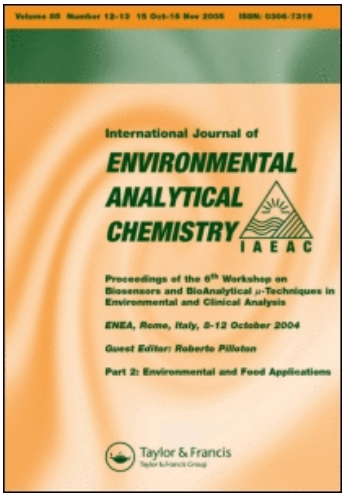

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Rapid screening of alkylphenol exposure in fish bile using an enzymatic peroxidase biosensor

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Rapid screening of alkylphenol exposure in fish bile using an enzymatic peroxidase biosensor

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This article deals with the development and novel application of an amperometric peroxidase biosensor for monitoring fish exposure to petroleum-related discharges, in particular, to alkyl phenols (AP) using fish bile as the main sample material. The biosensor consisted of a screen-printed electrode coupled with peroxidase immobilized by glutaraldehyde cross-linking. The sensor was optimized with regards to factors such as immobilization procedures, substrate selectivity, and matrix effects. The biosensor was used for the analysis of fish bile samples from Atlantic cod (*Gadus morhua* L.) exposed in the laboratory during a 2 week period to different petroleum related compounds. The biosensor could distinguish between bile samples of fish exposed to water containing high concentrations (a mixture of C_4–C_7) to moderate levels (mainly C_0–C_5) of alkylphenols and that of the control group.

Keywords: Enzyme-based biosensor; Screen-printed carbon electrodes; Alkylphenols

1. Introduction

Alkylphenols (APs) are an extensive range of compounds with at least one aromatic ring and with one or more alkyl substitutes. In the North Sea, a major source of alkylphenols to the marine environment is coming from the discharge of produced water from oil and gas offshore installations. During oil production, water that is extracted together with the oil has to be removed before the oil can be further processed. The discharged water is referred to as *produced water* (PW). Produced water is a complex mixture of dispersed oil and various chemicals. The exact chemical composition differs between different production fields. However, certain chemical classes of organic contaminants such as alkylphenols and polycyclic aromatic hydrocarbons are typically present [1]. The amount of produced water generally increases with the age of the oil field. In the North Sea, the annual discharge of produced water has increased in recent years to reach over 100,000 tons per year [2],

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making PW the largest wastewater stream in the oil-exploration and production process. The concentrations of alkylphenols in PW generally decrease with increasing size of the alkyl chain [3]. Concentration levels of shorter-chain alkylphenols (one to five carbons, C_1–C_5 APs) in the produced water from North Sea production have been reported to be as high as $300\,\mu g\,L^{-1}$ [4]. Acute toxic effects have been reported for alkylphenols in marine life living in close proximity to oil and produced water effluents [5]. However, the main concern remains chronic toxicity. Alkylphenols have a potential estrogenic mimicking capacity which increases with the increasing length of the alkyl chain [6]. Furthermore, increasing evidence suggests that exposure to alkylphenols may lead to effects on the reproduction and development of the fish. For example, in rainbow trout (*Oncorhynchus mykiss*), the exposure of nonylphenol has been shown to inhibit testicular growth [7]. The potential of a chemical to produce a chronic toxic response is strongly correlated with its bioaccumulation and biomagnification properties. Sundt and Baussant [8] have reported that the main uptake route for fish exposed to alkylphenols is through the gills. From the gills, the alkylphenols are transported via the bloodstream to the liver where the compounds are biotransformed (metabolized) into more water-soluble compounds that hence can be excreted [9]. In the case of alkylphenols, the metabolism occurs through the action of Phase II conjugation enzymes to form glutathione and glucuronic acid conjugates [2]. This has been confirmed by studies of nonylphenol metabolites shown to be mainly present as glucuronic acid conjugates [10]. The conjugated compounds can be excreted through the urine via the kidney or through the bile via the intestines. In fish, the major part of the metabolites is secreted into the bile and stored in the gall bladder before further excretion [8, 11, 12]. Consequently, bile metabolites can be used as a biomarker of exposure to petroleum compounds in marine biota [13–16]. Several studies have reported the quantification of alkylphenols in water and biological tissues, using fluorescence-based or gas chromatography mass spectrometry (GC-MS) techniques [17, 18]. Despite providing detailed information and with the possibility of identifying a large number of compounds, these methods require skilled personnel and to date do not offer the possibility of *in situ* use. The increasing demand for rapid environmental monitoring has in recent years driven the development of faster screening methods. For this purpose, enzyme-based biosensors, due to characteristics such as small size and low cost, have proven to be an important complement to already existing techniques. This article deals with the development and novel application of an amperometric peroxidase biosensor for monitoring fish exposure to petroleum-related compounds in fish bile samples. The detection principle of peroxidase is based on the catalysed oxidation of organic substrates (AH_2) involving two enzyme intermediates (compounds **I** and **II**) as illustrated by the following scheme [19].

$$\text{Native HRP } (Fe^{3+}) + H_2O_2 \rightarrow \text{HRP} - \text{comp. } \mathbf{I} + H_2O$$
$$\text{HRP compound } \mathbf{I} + AH_2 \rightarrow \text{HRP} - \text{comp. } \mathbf{II} + AH^*$$
$$\text{HRP compound } \mathbf{II} + AH_2 \rightarrow \text{Native HRP } (Fe^{3+}) + AH^* + H_2O.$$

Compounds **I** and **II** can be reduced back electrochemically through direct or mediated electron transfer (ET). By mediated ET, organic compounds such as phenols act as electron donors. The oxidized substrates are electrochemically regenerated, and the reduction current can be related to the concentration of the donor. To build up the biosensor, horseradish peroxidase was immobilized onto the working electrode

of disposable three electrode screen-printed strips using glutaraldehyde as a cross-linker. Different immobilization procedures, substrate selectivity, and matrix effects were investigated in order to optimize the amperometric biosensor. Also, the use of the system for monitoring alkyl phenol exposure in fish was tested with organisms exposed in the laboratory to seawater containing various types and concentrations of alkylphenols. Results obtained from analysis of alkylphenol exposed and reference bile samples are reported.

2. Experimental

2.1 *Material and reagents*

Horseradish peroxidase (EC 1.11.1.7) (1100 U mg^{-1} solid) was purchased from Sigma (Milan). Standard solutions were prepared daily from 4-methylphenol (99% pure) purchased from Aldrich (Milan). Stock standard 4-methylphenol solutions were prepared in a water/methanol mixture (1 : 4). Saline phosphate buffer, PBS (Na_2HPO_4/NaH_2PO_4/NaCl), 0.1 M pH 7.4, was prepared from disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate, and sodium chloride purchased from Merck (Milan). Hydrogen peroxide (30%) was purchased from Merck (Milan). Glutaraldehyde 25% aqueous solution (grade I), β-glucuronidase from *Helix pomatia* (type HP-2) and phenyl-β-D-glucuronide were purchased from Sigma (Milan). All solutions and buffers were prepared with Milli-Q water, obtained from a Milli-Q system (Millipore, Milford, MA). Phenol standards (4-methylphenol, phenol, 3,5-dimethylphenol, 4-pentylphenol, 2,4,6-trimethylphenol, 2,4-dimethylphenol, 4-*t*-butylphenol, 4-pentylphenol, 4-octylphenol and 4-nonylphenol) were obtained from Sigma (Milan). Polycyclic aromatic hydrocarbon (PAH) metabolite standard solutions (1,2-chrysene dihydroxydiol, 1-OH-pyrene, 1-OH-phenanthrene, 2-OH-naphthalene, 1-OH-naphthalene, 1-OH benzo[a]pyrene and benzo[a]pyrene) were kindly supplied by Akvamiljø (Norway).

2.2 *Biosensor preparation*

2.2.1 Screen-printed electrode transducers. The biosensor was assembled using a planar three-electrode strip, based on a carbon working electrode, a carbon counter electrode, and a silver pseudo-reference electrode (figure 1). The electrode strips were prepared using a DEK 248 screen-printer (DEK, Weymouth, UK). Graphite-based (Electrodag 423 SS), silver-based (Electrodag PF-410) polymeric inks were purchased from Acheson Italiana (Milan) and insulating ink (Vinylfast 36-100) from Argon (Lodi, Italy). A polyester flexible film (Autostat CT5) obtained from Autotype (Milan) was used as the printing substrate. The silver ink was used for the conductive tracks and for the silver pseudo-reference electrode, whereas the carbon ink was used to obtain the working and counter electrode. After each printing step, the inks were cured at 120°C for 10 min. The insulating ink was used to define the surface of the working electrode ($Ø = 3$ mm). After printing the insulating layer, the electrodes were cured for 20 min at 70°C.

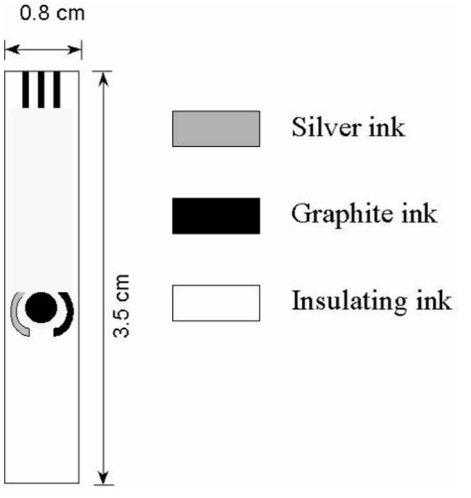

Figure 1. Design of a screen-printed electrode used as electrochemical transducer for the biosensor construction; for details, see text.

2.2.2 Horseradish peroxidase immobilization. Horseradish peroxidase was stored in PBS buffer at −20°C. Glutaraldehyde (25%) was diluted in PBS (0.1 M, pH 7.0). The enzyme aliquots were allowed to reach room temperature and subsequently diluted using the glutaraldehyde solution to give a final cross-linker concentration of 0.2%. Thereafter, the enzyme solution (5 µL) containing glutaraldehyde was deposited on the working electrode, giving a final enzyme concentration of 13 U (nominal concentration). Modified electrodes were stored dry at +4°C.

2.3 *Substrate simulation using phenyl-β-D-glucuronide*

In a first experiment, phenyl-*β*-D-glucuronide was used as a model compound to study the possibility of detecting alkylphenols present in the bile as phenolic conjugates. Deconjugation was carried out using the enzyme *β*-glucuronidase. Glucuronidases catalyses the hydrolysis of *β*-glucuronide conjugates to yield aglycones and free glucuronic acid. The feasibility of this enzyme substrate combination has been reported in [20] by Di Marco *et al.*, who performed on-line deconjugation of phenyl-*β*-D-glucuronide using an immobilized enzyme reactor based upon *β*-glucuronidase. The prepared standard solution of phenyl-*β*-D-glucuronide was added to various concentrations in the fish bile matrix. The whole sample as well as its single components (bile, phenyl-*β*-D-glucuronide, *β*-glucuronidase and buffer) were analysed, and the results expressed as phenol equivalents (µM) using phenol calibration curves.

2.4 *Fish bile samples*

The fish species investigated was Atlantic cod (*Gadus morhua* L.). Farmed Atlantic cod from Marine Troms Yngel (Tromsø, Norway) were transported to the RF/Akvamiljø laboratory (Randaberg, Norway). In the laboratory, the fish were divided into different groups and thereafter exposed to different petroleum related mixtures. To obtain an

Table 1. Overview of exposure treatments for eight groups of Atlantic cod (*Gadus morhua* L.).[a]

Group	Exposure solution	Concentration
1	None	–
2	C_4–C_7 low	$2\,ng\,L^{-1}$
3	C_4–C_7 medium	$10\,ng\,L^{-1}$
4	C_4–C_7 high	$2000\,ng\,L^{-1}$
5	Produced water *Oseberg C* field	1 : 200
6	Produced water *Oseberg C* field	1 : 1000
7	Crude oil	$200\,ng\,L^{-1}$
8	AP mix/PAH mix/crude oil	$200\,ng\,L^{-1}$ oil

[a] The compounds present in groups 2, 3, and 4 were 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol, and 4-*n*-heptylphenol. The concentration reported is referred to each single compound in the group.

even size distribution in all groups, the fish were divided according to weight, and eight groups (553 ± 93 g) were obtained. The fish were allowed to acclimatize in 600 L tanks with flow-through, temperature-controlled (12–14°C) seawater for 1 week prior to exposure. The exposure experiments lasted for 14 days, and the fish were not fed during this period to avoid the excretion of the bile content into the intestine. The exposure groups are described in table 1. The composition of the exposure mixtures was designed to mimic the chemical profiles typically found in crude oil and produced water. Group 1 represented a control group were the tank contained only seawater. In groups 2, 3, and 4, the fish were exposed to a C_4–C_7 alkylphenol mixture, (4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol). Based on measured bioconcentration factors in a study conducted by [8], the nominal water concentrations were 2, 10, and $2000\,ng\,L^{-1}$, respectively, for each compound in the group. Groups 5 and 6 contained produced water tapped at the *Oseberg C* field offshore installation in the North Sea and diluted with sea water in a ratio of 1 : 200 (estimated to correspond to a platform distance of 200 m) and 1 : 1000 (1000 m from the platform). Group 7 was exposed to dispersed crude oil ($200\,ng\,L^{-1}$) from the *Oseberg C* field. Oil from the *Oseberg C* field was chosen as representative of the North Sea. Group 8 was exposed to a mixture of alkylphenols, polycyclic aromatic hydrocarbons (PAH), and *Oseberg C* crude oil. During the exposure, a continuous flow-through system ensured a stable concentration of dispersed crude oil or produced water [21]. At sampling, the fish were sacrificed by a head blow and scored for weight and length. The gall bladder was carefully excised, and the bile was collected in a 1 mL Eppendorf cryotube. The bile was then frozen and stored at −20°C.

2.5 *Sample preparation*

All fish bile samples were deconjugated prior to analysis. Bile (10 μL), β-glucuronidase (15 μL), and acetate buffer (125 μL, 0.4 M, pH 5.0) were added to Eppendorf tubes. The samples were shaken and then placed in an oven at 40°C for 2 h [22]. β-Glucuronidase from *H. pomatia* (type HP-2) was chosen for the deconjugation, as it has both glucuronidase and sulphatase activity. Thereafter, the samples were centrifuged (5 min, 9660*g*), and the supernatants were transferred to new vials and stored at 4°C. Before analysis, a calibration curve using 4-methylphenol was produced. The deconjugated samples were analysed by injecting a fixed volume of the sample into

the electrochemical cell. After injection, the steady-state current was recorded, and the current values were expressed as 4-methylphenol equivalents calculated from the calibration curve. 4-Methylphenol was chosen as a working standard as it is one of the major compounds present in produced water.

2.6 *Biosensor measurement*

Amperometric experiments were performed with a PalmSens (Palm Instrument BV, Hoten, Netherlands). The applied potential for calibrations was −0.05 V *versus* Ag-pseudoreference. The sensor was immersed in PBS (2 mL) under stirring conditions with direct additions of standard substrate solutions using a micropipette. The amperometric signal was recorded when the current response had reached a steady state. In all experiments, hydrogen peroxide was added to a final concentration of 100 μM.

2.7 *Statistical analysis*

The results from different sample groups were compared using one-way ANOVA. When ANOVA indicated a significant difference between sample groups, Fisher's 'least-significant difference' (LSD) multiple comparison test was employed. Statistical tests were performed with Excel software. A significance level of $P < 0.05$ was applied to all statistical tests.

3. Results and discussion

3.1 *Biosensor characterization*

Several types of enzyme immobilizations were investigated, including using Nafion, polyethyleneamine, glutaraldehyde, and direct adsorption. The higher current response was achieved when 4-methylphenol was used as a substrate, using 13 enzyme units (U) and with glutaraldehyde as a cross-linker. Calibration curves carried out using 4-methylphenol showed a good reproducibility with an average coefficient of variance (CV) from the slope of the calibration curve of 3.6% ($n = 3$). The inter-electrode variation was larger with a CV of 6.9% ($n = 3$). It was possible to detect concentrations of 4-methylphenol with a limit of detection (LOD) of 50 nM ($y = 13.5$ (nA μM^{-1})x, $R^2 = 0.999$). The measured linear range was 0–5 μM (results not shown). The influence of pH on the biosensor response was also studied, and the optimal pH, in accordance with previous studies, e.g. [23], indicates that the enzyme is most active around pH 7. The storage stability of the peroxidase-glutaraldehyde modified electrodes was investigated by comparing the slope of the calibration curves using 4-methylphenol standard solutions carried out at different times from the immobilization day with electrodes stored dry at 4°C. The prepared sensors were stable for up to 10 days of dry storage when 4-methylphenol was used as a standard (figure 2).

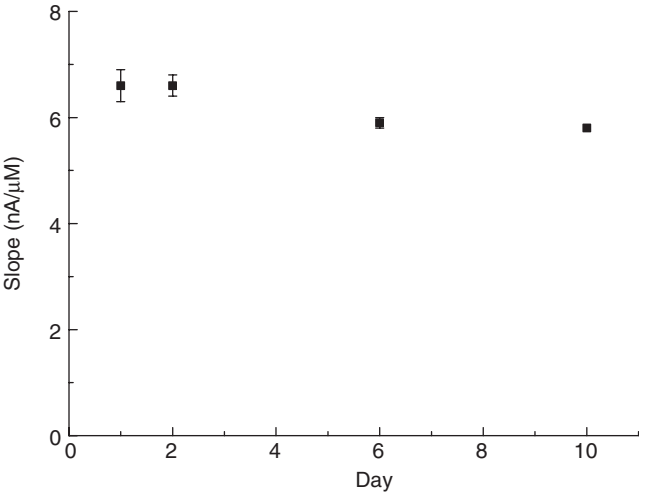

Figure 2. Storage stability of HRP-glutaraldehyde modified electrodes stored dry at 4°C ($n = 3$). Substrate: 4-methylphenol, measuring buffer 0.1 M PBS (pH 7.0). $E_{apl} = -0.05$ V *vs.* Ag-pseudoreference, $[H_2O_2] = 100\,\mu M$.

3.2 *Tests with standard solutions of APs and PAHs*

The relative sensitivity of the sensor was tested using various standard solutions of alkylphenols. Calibration curves were performed with the different compounds in the range 0.1–0.4 μM. The slopes of the calibration curves were normalized with respect to 4-methylphenol and expressed as relative sensitivity (%). The results are shown in figure 3. The sensor was most sensitive to 4-methylphenol. Interestingly, it appears that *p*-substitution, as in the case of 4-methylphenol, 4-pentylphenol, and 4-octylphenol (figure 3), leads to a higher sensitivity. On the contrary, none as well as two or more substitutions in other ring positions seem to decrease the sensitivity. The sensor was also tested using a series of PAH metabolites in the concentration range 0.1–0.4 μM. Among the tested compounds, the sensor responded to 1-OH-pyrene, 1-OH-phenanthrene, 2-OH-naphthalene, and 1-OH-naphthalene.

3.3 *Tests with phenyl-β-D-glucuronide*

The results from the experiments carried out using phenyl-*β*-D-glucuronide (table 2) showed that the glucuronide in buffer solution did not produce a biosensor signal, even at high concentrations. Moreover, the signal from the bile was not influenced by the addition of glucuronide (1.4 μM) in the absence of glucuronidase. However, when the bile was treated with glucuronidase, in the absence of the phenolic glucuronide, the resulting signal increased. Due to the complicity of the bile matrix, it was not possible to attribute this signal to a specific compound. Furthermore, the high blank signal might be a limitation for the detection of low concentrations of alkylphenols. Nevertheless, in the presence of glucuronidase, the signal of the glucuronide was clearly distinguished from the blank (bile and glucuronidase). Hence, the enzyme glucuronidase did not influence the action of the peroxidase enzyme. Moreover, the results confirmed that the biosensor was able to detect only the liberated phenolic compounds and not the

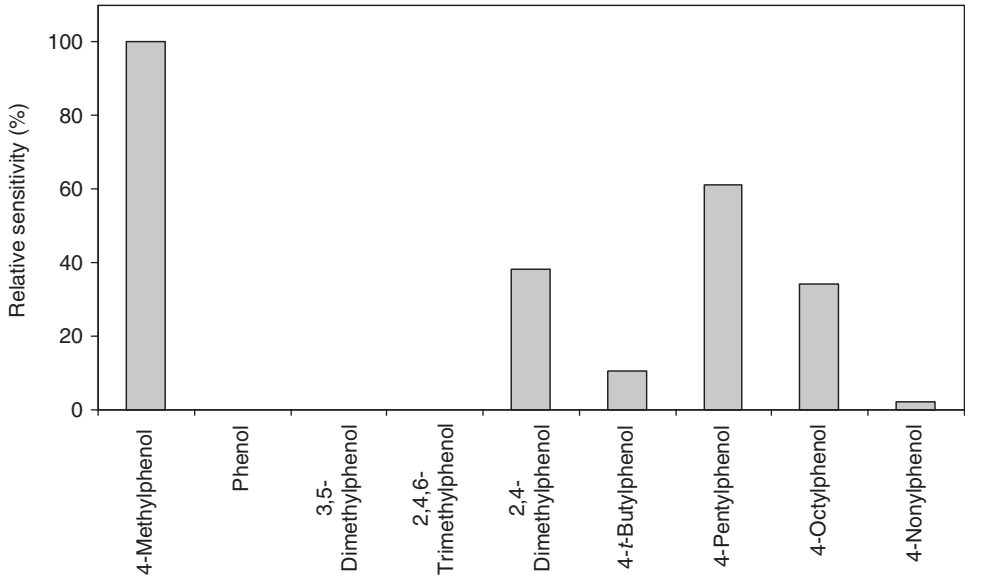

Figure 3. Relative sensitivity of HRP-glutaraldehyde modified electrodes towards 4-methylphenol, phenol, 3,5-dimethylphenol, 2,4,6-trimethylphenol, 2,4-dimethylphenol, 4-*t*-butylphenol, 4-pentylphenol, 4-octylphenol and 4-nonylphenol. Experimental conditions as in figure 2.

Table 2. Phenol equivalents of bile, glucuronide, glucuronidase, and combinations after deconjugation.[a]

Sample	Biosensor response phenol eq. (μM)
Bile	0.1
Phenyl-β-D-glucuronide	0
β-Glucuronidase	0.05
Bile + phenyl-β-D-glucuronide	0.04
Bile + β-glucuronidase	0.45
Bile + phenyl-β-D-glucuronide + β-glucuronidase	1.4

[a] Experimental conditions as in figure 2.

glucuronide form, hence emphasizing the need for the deconjugation of the bile samples.

3.4 *Fish bile samples from laboratory exposure*

Bile samples ($n = 97$) from Atlantic cod exposed in the laboratory to different petroleum-related solutions were analysed with the biosensor (figure 4). Comparing the exposed groups with that of the control, the signals obtained from bile samples exposed to low and medium concentrations of C_4–C_7 alkylphenols were not statistically different. However, the biosensor could clearly distinguish between the positive groups where the fish had been exposed to C_4–C_7 alkylphenols at

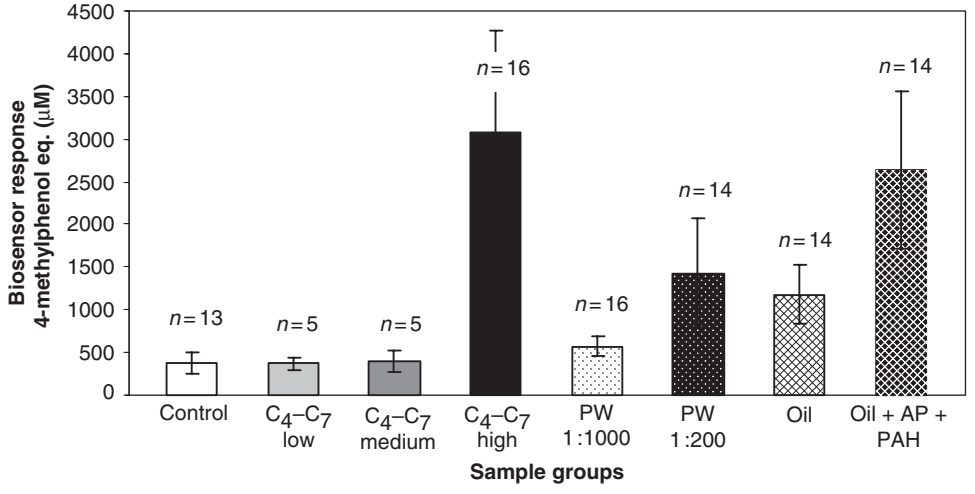

Figure 4. Results of the analysis of fish bile samples from different exposure groups. Experimental conditions as in figure 2.

high concentrations (2000 ng L^{-1}) from that of the control group (no exposure). Furthermore, the biosensor signal was significantly higher in bile samples from fish in group 8 (oil + PAH + AP). Also, the signal measured in fish that had been exposed to produced water at a concentration of 1 : 200 (Group 5) was significantly reduced from that of group 8 but significantly higher than the control group (Group 1). Finally, the group exposed to oil only (Group 7) was significantly different from Group 8 as well as from the control group.

Due to the broad substrate selectivity of the sensor as well as the complexity of these exposure solutions, the biosensor signal could, however, not be attributed to a specific compound. For example, the presence of metabolites resulting from organophosphate pesticide exposure in both control and exposed samples could not be excluded. Despite this, the results indicate that the biosensor could offer a future interesting application as a screening tool of bile samples for the exposure of oil-related discharges in fish. The variability in each group could be explained by intraspecies variability, where factors such as sex and age of the fish might be of importance.

4. Conclusions

A peroxidase-based biosensor was optimized and employed for the rapid analysis of bile samples from Atlantic cod (*Gadus morhua* L.) exposed to a different dose and content of alkyl phenols, and crude oil during a 2 week period. The results indicate that the sensor could successfully differentiate between bile samples exposed to high concentrations of alkylphenols (2000 ng L^{-1}) and the control fish. Moreover, the biosensor could detect elevated signals of organic compounds in fish simulated to be living 200 m from a platform. Due to advantages such as low cost and a short analysis time, this novel application of a peroxidase biosensor could offer a useful tool for rapid on-site environmental monitoring of petroleum-related discharges.

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