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Aquatic Toxicology 69 (2004) 371–383

AQUATIC TOXICOLOGY

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Contrasting effects of coplanar versus non-coplanar PCB congeners on immunomodulation and CYP1A levels (determined using an adapted ELISA method) in the common sea star *Asterias rubens* L.

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Received 25 February 2004; received in revised form 8 June 2004; accepted 20 June 2004

Abstract

Biological effects of two structurally contrasting PCB congeners (coplanar 77 and non coplanar 153) were investigated by measuring the induction of CYP1A immunopositive protein (CYP1A IPP) in the pyloric caeca and the production of reactive oxygen species (ROS) by amoebocytes in the common sea star *Asterias rubens*. CYP1A IPP was quantified using a specially designed ELISA which uses competitive binding between sea stars and trout CYP1A IPPs. Only the coplanar congener had a significant effect on the two considered biological responses. Intensity of the effects was dose-dependent. However, the highest dose of PCB 77 induced a dramatic decrease of ROS production. It is concluded that coplanar PCBs straightforwardly affect key biological processes such as the immune system and mixed-function oxidase (MFO) system.

Keywords: CYP1A; ELISA; PCBs; Echinoderms; ROS; Immune system; *Asterias rubens*

1. Introduction

Biological effect monitoring is essential in determining the spatial and temporal distribution of risks caused by contaminants in the marine environment (Huggett, 1992; Stegeman et al., 1992). In the marine environment, biomarkers can offer early warning sig-

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doi:10.1016/j.aquatox.2004.06.008

nals of exposure to contaminants and may be used to diagnose and interpret observed effects, for example by testing sublethal toxicity in water or sediment samples in laboratory bioassays using selected test species (den Besten, 1998). In this study, biological effects of PCBs were investigated in the sea star *Asterias rubens*, a widely distributed and abundant key benthic species (*sensu*, Lewis, 1978) in the North Sea and NE Atlantic, which has been used as bioindicator for monitoring contamination in the field (e.g., den Besten et al., 1993, 2001; Everaarts et al., 1998; Temara et al., 1998, Coteur et al., 2003a; Stronkhorst et al., 2003). Two biological responses were specifically addressed: reactive oxygen species (ROS) production by amoebocytes and cytochrome P450 immunopositive protein (CYP1A IPP) induction in the pyloric caeca.

ROS production is one of the main immune responses of echinoderms and plays a key role in the destruction of microorganisms through cytotoxic mechanisms (Chia and Xing, 1996). This immune response is triggered by amoebocytes which are the most active, free-circulating cells found in echinoderm coelomic cavities. Its amplitude was reported to be modulated by contaminants in *A. rubens* (Coteur et al., 2003a, 2003b).

CYP1A induction is probably one of the most commonly studied biochemical markers in environmental monitoring (e.g., Bucheli and Fent, 1995; Stegeman, 1995; Hahn, 2002). The main function of the CYP1A-dependent monooxygenase system is to convert relatively insoluble organic compounds to soluble metabolites; however, the resulting products are often more toxic than the parent compounds (Walker and Peterson, 1994). Total cytochrome P450 concentrations were reported in *A. rubens* using the carbon monooxide (CO) difference spectra of sodium dithionite-reduced samples (den Besten et al., 1991, 1993). During the last decade, a shift from analyzing total cytochrome P450 towards measuring amounts of dioxin-inducible isoenzyme of CYP1A has taken place (Bucheli and Fent, 1995). More specifically, in the analysis of fish cytochrome P450, isoenzyme content has been measured using enzyme-linked immuno sorbent assays (ELISAs) (Celander and Förlin, 1991; Goksøyr, 1991; Förlin et al., 1992). However, this method has never been used before for such measurements in echinoderms. Because of the high conservation of CYP1A sequence throughout the animal kingdom, the ability of antipeptide antibodies (developed with a high affinity for trout CYP1A) to cross react with sea star cytochrome P450-like proteins is worthwhile testing in order to optimize CYP1A IPP measurement in these invertebrates.

In this study, we set up, test and apply a customized ELISA for the determination of CYP1A IPP in sea stars. Intracoelomic injections were used as an exposure route in order to test the toxicity of PCBs. The doses considered were chosen to include the higher range of those reported in previous bioaccumulation experiments (Danis et al., 2003). The effect of two structurally contrasting PCB congeners (viz. the coplanar congener 77 and the non-coplanar congener 153) on the immune system and on CYP1A IPP induction in the sea star *A. rubens* was then determined.

2. Materials and methods

2.1. Organisms

The sea stars *A. rubens* (*Linnaeus* 1758) were collected in April 2002 from the intertidal zone at Audresselles (Pas-de-Calais, France). Prior to experimentation, 50 specimens of similar size (5–7 cm arm radius) and weight (36 ± 3.5 g) were acclimated to laboratory conditions for 1 month (constantly aerated closed circuit aquaria; salinity 34 psu; 16 ± 0.5 °C; 12/12 h dark/light cycle) during which time they were fed mussels (*Mytilus edulis* L.) ad libitum.

2.2. Chemicals and solutions

Stock powders of 2,2′,4,4′,5,5′-hexachlorobiphenyl (non-coplanar PCB 153), 3,3′,4,4′,5-pentachlorobiphenyl (coplanar PCB 126) and 3,3′,4,4′-tetrachlorobiphenyl (coplanar PCB 77) purchased from Promochem (Germany) were dissolved in ultrapure acetone (Sigma) to reach a concentration of 1 μg ml^{-1}. Stock solutions were stored at −20 °C until final dilutions were prepared. A few minutes before injecting the animals, successive dilutions were made in glass haemolysis tubes using ultrapure acetone as a solvent.

The polyclonal rabbit anti-trout CYP1A antibody (0.4 mg ml^{-1}) and the β-naphtoflavone (BNF)-induced trout microsomal fraction were purchased from Biosense (Norway). Phosphate-buffered saline (PBS), bovine serum albumin (BSA), horseradish per-

oxidase (HRP) coupled to Extravidin and Coomassie blue were obtained from Sigma (USA). The substrate chromogen (tetramethylbenzidine, TMB) was purchased from Biosource (UK).

2.3. Preliminary PCB exposure

In order to set-up and test the ELISA, four batches of sea stars (n = 3) were injected into the coelomic cavity with 50 μl of the coplanar PCB 126 solution (6 ng g^{-1} whole body FW) or seawater (control). Injected sea stars were maintained in separate compartments of a 70 l aquarium under flowing seawater (flow rate 30 l h^{-1}, salinity 34 psu, temperature 16 ± 0.5 °C, light/dark cycles 12 h/12 h). Animals were sampled 48 h after being injected and pyloric caeca were dissected and immediately frozen in liquid nitrogen (−196 °C). Samples were then stored at −80 °C.

2.4. PCB exposure

For the toxicity test, five batches of three sea stars were injected with 50 μl of PCB solutions (at five different concentrations) using a 50 μl Hamilton glass syringe. In addition, a control group (n = 5) was injected with acetone alone, and another one (n = 5) with seawater; a last group of blank animals (n = 5) was not injected. Injected doses were 15.2, 2.82, 0.56, 0.15 and 0.02 ng PCB 153 g^{-1} whole body (FW) and 5.96, 1.51, 0.18, 0.04 and 0.008 ng PCB 77 g^{-1} whole body (FW). After injections were performed, individuals were maintained as described above (preliminary PCB exposure). Animals were sampled 48 h after being injected and pyloric caeca were dissected, frozen in liquid nitrogen and stored at −80 °C until making biomarker measurements.

2.5. Protein normalisation

Frozen pyloric caeca were homogenized in PBS buffer using an ultraturrax (22,500 rpm; 20 s) and the homogenate was centrifuged (12,500 × g; 4 °C; 15 min). The supernatant (post-mitochondrial supernatant, PMS) was transferred to new centrifugation tubes and re-centrifuged using the same protocol. In order to quantify the total protein content, a sub-sample of the PMS was transferred to 96-well plates and mixed with Coomassie blue (Bradford, 1976). Absorbance at 620 nm was measured using a Tecan Spectrafluor Plus plate-reader. Total protein concentration of the samples was then normalised to 100 μg ml^{-1}.

2.6. Protein biotinylation

Protein biotinylation of the trout microsomal extract was performed using Pierce Endogen (UK) biotinylation kit. Sulfo–NHS–Biotin was added to protein solutions to give a 20-fold molar excess of Sulfo–NHS–Biotin in a 10 mg ml^{-1} protein solution. After incubating at room temperature for 30 min, a desalting column was equilibrated with 30 ml PBS. After application of the trout microsome solution, an aliquot of buffer was applied and fractions containing variable protein concentrations were collected in separate test tubes. Fractions containing the highest protein concentration (as determined using the Coomassie blue assay, Bradford, 1976) were then pooled together. The biotin incorporation efficiency in this pool was tested using the HABA method (Green, 1965).

2.7. Western blot

Western blot was performed to test the cross-reactivity of the anti-trout CYP1A antibody with sea star homologue proteins. After being cooled at 4 °C, 0.1% bromophenol blue was added to the samples. Protein samples were combined with equal parts of treatment buffer (0.125 M Tris(hydroxymethyl)aminomethane (Tris)–Cl, 2% Sodium dodecyl sulphate (SDS), 10% glycerol, 5% 2-mercaptoethanol) and heated in a water bath (60–70 °C) for 2 min. SDS polyacrylamide gel electrophoresis (SDS–PAGE, 10% polyacrylamide) was used for this assay. After electrophoretic migration (200 V, constant voltage, 2 h), proteins were transferred to nitrocellulose sheets using a Genie® system (BioRad). Transfer papers were rinsed and blocked for 30 min using a blocking buffer (10^{-2} M Tris–SDS). Anti-trout CYP1A antibody was added to a fresh change of blocking buffer at a 1:1000 dilution, and left overnight at 4 °C. After thorough rinsing, transfer papers were placed in blocking buffer (Tris–ethyldiaminetetraacetic acid (EDTA)–BSA) containing anti-rabbit IgG-alkaline phosphatase (1:1000 dilution). The papers were then washed, rinsed and transferred to 100 mM Tris–HCl with

1 mM $MgCl_2$. Enzyme-substrate chromophore cocktail (0.15 M bicarbonate/carbonate–BiCP–nitroblue tetrazolium) was finally added.

2.8. Enzyme-linked immuno sorbent assay (ELISA)

Cytochrome P450 IPP content was quantified using competitive-ELISA (Fig. 1). The ELISA was carried out using competitive binding to an anti-trout CYP1A antibody between the CYP1A IPP contained in the sea star samples and the biotinylated CYP1A from BNF-injected trout. Because the concentration of biotinylated trout CYP1A remains constant while the concentration of sea star CYP1A IPP varies between samples, the amount of bound biotinylated trout CYP1A is inversely proportional to the concentration of CYP1A IPP in sea star samples. In order to determine the optimal quantity of biotinylated trout CYP1A to be used in our setting, a sigmoid-shaped fixation curve was resolved experimentally. The optimal biotinylated trout CYP1A quantity to be used was chosen in the linear portion of the fixation curve, corresponding to a concentration of 1 mg ml^{-1} total protein. For the ELISA, multiwell plates (96 wells) were coated at 4 °C with 100 μl of anti-CYP1A (rabbit anti-fish CYP1A peptide, polyclonal antibody; dilution 1:1000 in PBS-BSA 0.1%). After 12 h, wells were washed five times with PBS and nonspecific binding sites were blocked with 200 μl PBS-BSA 2% agitated for 2 h at room temperature. Wells were washed again, and 100 μl of biotinylated trout were added along with protein-normalised samples. This operation was performed as fast as possible, using a template multiwell plate (with low sorption characteristics) containing the samples. Competition between the two antigens was allowed to take place for 2 h at room temperature on an agitation plate. At this stage, five washing steps with PBS were performed and Extravidin-HRP (Sigma) in PBS-BSA 0.1% (dilution 1:2000) was added to all the wells. The plate was then incubated at room temperature for 45 min and the wells washed again using PBS. TMB chromogen cocktail (dilution 1:105 in substrate buffer; Biosource, UK) was added to all the wells and the plate was left in the dark for approximately 10 min at room temperature. The reaction was stopped by adding 50 μl of 95–97% sulfuric acid (Sigma) to the wells. Absorbance was measured in the 96-well plates at 450 nm using a microplate reader (Spectracount, Packard), assuming that absorbance is proportional to the amount of biotinylated trout CYP1A bound to the antibody.

2.9. Reactive oxygen species (ROS) production measurements

Amoebocyte ROS production was measured by the peroxidase, luminol-enhanced method optimized by Coteur et al. (2002). Briefly, 3 ml of coelomic fluid were collected in the same volume of anticoagulant buffer. The cell concentration was measured by absorbance at 280 nm using a Tecan Spectrafluor Plus

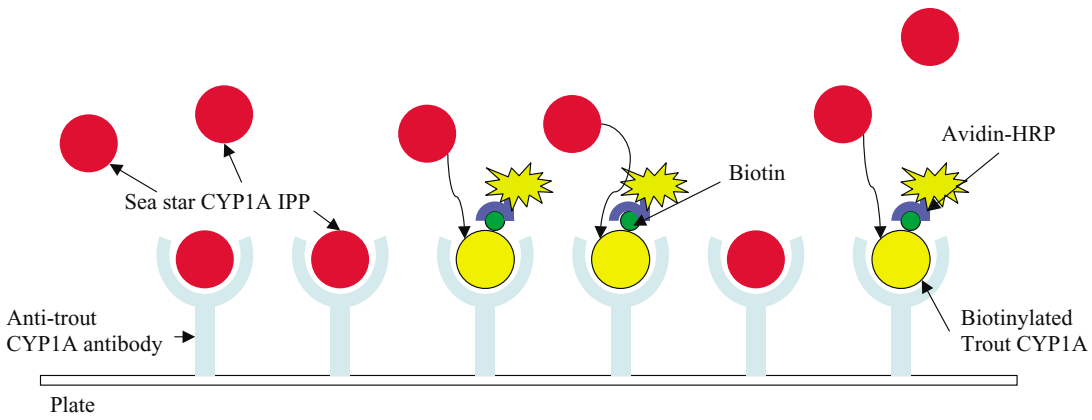

Fig. 1. Schematic representation of CYP1A IPP competitive ELISA method.

plate-reader. This suspension was then centrifuged for 10 min at 400 g and resuspended in Ca^{2+}-, Mg^{2+}-free artificial seawater (ASW). ASW volume was adjusted to obtain a final amoebocyte concentration of 10^6 cells ml^{-1}. A stock solution of luminol and HRP in dimethylsulphoxide (DMSO) was freshly diluted 100-fold in ASW (final concentrations of HRP and luminol were 500 μg ml^{-1} and 250 μg ml^{-1}, respectively). The reaction was begun by adding 200 μl of amoebocyte suspension in 100 μl of luminol/HRP solution and 20 μl of a *Micrococcus luteus* suspension (2.5×10^9 bacteria ml^{-1}) (stimulated amoebocytes) or 20 μl of ASW (non-stimulated amoebocytes). The chemiluminescence was measured every 10 min over a 2 h period using a Tecan Spectrafluor Plus plate-reader previously placed in an thermostabilized incubator (14 ± 0.5 °C). Results were expressed as the sum of all 10 min interval measurements for 10^6 cells ml^{-1} (total chemiluminescence) of bacteria-stimulated amoebocytes or non-stimulated amoebocytes.

2.10. Data analysis and statistics

Experiments were performed with at least three biological replicates. Data was expressed as arithmetic means ± S.D. CYP1A induction was determined using dilution curves performed with highly responsive samples (PCB 126-injected sea star). Dilution curves were best modelled using exponential equations (Eq. (1)). These data were then expressed as CYP1A inhibition percentages (Eq. (2)). Finally, inhibition percentages were converted to CYP1A inhibition percentages measured in control (acetone-injected) sea stars (Eq. (3)) to obtain CYP1A induction folds in sea star samples.

$$y = a\, e^{b \cdot \mathrm{CYP_i}} \quad (1)$$

$$\mathrm{CYP_i} = 100 - \frac{100\ A}{A_{\max}} \quad (2)$$

$$\mathrm{CYP_I} = \frac{(a\, e^{b \cdot \mathrm{CYP_i}})}{(\mathrm{CYP_{ic}})} \quad (3)$$

where CYP_i is CYP1A inhibition percentage, A is absorbance at 450 nm in wells, A_{max} is maximum absorbance in the absence of competitor (sea star samples), CYP_I is the measure of CYP1A induction (induction folds), a and b are the parameters of the exponential equation fitting the dilution curve, and CYP_{ic} is the inhibition percentage in control sea stars (viz. acetone-injected animals).

Dose–response relationships were tested by linear and non-linear regressions, using the Systat 5.2.1 software (Wilkinson, 1988). Differences between ROS production and P450 levels in the different exposure conditions were tested for significance using 1-way ANOVA followed by the multiple comparison test of Tukey (Zar, 1996). The level of significance was always set at $\alpha = 0.05$.

3. Results

3.1. ELISA set-up

In the set-up phase of the novel ELISA for measuring CYP1A IPP in sea stars, a first step was to carry out western blot immunoassays to check the cross-reactivity of anti-trout CYP1A antibody with sea star samples. For this purpose, post-mitochondrial supernatant (PMS) extracts from sea stars injected with PCB 126 or seawater alone were run on gels (Fig. 2). BNF-induced trout microsomes were used as positive controls (lane B) and molecular weight standards were also run in the wells (lane A). Results show that a band appears in the case of PCB 126-exposed sea stars (lane C). The sea star band is in the range of CYP1A molecular weights reported in other species (between 45 and

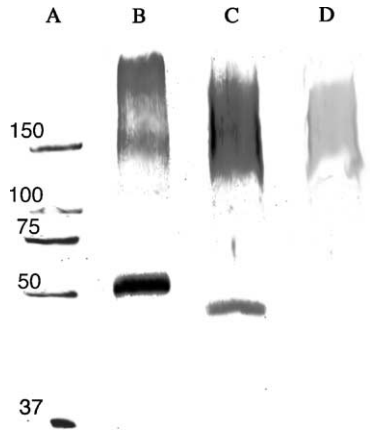

Fig. 2. Western blot SDS–PAGE gel. Lanes are: (A) molecular weight standards (kD), (B) BNF-injected trout microsomes, (C) PCB 126-injected sea star PMS extract and (D) seawater-injected sea star PMS extract.

Table 1
Optical densities measured in a representative ELISA (mean ± S.D.)

Ab	Sample/treatment	B*Tμ	Av-HRP	OD	*n*
+	–	–	+	0.06	
+	–	+	+	0.93	
+	SW	+	+	0.75 ± 0.05	5
+	AC	+	+	0.71 ± 0.13	5
+	PCB 153	+	+	0.65 ± 0.09	15
+	PCB 126	+	+	0.26 ± 0.05	15
+	Tμ	+	+	0.16 ± 0.05	5

Ab, rabbit antitrout CYP1A1 antibody; SW, sea water injected sea stars (100 μg ml^{-1}); AC, acetone injected sea stars (100 μg ml^{-1}); Tμ, trout microsomes (100 μg ml^{-1}); B*Tμ, biotinylated trout microsomes; Av-HRP, avidine-coupled horse radish peroxidase; OD, optical density (mean ± S.D., biological triplicates); –, no sample/treatment.

60 kD; Lewis, 1996) and was induced only in individuals exposed to the coplanar PCB: control sea stars (lane D) showed no band. Therefore, the antibody was used for further testing and ELISA procedures.

The competitive binding-based ELISA was carried out on PMS extracts of sea star pyloric caeca using biotinylated BNF-induced trout microsomes as a competitor. The linear zone of the fixation curve was between 1 and 10 mg total protein per ml, justifying the use of a 1 mg total proteins per ml solution for the competitor (being in the linear zone and representing the lesser quantity to be used for the assay). The test for specificity (values of a representative run) are presented in Table 1. The optical density (OD) measurement in blank wells was 0.06. Maximum absorbance, measured in the absence of sample, produced an OD of 0.93. Weak competition occurred with seawater and acetone-injected sea star PMS extract (OD = 0.75 and 0.71, respectively). PCB 153 exposure induced a competition of medium intensity (OD = 0.65) while samples from PCB 126-exposed sea stars induced significant competition for the anti-CYP1A antibody (OD = 0.26). The most intense competition was found for BNF-injected trout microsomes (OD = 0.16). As mentioned in the methods section, the dilution curve was used to calculate CYP1A IPP induction folds (as compared to controls) in the different samples using Eq. (3). Significant variation of CYP1A IPP content was observed only in animals injected with the dioxin-like, coplanar congener 126 (Fig. 3). Reproducibility among different experiments was good when results were converted to induction factors, with a 73-fold mean increase in CYP1A IPP levels. The sensitivity of the test, defined as the lowest protein concentration at which a significant variation of CYP1A IPP can be detected, is 1.005-fold (5%) in the case of PCB 126-exposed sea stars (calculated as 3 S.D. of blank measurements).

3.2. *Comparative effects of structurally contrasting PCB congeners on CYP1A induction*

Sea stars were injected with increasing doses of structurally contrasting congeners (PCB 153 versus 77). CYP1A IPP induction was measured in the dif-

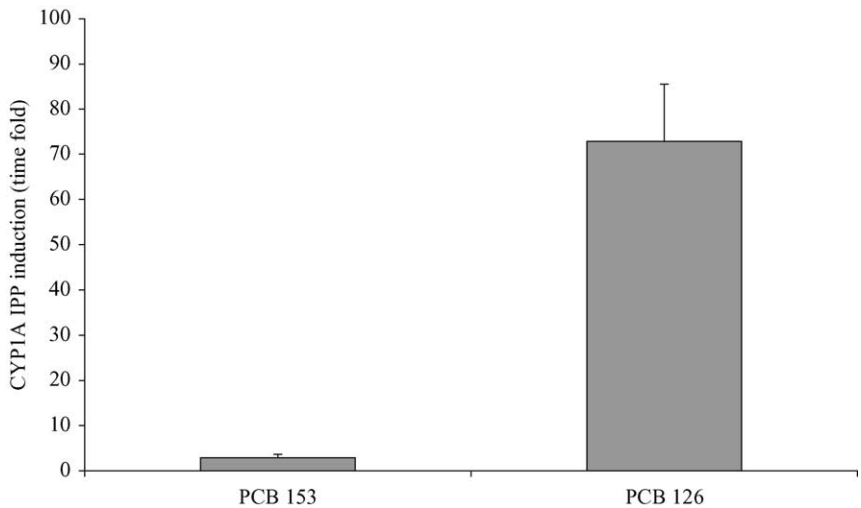

Fig. 3. CYP1A IPP induction (induction fold, mean ± S.D., *n* = 3) in sea stars injected with coplanar (126) or non-coplanar (153) PCB.

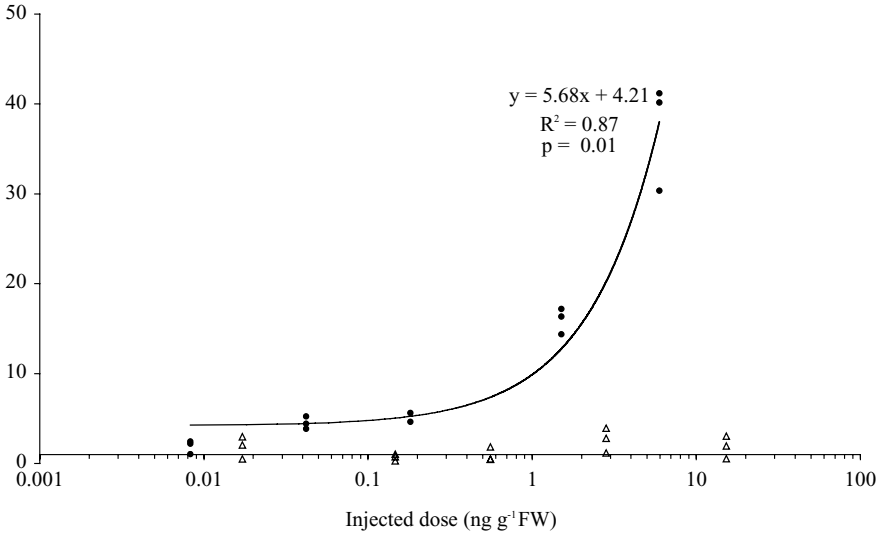

Fig. 4. CYP1A IPP induction (ratio of experimental response to control group) as a function of injected PCB dose (ng g^{-1} FW; log scale) for two structurally contrasting congeners. Curve fitting: linear regression of CYP1A induction as a function of injected dose.

ferent groups of individuals. Control groups (acetone-injected, seawater-injected and non-injected) displayed no significant differences ($P = 0.36$) among each other. Thus, all control individuals were considered as a single control group for the purpose of comparison.

For PCB-exposed sea stars, the results showed a contrasting response between individuals exposed to the coplanar versus the non-coplanar congener (Fig. 4). PCB 153 did not significantly induce CYP1A IPP compared to controls, whereas PCB 77 induced a

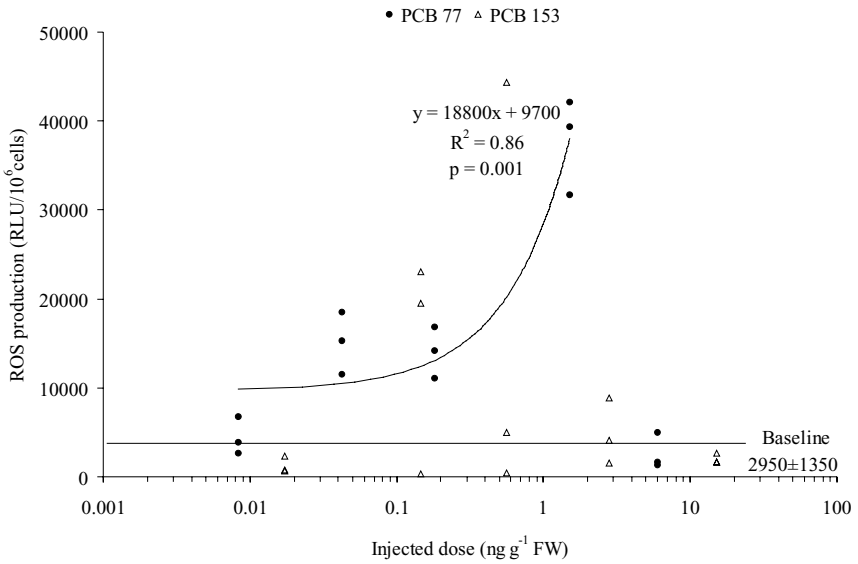

Fig. 5. ROS production (total chemiluminescence) by *non-stimulated* amoebocytes as a function of injected PCB dose (ng g^{-1} FW; log scale) for two structurally contrasting congeners. Curve fitting: linear regression of ROS production as a function of injected dose (excluding data of the highest dose); baseline: ROS production value in control group ($n = 15$).

steep increase of CYP1A IPP content which displayed a significant, linear dose–response relationship (R^2 = 0.87, P = 0.01).

3.3. Effects of PCB congeners on ROS production

ROS production was measured in the different control groups (non-injected, seawater-injected or acetone-injected sea stars) and no significant differences were found among these three groups (P = 0.59 and 0.16 for non-stimulated and bacteria-stimulated amoebocytes, respectively). Therefore, all control individuals were pooled together as a single global control group for further comparisons.

ROS production by non-stimulated amoebocytes is presented in Fig. 5. While PCB 153 induced some increase in ROS production in a few individuals compared to controls, no statistically significant relationship was observed between ROS production and injected PCB 153 dose. In contrast, coplanar congener PCB 77 induced a significant increase in ROS production, i.e. up to 20 times the control values in individuals exposed to a dose of 1.51 ng g^{-1} FW. At the highest PCB 77 dose (5.96 ng g^{-1} FW), the level of ROS production dramatically dropped to control values (baseline in Fig. 5).

The same behaviour was observed for bacteria-stimulated amoebocytes exposed to PCB 77 (Fig. 6). Considering only the results at doses lower than the highest one, a very clear, linear dose–response relationship was found between ROS production and the dose of PCB 77 (R^2 = 0.86, P = 0.001 and R^2 = 0.95, P = 0.01 for non-stimulated and bacteria-stimulated amoebocytes, respectively). In the case of PCB 153, no significant differences were found for ROS production by stimulated or non-stimulated amoebocytes in control versus exposed sea stars.

Finally, in order to check for a relationship between the responses of the two biological effects (viz. ROS production and CYP1A IPP induction) in PCB 77-exposed sea stars, both factors were plotted as a linear function of either non-stimulated or bacteria-stimulated amoebocytes (Fig. 7). Very strong determination coefficients were found for linear regressions in both cases, i.e. R^2 = 0.95, P = 0.03 and R^2 = 0.96, P = 0.02 respectively. The strongest relationship was obtained between ROS production and CYP1A induction in the presence of bacteria-stimulated amoebocytes.

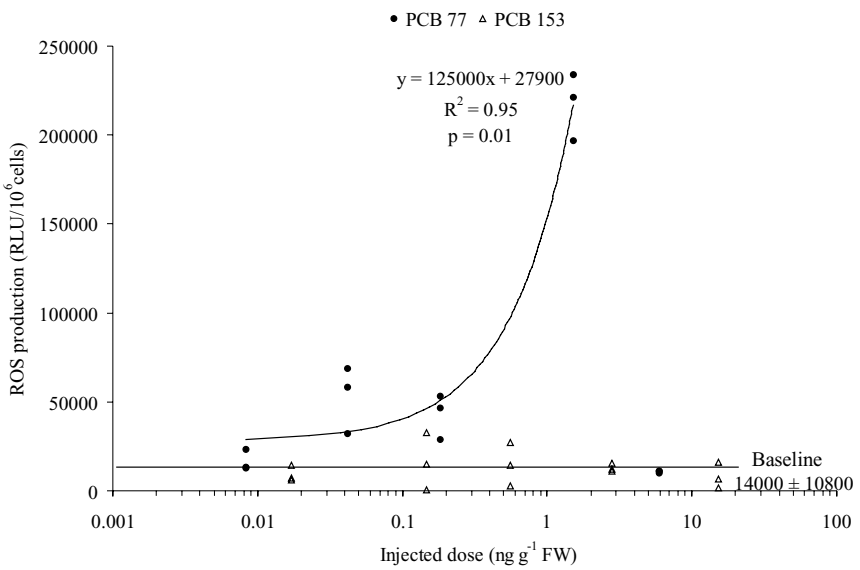

Fig. 6. ROS production (total chemiluminescence) by *bacteria-stimulated* amoebocytes as a function of injected PCB dose (ng g^{-1} FW; log scale) for two structurally contrasting congeners. Curve fitting: linear regression of ROS production as a function of injected dose (excluding data of the highest dose); baseline: ROS production value in control group (n = 15).

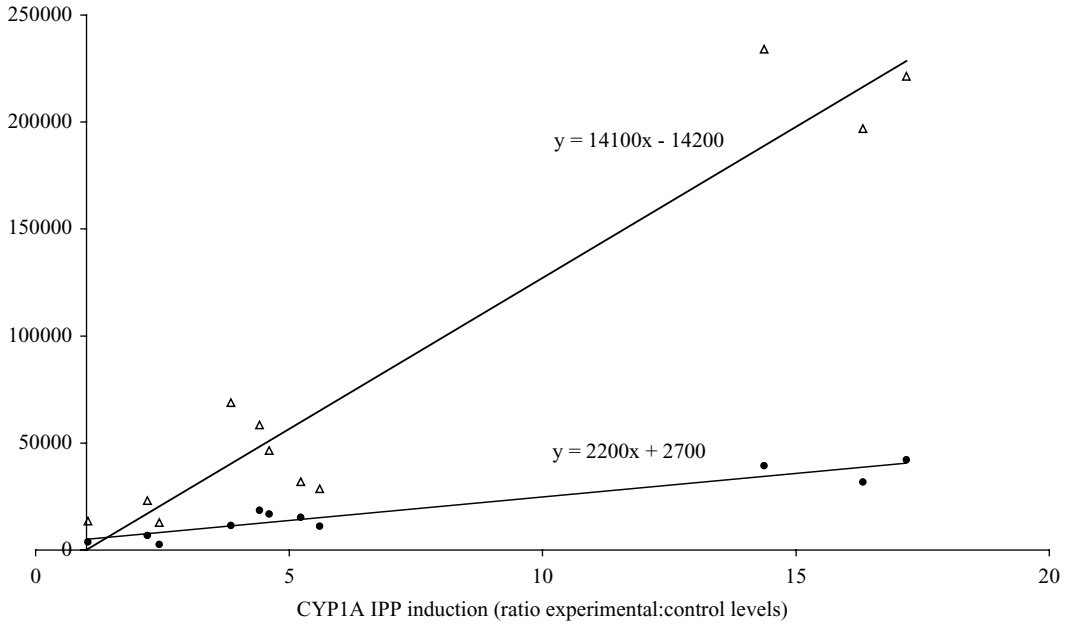

Fig. 7. Regressions between ROS production (total chemiluminescence) and CYP1A IPP induction (ratio of experimental response to control group) in the case of non-stimulated and bacteria-stimulated amoebocytes from PCB 77-injected sea stars. Curve fitting: linear regression of CYP1A IPP induction as a function of ROS production; R^2: determination coefficient.

4. Discussion

In this paper, a novel, rapid and sensitive competitive ELISA for assessing CYP1A IPPs in sea stars is described. This assay does not require long ultracentrifugation steps, or cell culture maintenance and is less time-consuming than other widespread methods such as Western blot, or the CO-difference spectrum for determining total P450 contents. The only pre-requisite of the method is to determine the optimal quantity of biotinylated competitor to be used in the test. Another advantage of the method is the need of only minute quantities of tissues for analysis, as the relationship between CYP1A levels and sample dilution remained linear over a wide range of concentrations. The number of samples that can be processed simultaneously is also greatly enhanced, allowing fast and efficient screening.

Purified sea star CYP1A protein is not available commercially and a true calibration curve could not be established. Therefore, for further testing, it is recommended to establish a dilution curve using PMS extracts of individuals exposed to a CYP1A-inducing compound, such as coplanar PCBs, dioxins, furans or polyaromatic hydrocarbons. Obviously, using this curve implies comparing treated samples to control individuals. Nevertheless, overall, the method is very satisfactory for fast screening of CYP1A IPP variations. In addition, the high degree of inductibility of the CYP1A (up to 73-fold mean increase) is encouraging with respect to applying this method to samples coming from low-contrasting field situations. The exact affinity of the antibody used in this study for sea star CYP1A could not be determined, since no pure sea star CYP1A is available. Also, absolute values of absorbance at 450 nm varied among the different experiments, but this observation is generally considered as typical of ELISA testing (Kennedy, 1991).

The induction of CYP1A is one of the most frequently used biomarkers in the field of environmental contamination (Bucheli and Fent, 1995). Quantification of CYP1A protein levels in field and laboratory samples can provide important and complementary information about the regulation of this protein and toxicity mechanisms in general. Fast and simple methods are required for this purpose and the ELISA described here seems to be a very promising tool. Provided that

the cross-reactivity of the antibody and that the responsiveness to the compound is tested, the present method can probably be applied to a certain range of marine invertebrate species.

In the present study, the adapted ELISA method allowed the observation of PCB congener-dependent behaviour for CYP1A IPP induction in sea stars. Exposure of congener 153 did not result in any significant induction of CYP1A IPP whereas coplanar PCB 77 induced a sharp increase in this protein content. In a previous study, den Besten et al. (1993) measured total cytochrome P450 in sea stars that received single injections of different PCB congeners (PCBs 118, 126 and 153). In this work, total cytochrome P450 was determined using the carbon monoxide difference spectrum of dithionite-reduced samples. Morever, PCB 126 was used at doses ranging up to $2\,\mu mol\,kg^{-1}$ (approximately $650\,ng\,g^{-1}$), which is two orders of magnitude greater than the highest dose used in our experiments. No dose-dependent effects of PCBs were found on the cytochrome P450 content by den Besten et al. (1993). The discripancy between the latter results and those obtained in the present study can be explained by the lower specifity of measuring total cytochrome P450 compared to measuring only CYP1A IPP isoforms. Other workers found results that were similar to ours by exposing cell cultures of trout hepatocytes to different PCB congeners: only coplanar congeners 77 and 126 induced significant CYP1A responses (Bruschweiler et al., 1996). As suggested by Coteur et al. (2001), similarities found in responsiveness to dioxin-like compounds measured in sea stars and vertebrates could indicate the existence of a similar detoxification mechanism in echinoderms and vertebrates. The intensity of CYP1A IPP induction measured in this study was comparable to that described in human cells (Jones and Anderson, 1999) and in mussels (Livingstone et al., 2000). The occurrence of a CYP1A-like protein that can be induced by PAHs and PCBs in molluscs has been shown in various studies (Livingstone and Goldfarb, 1998; Peters et al., 1998), and its induction in mussels has been shown experimentally (Michel et al., 1993). However, there is still a lot of discussion about the mechanisms underlying this induction in invertebrates (see Hahn, 2002 for a review). In vertebrates, CYP1A induction is known to be linked to the exposure to dioxin-like compounds. Mechanisms underlying this induction have been investigated and many studies have drawn the Aryl hydrocarbon Receptor (AhR) in the production of dioxin-like toxicity (e.g., Fernandez-Salguerro et al., 1996; Schmidt et al., 1996). Although the occurrence of the AhR has not been demonstrated in echinoderms, our data and those of Coteur et al. (2001) strongly suggest that a vertebrate-like, CYP1A-triggering mechanism may actually occur in echinoderms. The strongest arguments for this occurrence are: (i) the conservative evolution of the AhR sequence (Lewis, 1996), (ii) a similar structure-induction relationship, and (iii) the dose–response relationship found with cPCBs. Nevertheless, further research is needed to demonstrate the existence of the AhR in echinoderms, by addressing, e.g. the induction of echinoderm CYP1A by vertebrate AhR ligands, the relative structure–activity relationship (SAR) of the potential inducers, and finally 2,4,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) interaction with the receptor leading to its potency to induce CYP1A expression.

The effect of the two structurally contrasting PCBs on the immune system activity was assessed by measuring ROS production in sea stars. In these organisms, ROS production by amoebocytes constitutes one of the main defence line against non-self-material, but the efficiency of this process has been shown to be modulated by xenobiotic exposure (Anderson et al., 1997; Coteur et al., 2001). For example, immunomodulation induced by coplanar PCBs can lead to altered defence against infections (Livingstone et al., 2000). One of the key actors in ROS production is the activation of a membrane-associated enzyme, the NADPH-oxidase, which reduces molecular oxygen to superoxide anion ($O_2^{\cdot -}$), which leads to the production of other oxidants (Babior, 1984). In our study, non-coplanar PCB 153 did not induce significant variation in ROS production whichever dose was applied (except at one concentration). In contrast, coplanar PCB 77 induced a significant increase in ROS production at all doses tested except the highest one, where ROS production dramatically dropped to control levels. This type of response has also been observed in sea urchins (Coteur et al., 2001). Sea urchins injected with increasing doses of selected PCBs (congeners 77, 126 and 153) showed no significant effect in ROS production with PCB 153, whereas PCB 77 and PCB 126 caused a large increase in ROS production. As in the present experiment, ROS production in sea urchins also dropped at the highest

PCB concentration (12.5 ng l^{-1}) tested by Coteur et al. (2001). In our study, induction of ROS production was observed in bacteria-stimulated amoebocytes as well as in non-stimulated cells, an observation which is in contrast with that reported by Coteur et al. (2001) in the case of non-stimulated amoebocytes. This is most probably due to differences in composition of the coelomocyte population between sea urchins and sea stars. While amoebocytes (which are responsible for ROS production) are the sole free-circulating coelomocyte type in *A. rubens*, there are at least six different morphological and functional coelomocyte types co-existing in sea urchins (Chia and Xing, 1996). As ROS production is measured on a per 10^6 cells per ml basis, this difference in coelomocyte population composition could result in an underestimated response when ROS production is measured in sea urchins compared to the same response in *A. rubens*.

In the present study, ROS production increased with injected cPCB doses, which indicates immunomodulation by this xenobiotic, but could also be due partly to the production of ROS as by-products of other mechanisms related to cPCB exposure, viz. detoxification mechanisms. For instance, PCB metabolites produced by CYP1A (quinones, hydroquinones) can lead to the formation of superoxide anion radicals (Aryoshi et al., 1993). Therefore, CYP1A induction can trigger an oxidative stress, which is also a major signal in apoptosis processes (Zahner et al., 1998; White and Prives, 1999). In our study, the possible occurrence of ROS sources other than those produced in amoebocytes could be supported by the fact that CYP1A IPP induction was directly proportional to ROS production (Fig. 7), at least up to the second highest injected dose, but also by the fact that ROS production increased regardless amoebocytes were bacteria-stimulated or not. Whether the measured ROS were produced only by amoebocytes or were also the result of PCB metabolisation processes (both sources probably coexist), it is however a fact that ROS production dropped down to control levels in sea stars exposed to the highest dose of PCB 77. This drop could be explained by the death of amoebocytes either through direct cytotoxicity of PCB 77 at high concentration or through indirect PCB cytotoxicity via a ROS-triggered oxidative stress. Massive amoebocyte death directly geopardizes the efficiency of sea stars immune system, leaving them vulnerable to microbial attack. However, as ROS production did not drop down to zero but to control levels, one could alternatively suggest that the drop in ROS production could also be due to the inhibition of this immune response once a certain treshold of PCB 77 concentration is reached in the coelomic fluid (this treshold would be comprised between the two highest doses tested, viz. 1.51 and 5.96 ng g^{-1} whole-body fresh weight).

We conclude that cPCBs have the potency to induce detoxification mechanism and to impair immune system of *A. rubens*, and therefore represent a threat for sea star populations and in extenso for benthic ecosystems. However, observed effects at the immune level could be also indirectly enhanced by CYP1A IPP activity, which can potentially produce toxic cPCB metabolites, as occurs in the vertebrates. Measuring CYP induction and ROS production in parallel provides valuable information on the organisms health status and thus could serve as an undissociable tool in environmental monitoring studies.

Acknowledgements

The IAEA Marine Environment Laboratory operates under a bipartite agreement between the International Atomic Energy Agency and the Government of the Principality of Monaco. B.D. and S.G. are respectively holders of a FRIA and NFSR doctoral grant. V.F. and P.D. are Research Associates and M.W. is a Honorary Research Associate of the National Fund for Scientific Research (NFSR), Belgium. Research is partly supported by an Agathon De Potter (Belgian Royal Academy of Sciences) grant, a NFSR equipment funding (Tecan Spectrafluor Plus) and a Belgian Federal Research Programme (SSTC, Contract MN/11/30 and EV/11/23A). Special thanks are due to Geoffroy Coteur (Laboratoire de Biologie Marine, ULB) for fruitful discussions on ROS mechanisms.

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