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Unidirectional Sodium Fluxes and Gill CYP1A Induction in an Amazonian Fish (*Hyphessobrycon erythrostigma*) Exposed to a Surfactant and to Crude Oil

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Surfactants are organic compounds used largely as detergents or dispersant agents. Synthetic surfactants are ubiquitous in the aquatic environment as a result of water runoff from industrial and urban effluents, which make them a significant contaminant in water bodies around the world (Lewis 1991). Common uses of surfactants are in wastewater treatment and oil spill mitigation. The effectiveness of surfactants in clean up processes is related to their surface-active properties, which allow them to be adsorbed onto surfaces. Although anionic and nonionic surfactants are regularly used to clean up oil spills, they have been shown to be toxic to aquatic organisms. Sodium dodecyl sulfate (SDS) is an anionic akyl sulphate-type surfactant present in a number of commercial petroleum dispersants and it is often used as a reference substance recommended by the EPA for aquatic toxicity tests. SDS has been reported to induce changes in fish gill morphology, such as clubbing and fusion of the secondary lamellae, hyperplasia of the respiratory epithelium, and destruction and shortening of gill filaments, which most likely affect the ionoregulatory and respiratory function (Rosety-Rodriguez et al. 2002). Disturbances in ionoregulatory function resulting from exposure to surfactants, however, have not been documented in terms of changes in ion transfer across the gill epithelium, a major factor involved in the control of electrolyte concentrations in body fluids. Measurement of ion fluxes in fish provides an effective way to assess the toxicity of waterborne chemicals before they are reflected as changes in internal ion concentration and sometimes long before they cause irreversible damage to the fish (Wood 1992). Sodium is one of the most important electrolytes in the blood plasma, and its uptake in fish occurs at branchial apical sites, compensating for constant loss occurring through diffusion. Analysis of unidirectional flux (influx, efflux, net flux) is an easy way to determine whether a pollutant is affecting the sodium balance and it also provides an indication of disruption in gill membrane permeability. Because of their amphiphilic character, i.e., the presence of both hydrophobic and hydrophilic sites, surfactants have been reported to disturb the structural organization of membrane lipid bilayers, generally by increasing permeability (Helenius and Simons 1975).

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The use of surfactants or dispersants in oil spill remediation also represents a major concern for environmental authorities because the chemically dispersed oil may be more toxic than the crude oil itself (e.g., Gulec and Holdway 2000; Ramachandran et al. 2004). The polycyclic aromatic hydrocarbons (or PAHs) are considered to be the most toxic components present in a crude oil fraction and their bioavailability increases when surfactants are employed in spill mitigation. One way to assess increased exposure of fish to PAHs is by assessing the inducibility of proteins from the cytochrome P450 system. Cytochrome P450s are a diverse group of enzymes involved in the metabolism of both endogenous and exogenous compounds, especially xenobiotics. CYP1A is among the P450 forms best studied in environmental monitoring because of its applicability as a biomarker of exposure to PAHs, polychlorinated biphenyls (PCBs), and polychlorinated dioxins (TCDDs). The toxicity of PAHs results from their biotransformation by CYP1A enzymes, which leads to the formation of more toxic, highly reactive metabolites known to be genotoxic or carcinogenic because of their ability to interact with DNA and promote cancer (Stegeman and Lindström-Seppä 1997). Most studies have focused on CYP1A induction in the liver, the major site for detoxification in vertebrates. The primary route of PAH uptake in fish, however, occurs through the gills. Studies of CYP1A expression can be useful to estimate an organism's susceptibility to toxic forms present in crude oil (Stegeman and Lindström-Seppä 1997). Our goals for this study were to evaluate changes in the dynamics of branchial sodium transport and to evaluate gill CYP1A induction in an Amazonian fish species, *Hyphessobrycon erythrostigma*, upon acute exposure to a surfactant and to crude oil.

MATERIALS AND METHODS

Adult specimens of *Hyphessobrycon erythrostigma* (1.24 ± 0.05 g), native to the ion-poor waters of the Rio Negro, a major tributary of the Amazon River, were obtained from an ornamental fish exporter. Fish were acclimated for two months in naturally soft water with an average composition of: $Na^+ = 34$, $Ca^{2+} = 11$, $K^+ = 15$, $Cl^- = 27$, $Mg^{2+} = 0.8$, all in $\mu mol \cdot L^{-1}$; pH 6.5; 28°C. Fish were fed Tetramin flakes throughout the acclimation period, but food was suspended 24 h before experimentation. For our experiments, fish were exposed to either a surfactant or crude oil alone, or to both pollutants combined (N = 8 per treatment). We used SDS (Sigma) as our surfactant and crude oil from the Urucu Reserve (Coari, Amazonas, Brazil). Acute exposure of fish to the treatments was as follows: a) control (soft water), b) SDS (10 $mg \cdot L^{-1}$), c) crude oil (2% v/v), and d) SDS + crude oil. SDS was added to the experimental chambers from a concentrated stock dissolved in deionized water, whereas crude oil was added in its commercial form, without previous separation of the water-soluble fraction. We refer to the treatments in nominal concentrations, given that analytical measurements of SDS (e.g., purity) and crude oil (as total petroleum hydrocarbons, TPH) were not possible.

For unidirectional sodium flux analysis, fish were individually transferred to experimental glass chambers 12 h prior to the onset of exposure and allowed to adjust to the container's environment. Water in the chambers was subsequently renewed and, after 1 h, fish were acutely exposed to the treatments under static conditions and constant aeration. To optimize sensitivity of the method, water volume in the chambers was kept at approximately 50 mL:1 g (water volume to fish mass ratio) during flux experiments. Radio-labeled sodium (^{22}Na, 0.05 $\mu Ci \cdot L^{-1}$, Amersham Pharmacia) was added to each chamber in the designated treatments, and water samples (5 ml) were taken at 0, 1, 3, and 6 h. Analysis of sodium uptake was based on the disappearance of the isotope from the water into the fish over time. Influx, efflux, and net flux were calculated using the radioactivity of the sample, as determined by liquid scintillation counting (LS6500, Beckman & Coulter), and the sodium concentration, as determined by atomic absorption spectrophotometry (AAnalyst, Perkin Elmer), following Wood (1992). At the end of the flux measurements, four fish from each treatment were sacrificed to sample gills. For the remaining individuals, experimental solution in the chambers was restored and they were then sampled at 24 h. Gills were fixed in 10% neutral buffered formalin for CYP1A analysis. Sodium flux measurements in the treatments were analyzed relative to the control using one-way ANOVA, followed by Dunnett's multiple-comparison tests. Statistical significance was set at the level of $\alpha<0.05$ throughout.

CYP1A induction in *H. erythrostigma* was analyzed by immunohistochemistry at the Woods Hole Oceanographic Institution (Woods Hole, Massachusetts, USA). Gill samples (N = 4 per treatment) were decalcified using a formic acid - sodium citrate solution prior to being embedded in paraffin. Sections of 5 μm were cut on a microtome, mounted on slides, and let dry at room temperature for a minimum of two days before staining. The immunohistochemistry staining procedure was based in part on the protocol described by Smolowitz et al. (1991). Slides were run as a positive and a negative control for each sample, with the positive slides being those incubated for CYP1A antibodies. CYP1A detection in the cells resulted from staining of the antigen-antibody complex made visible by a chromogenic substrate (AEC Chromogen Systems, Signet). Red staining in the positive slides indicated sites where the CYP1A proteins were located in the cells. All slides were counterstained with Mayer's hematoxylin solution (Sigma), covered with mounting medium (Crystal/Mount, Biomeda), and photographed.

RESULTS AND DISCUSSION

Gills are the first and the major target sites for waterborne pollutants in fish, so their physiological function is highly susceptible to the presence of chemicals in the environment (Wood 1992). Flux measurements revealed that exposure to both the anionic surfactant (SDS) and the Urucu crude oil caused inhibition of branchial sodium influx in *H. erythrostigma* (Figure 1, white bars). Fish exposed to SDS at 10 $mg \cdot L^{-1}$ were unable to restore sodium influx throughout exposure, indicating an average inhibition of 48% in sodium uptake relative to the control.

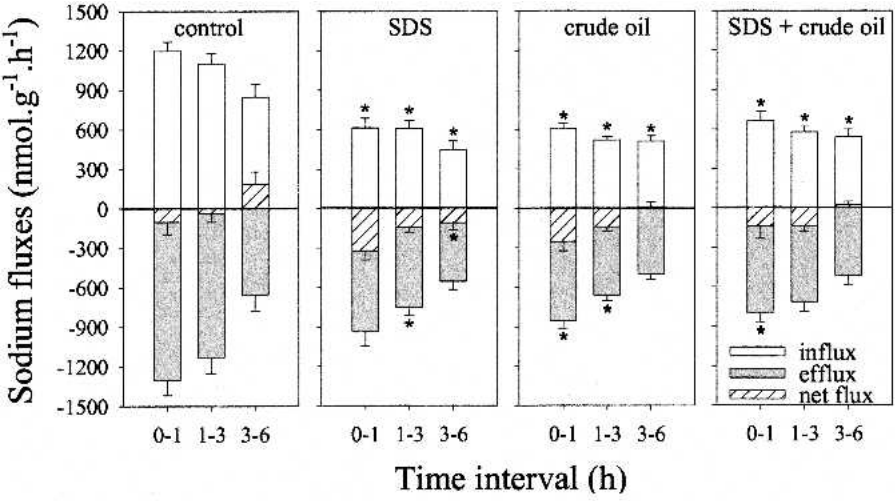

Figure 1. Unidirectional sodium fluxes in *H. erythrostigma* acutely exposed to various treatments. Exposure to both SDS and crude oil resulted in inhibition of sodium uptake up to 48%, and a comparable decrease in the loss of the ion by diffusion (efflux). Therefore the resulting net sodium flux in fish exposed to the pollutants did not differ from that of control group, revealing a tight control of sodium transport at the gills of *H. erythrostigma*. Asterisks (*) indicate significant differences relative to the control at each time interval ($\alpha<0.05$).

Exposure to crude oil, alone or in combination with SDS, resulted in a similar degree of inhibition on sodium influx in *H. erythrostigma*. Inhibition of sodium uptake to this degree can quickly lead to a decreased concentration of sodium in the plasma, as was found in rainbow trout exposed to anionic surfactants (McKeown and March 1978). Anionic surfactants not only bind to phospholipids but also to proteins, thus affecting enzyme activity (reviewed in Cserháti et al. 2002). McKeown and March (1978) suggested that surfactants directly inhibit the activity of Na^+K^+ATPase, the enzyme responsible for pumping sodium into the blood in exchange for potassium. Our results support this hypothesis, given that inhibition of sodium uptake ultimately leads to a decreased intracellular sodium concentration, which consequently suppresses branchial Na^+K^+ATPase. Inhibition of sodium influx upon exposure to crude oil may affect *H. erythrostigma* in a similar way, based on the information that *Leptocottus armatus*, a euryhaline fish, had decreased Na^+K^+ATPase activity when exposed to petroleum fractions (Boese et al. 1982). Fish exposed to SDS + crude oil were unable to stimulate sodium influx anywhere close to control values. Despite the strong influx inhibition, sodium efflux remained unchanged or even lower relative to the control (Figure 1, gray bars). Increased efflux of ions is usually associated with increased membrane permeability, which was not verified for *H. erythrostigma* exposed to the surfactant or to the crude oil. Crude oil is not known to affect membrane

permeability, but due to the lipophilic nature of its components we cannot rule out the occurrence of direct interactions at the gill membrane. The control of diffusive sodium loss (efflux) in the presence of SDS and/or crude oil also counteracted the negative impact that sodium uptake inhibition would have caused in *H. erythrostigma*. This resulted in net sodium fluxes that were not significantly different from the control values (Figure 1, hatched bars), indicating that inhibition of sodium influx generated by either SDS or crude oil is unlikely to result in lower plasma sodium concentrations in *H. erythrostigma* (see review of surfactant effects in Wood 2001). Although chronic studies are still necessary to address long-term effects of these pollutants on sodium balance, we can say that *H. erythrostigma* is highly specialized. This species, which is native to ion-poor waters, appears to have a very tight control of branchial sodium regulation, which is easy to understand when we examine the demands imposed on it by the environment. Fish living in ion-poor waters are more hyperosmotic to the environment than average freshwater fish. Therefore, they face even higher rates of diffusive ion losses in association with increased water inflow into their tissues, these demanding physiological adjustments to ensure survival. Such adjustments enable *H. erythrostigma* to respond quickly to exposure to pollutants and avoid disturbances in such a way that net losses are kept to a minimum.

The toxicity of petroleum fractions to fish appears to be higher in the presence of anionic surfactants than when petroleum compounds are alone involved (Gulec and Holdway 2000; Ramachandran et al. 2004). This is often explained by the fact that surfactants increase the solubility of the toxic crude oil fraction (therefore increasing bioavailability of PAHs to fish), and also, by the potential of surfactants to directly interact with membrane bilayers (Singer et al. 1998; Cserháti et al. 2002). We found that gill CYP1A expression in *H. erythrostigma* was inducible upon exposure to crude oil, as expected, but it was also inducible upon exposure to SDS (Figure 2). The induction was mild in fish exposed to the treatments in the first 6 h, but became slightly stronger at 24 h, as indicated by the intensity of the red color in gill sections. Exposure to SDS + crude oil resulted in CYP1A induction being moderately higher at 24 h, relative to all other treatments. The control group did not exhibit any degree of gill CYP1A induction, at least as detected by immunohistochemistry.

Whereas the mechanism of CYP1A induction in fish exposed to PAHs present in crude oil is well known, the nature of CYP1A induction in fish exposed to SDS remains unclear. CYP1A expression occurs through the activation of the cytosolic aryl hydrocarbon receptor (AhR) by specific ligands, such as PAHs. Anionic surfactants like SDS can be bioreactive, interacting with protein, DNA, and lipid bilayers (reviewed in Cserháti et al. 2002). Our results suggest that SDS may carry a ligand for the AhR that stimulates CYP1A expression, although the possibility that SDS had been indirectly contaminated with PAH cannot be ruled out. Ramachandran et al. (2004) found that Corexit, a commercial surfactant used in spill remediation, was not an inducer for CYP1A expression in rainbow trout (*Oncorhynchus mykiss*). These authors found, however, that exposure to both the

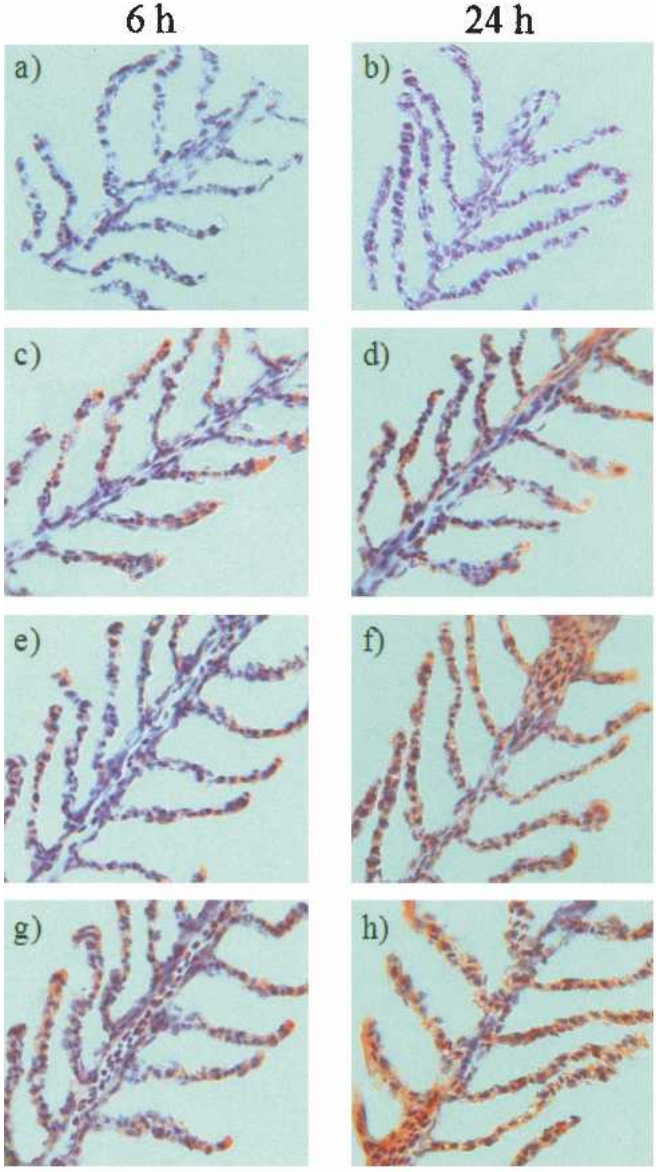

Figure 2. Immunohistochemical detection of CYP1A proteins in the gills of *H. erythrostigma* (control: *a-b*, SDS: *c-d*; crude oil: *e-f*; SDS + crude oil: *g-h*). Intensity of red coloration indicates the degree of CYP1A induction. Induction at 24 h was relatively more intense than induction at 6 h in fish exposed to SDS and crude oil (400 × magnification).

surfactant and PAHs resulted in increased levels of CYP1A catalytic activity (represented by EROD, ethoxyresorufin O-deethylase), because the surfactant increased the availability of PAH in solution. Gagnon and Holdway (2000) also found similar results for Atlantic salmon (*Salmo salar*). Interpretation of the results for SDS + crude oil exposure was complicated by the fact that we did not measure the actual concentrations of PAHs in the treatment solutions and because CYP1A induction was detected in exposure to SDS alone. We therefore cannot assume that increased CYP1A expression in the SDS + crude oil treatment was attributed to the higher bioavailability of PAHs in solution by surfactant action.

Gill CYP1A immunohistochemistry proved to be a sensitive tool to assess CYP1A inducibility. It may take up to 2 days to detect measurable changes in CYP1A induction by measuring EROD activity in the liver (Gagnon and Holdway 2000; Ramachandran et al. 2004), but gill CYP1A is quickly inducible and detectable even in short-term studies (6 to 24 h). Moreover, in small fish, for which liver sampling yields limited amounts of tissue for isolation of microsomes for EROD determination, gill CYP1A immunohistochemistry is a reliable way to estimate the impact of certain waterborne pollutants.

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