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Multixenobiotic resistance, acetyl-choline esterase activity and total oxyradical scavenging capacity of the Arctic spider crab, *Hyas araneus*, following exposure to bisphenol A, tetra bromo diphenyl ether and diallyl phthalate

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ABSTRACT

The Arctic has become a sink for persistent organic pollutants (POPs) originating from lower latitudes, and relatively high levels have been found in different biota. Recent studies have identified detrimental effects on wildlife including endocrine disruption, impairment of enzyme activity, and reduced immune function. The Arctic spider crab, *Hyas araneus*, shown interesting potential for its use as sentinel organism in polar ecosystems. This study investigated the effect of 2,2′,4,4′-tetra bromo diphenyl ether (BPDE), bisphenol A (BPA), and diallyl phthalte (DPA) on *H. araneus* in a three weeks exposure study. Expression of multixenibiotic resistance (MXR) proteins has been studied using the C219 monoclonal antibody which allows identifying an immunoreactive protein of 40 kDa in the digestive gland while no such protein could be observed in the gills. Expression of this protein was increased by exposure to DPA (+75%; $p < 0.05$, $n = 10$). All compounds significantly affected muscle acetylcholine esterase (AChE) activity ($p < 0.05$, $n = 10$) with 50 μg/L DPA having the strongest effect by lowering the value to 37% of control. The total oxyradical scavenging capacity measured in the digestive gland toward peroxyl, hydroxyl and peroxynitrite was also significantly reduced indicating a decreased resistance to oxidative stress generated by DPA ($p < 0.05$, $n = 5$). These results thus suggest the potential detrimental effects of DPA even at concentration as low as 50 μg/L on *H. araneus*.

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1. Introduction

The Arctic has become a sink for persistent organic pollutants (POPs) originating from lower latitudes, and relatively high levels have been found in biota (Bang et al., 2001; Derocher et al., 2003). Recent studies have identified detrimental effects on aquatic wildlife including endocrine disruption, impairment of enzyme activity, and reduced immune function in the deep-sea fish (Porte et al., 2000) and crustaceans (Hargrave et al., 1992). In addition, pollutants may have potential long-term adverse effects in polar marine ecosystem (AMAP, 1997) especially when they affect the endocrine system. Phthaltes, bisphenol A and bromo-diphenyl-esters belong to this new class of chemicals defined as endocrine disrupting compounds (EDC) that can alter the reproduction or behaviour of wild populations (WHO, 2002).

Pollutant entering the cells can potentially induce an oxidative stress in marine organisms. Indeed, numerous pollutants are reported to enhance the formation of reactive oxygen species (ROS) which naturally originate from the partial reduction of molecular oxygen. Harmful effects of ROS include lipid peroxidation, DNA damage and cell death (Winston and Di Giulio, 1991). Nevertheless, the extent of such damages depends on the effectiveness of the antioxidant defence system. The TOSC assay allow to discriminate the different role of specific ROS in the oxidative stress syndrome (Winston et al., 1998) and their links with effects at higher levels of biological organisation (Regoli et al., 2002). Acetylcholine is the primary neurotransmitter in the sensory and neuromuscular systems in most species. The activity of this system is vital to the normal behaviour and muscular function and represents a prime target on which some toxicants can exert a detrimental effect. Inhibition of the AChE enzyme results in a build up of acetylcholine, causing a continuous and excessive stimulation of the nerve/muscle fibres, which leads to tetany, paralysis and eventual death. Measurement of AChE inhibition in aquatic organisms has gained recognition as important biomarker of effects of neurotoxic contaminants (Galgani and Bocquené, 1990; Payne et al., 1996; Kirby et al., 2000). The expression of the MXR system corresponds to a first line of defence against various structurally and functionally unrelated compounds (Bard, 2000).

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Resistant cells express high molecular weight membrane proteins that belong to the wide family of ABC (ATP-binding cassette) transporters (Zimniak et al., 1999). Those transporters can pump out several chemicals of the cell thus maintaining the intracellular level of a wide variety of structurally and functionally unrelated compounds below their toxic levels (Gottesman and Pastan, 1993). Elevated MXR proteins have been measured in polluted areas (Minier and Moore, 1996) and have been related to poor condition index of organisms such as mussels (Minier et al., 2006) but, to date, no ABC transporters have been characterised in *H. araneus*.

In this study we applied three different biomarkers to assess the potential effects of bisphenol A (BPA), 2,2′,4,4′-tetra bromo diphenyl ether (BPDE) and diallyl phthalte (DPA) on the Arctic spider crab, *Hyas araneus*. BPA is one of the estrogenic contaminant commonly found in rivers and coastal waters. Concentrations can be as high as 12 μg/L in surface water (Kolpin et al., 2002) and 200 μg/L in waste waters (Baugros et al., 2008; Peng et al., 2008). DPA belongs to the many phthalates esters found at total concentrations of 0.1–400 μg/L in surface waters around the world (Fatoki and Noma, 2002; Wang et al., 2008) while BPDE is only found at concentrations below 0.1 ng/L (Wurl et al., 2006). *H. araneus* is commonly found in Artic benthic environments although it has a wide geographical distribution spanning from northern Spain to northern Norway (Nadeau and Cliché, 1998). The high abundance and wide distribution of *H. araneus* make it a potential sentinel species for monitoring the Arctic marine ecosystem (Camus et al., 2002). This work aimed at studying the ability of the spider crab to manage with some new class of contaminant that have been shown to be detrimental to several aquatic species (Eriksson et al., 2001; Roepke et al., 2005). Measured endpoints include measurements of the expression of multixenobitic resistance (MXR) proteins, the total oxyradical scavenging capacity (TOSC) and the acetylcholine esterase (AChE) activity. As those end points are relatively new for an Arctic crustacean, the study would also provide a useful baseline characterization of these biomarkers, integrated with their responsiveness to tested molecules.

2. Materials and methods

2.1. Sampling and maintenance of crabs and exposure experiment

Specimen of *H. araneus*, collected in an unpolluted site at Krokanes, Karmsundet (Norway), were acclimated in a continuous flow-through system supplied with filtered seawater, at 10–12 °C, 34‰, as described in Sundt et al. (2006). Crabs were then exposed for three weeks to 50 μg/L bisphenol A (BPA Merck, EC No. 201-245-8, purity >97%), 5 μg/L 2,2′,4,4′-tetra bromo diphenyl ether (BPDE Chiron Product No. 1688.12, purity >95.6%) and 50 μg/L diallyl phthalte (DPA Fluka EC No. 2050163, purity >98%) through contaminated water for 3 weeks using a continuous flow system used for oil dispersions (Sanni et al., 1998). Exposure concentrations were selected based on previously reported LC50 values that were divided by a factor of 100. Concentration of acetone, the carrier for all compounds, in the exposure units was kept lower than 2 ppb (grade >99.5). Water concentration was monitored throughout the experiment and actual concentration were 59.4 (±10.6) μg/L BPA, 0.23 (±0.19) μg/L BPDE and 38.3 (±9.7) μg/L DAP (Sundt et al., 2006). Average water concentration of BPDE was only 4.6% of nominal concentration and thus likely adsorbed to particles, (e.g. food reminders and faeces) or the container walls. Nevertheless, BPDE was still highly bioavailable and lead to considerable amounts of the compound taken up by the exposed organisms (Sundt et al., 2006). No lethality of organisms was recorded during the experiment. After the exposure experiment, crabs were dissected out and tissues were immediately frozen at −80 °C until further processing.

2.2. MXR protein analysis

Gills and hepatopancreas were homogenized in a saline buffer (10 mM Tris/HCl, 0.25 M sucrose, 0.15 M KCl, 5% β-mercaptoethanol, pH 7.6, 3.5 μM leupeptin, 3 μM aprotinin, 0.1% triton X100). Twenty micrograms of protein extracts from *H. araneus* and from blue mussel (*Mytilus edulis*), used as positive control, were size fractionated on a 9% polyacrylamide gel and then transferred onto a nitrocellulose membrane (Hybond-C extra, Amersham). Membrane was blocked for one hour with Tris buffered saline (TBS: 20 mM Tris, 137 mM NaCl, pH 7.6) supplemented with 3% bovine serum albumin (BSA) and immunolabelling was performed using the C219 monoclonal antibody (AbCys, France) at 0.5 μg/mL in TBS-1%BSA for 2 h at room temperature, followed by the incubation with an anti-mouse Ig-G polyclonal antibody coupled to phosphatase alkaline diluted to 1:1500 in TBS-BSA1% for one hour. Chromogenic reaction was finally performed using bromo-chloro-indolyl-phosphate and nitro-blue-tetrazolium (Sigma) and staining intensity was measured by image analysis (ImageMasterTotalLab, Amersham, Piscataway, NJ, USA).

2.3. TOSC-assay

The method was based on Winston et al. (1998) and Regoli and Winston (1999), with the buffers adjusted for marine invertebrates. The digestive gland was homogenised with a Potter–Elvehjem glass/Teflon homogeniser in four volumes of 100 mM KH_2PO_4 buffer, 2.5% NaCl, pH 7.5. The homogenate was centrifuged at 100000*g* for 1 h at 4 °C, and cytosolic fractions were aliquoted and stored at −80 °C. Peroxyl radicals are generated by the thermal homolysis of 2-2′-azo-bis-(2 methyl-propionamidine)-dihydrochloride (ABAP) at 35 °C. The iron-ascorbate Fenton reaction was used for hydroxyl radicals, while peroxynitrite was generated from 3-morpholinosydnomine (SIN-1), a molecule that releases concomitantly nitric oxide and superoxide anion, which rapidly combine to form HOONO. Final assay conditions were: (a) 0.2 mM α-keto-γ-methiolbutiryc acid (KMBA), 20 mM ABAP in 100 mM potassium phosphate buffer, pH 7.4 for peroxyl radicals; (b) 1.8 μM Fe^{3+}, 3.6 μM EDTA, 0.2 mM KMBA, 180 μM ascorbic acid in 100 mM potassium phosphate buffer, pH 7.4 for hydroxyl radicals; and (c) 0.2 mM KMBA and 80 μM SIN-1 in 100 mM potassium phosphate buffer, pH 7.4 with 0.1 mM diethylenetriaminepentaacetic acid (DTPA) for peroxynitrite. Peroxyl, hydroxyl and peroxynitrite radicals can oxidize the substrate KMBA to ethylene gas which is measured with gas chromatography. With these assay conditions, the various oxyradicals induce a comparable yield of ethylene in the control reaction, thus the relative efficiency of cellular antioxidants is compared by their ability to counteract a quantitatively similar prooxidant challenge (in terms of KMBA oxidation). Reactions were carried out in 10 ml rubber septa sealed vials in a final volume of 1 ml. Ethylene production was measured by gas-chromatographic analysis of 200 μl taken from the head space of the reaction vials. Ethylene formation was monitored for 96 min with a Hewlett Packard (HP 5890 series II) gas chromatograph equipped with a supelco SPB-1 capillary column (30 m × 0.32 mm × 0.25 μm) and a flame ionization detector (FID). The oven, injection and FID temperatures were 35, 160 and 220 °C, respectively; helium was the carrier gas (1 ml/min flow rate) and a split ratio 20:1 was used. The data acquisition system was run by the software Millenium32® (Waters). Each analysis required the measurement of control (no antioxidant in the reaction vial) and sample reactions (biological fluid in the vial). In the presence of antioxidant, ethylene production from KMBA was reduced quan-

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