be associated with changes in ORAC, TBARS and AChE activity.

Table 1
Information about the location of sediment and animal collection sites as well as organic carbon content of the sediments and salinity at the collection sites.

	Location	Organic carbon (%)	Salinity (PSU)
Sediment 1	N 65.0733° E 21.5488°	1.4	3.6
Sediment 2	N 64.9158° E 22.146°	1.2	2.8
Sediment 3	N 65.2533° E 21.6721°	0.97	2.5
Sediment 4	N 65.2566° E 21.7531°	2.6	2.5
Animal collection site	N 58.889° E 17.649°		6.8

2. Material and methods

2.1. Collecting sediment and test animals

Surface sediments (0–5 cm) were collected in August of 2010 from four sites in the northern Baltic Sea. Two of the four sites are located in relatively pristine areas (sediment 1 and 2) and the two other sites in an area that receives effluent from a pulp mill plant (sediment 3 and 4). The top ~5 cm of the sediment was sieved through a 0.5 mm sieve to remove macrofauna and stored dark at 4 °C until the start of the experiment and chemical analyses. The chemical characterizations of the sediments are summarized in Appendix A (Table S1). Detailed information on the chemical characterizations is published in Löf et al. (2016). The locations of the sites, together with information about organic matter and salinity, are presented in Table 1. The amphipods were collected in the beginning of October 2010, before their breeding period, at a site in the northern Baltic Proper with no known local sources of pollution. Exact location and salinity at the animal collection site is presented in Table 1. The amphipods were kept at 4 °C in a 50 L container with continuous water flow (PSU 6.1) until the start of the experiment. Oxygen was measured daily.

2.2. Experimental setup

The amphipods were exposed to the sediments in 2 L microcosms (Erlenmeyer flasks) covered with green plastic film to simulate natural light conditions at 20–30 m depth (Sundelin, 1983). Water temperature was kept at 4 °C to simulate field conditions. In each microcosm, 400 mL of sediment and 1 L of brackish water (PSU 6.1) was added 1–2 days before adding 25 female amphipods with gonads and 25 male amphipods. Microcosms received a continuous flow of 3.6 mL water min^{-1}. Addition of food, apart from what is naturally occurring in the sediment, is not essential according to Segerstråle (1971). However, to further reduce the risk of food deficiency, 245 mg of algal-based fish food (Tetra-phyll, Melle, Germany), corresponding to ~98 mg C (Goedkoop et al., 2006), was added to each microcosm after 42 days exposure, which corresponds to a normal level of carbon settling in the region where the amphipods were collected (Blomqvist and Larsson, 1994). Temperature, oxygen levels, water flow, salinity and pH were measured twice weekly. Each sediment had 7 replicate microcosms and the exposure ended at late embryogenesis (mid-January). Because of logistic reasons and time-consuming embryo analyses, the termination of the experiment extended over an 8-day time period, resulting in 88–95 days exposure. To avoid bias, due to different exposure times, termination of the 7 replicates for each treatment were evenly distributed over the 8-day time period. All gravid females were weighed after being dried on medical wipes. Embryos were removed and classified under a stereomicroscope (80–100 × magnification) as normal or aberrant according to