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Effects of sequential exposure to water accommodated fraction of crude oil and chlorpyrifos on molecular and biochemical biomarkers in rainbow trout

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ABSTRACT

Fish can be simultaneously or sequentially exposed to various kinds of pollutants, resulting in combined effects. Polycyclic aromatic hydrocarbons induce cytochrome P450 monooxygenase 1A (CYP1A) expression, which catalyzes the conversion of the organophosphorus insecticide chlorpyrifos (CPF) into its most active derivative, CPF-oxon. CPF-oxon inhibits CYP1A and other enzymes, including carboxylesterases (CEs) and acetylcholinesterase (AChE). We studied the effects of an *in vivo* exposure to crude oil water accommodated fraction (WAF) followed by an *ex vivo* exposure of liver tissue to CPF on the expression of *Cyp1a*, *AhR and ARNT* mRNA, CYP1A protein and on the activity of biomarker enzymes in the rainbow trout (*Oncorhynchus mykiss*). Juvenile rainbow trout were exposed to WAF (62 μg L^{-1} TPH) for 48 h. Then, liver was dissected out, sliced and exposed to 20 μg L^{-1} CPF *ex vivo* for 1 h. Liver tissue was analyzed for mRNA and protein expression and for CEs, AChE, glutathione S-transferase (GST) and CYP1A (EROD) activity. WAF induced *Cyp1a* mRNA and CYP1A protein expression by 10-fold and 2.5–8.3-fold, respectively, with no effect of CPF. WAF induced *AhR* expression significantly (4-fold) in control but not in CPF treated liver tissue. *ARNT* mRNA expression was significantly lowered (5-fold) by WAF. CPF significantly reduced liver EROD activity, independently of WAF pre-treatment. CEs activity was significantly inhibited in an additive manner following *in vivo* exposure to WAF (42%) and *ex vivo* exposure to CPF (19%). CPF exposure inhibited AChE activity (37%) and increased GST activity (42%).

1. Introduction

In agricultural areas with growing oil extraction and industry such as those in North Patagonia, Argentina, exposure of aquatic organisms to hydrocarbons can be expected to occur throughout the year, while pesticides may reach dangerous concentrations mainly during the application season (Loewy et al., 2011; Monza et al., 2013). Exposure to a mix of different pollutants can produce combined effects which are challenging to predict due to chemical interactions and effects on detoxification mechanisms (Wassmur et al., 2012).

The North Patagonian region accounts for an important proportion of the Argentine gas and oil production (Monza et al., 2013). Conventional and nonconventional hydrocarbon production activities coincide with the Neuquén River basin (32,450 km^2), which in the lower basin locale includes irrigated areas with fruit production and the main cities of the region. Besides irrigation, the Neuquén River supplies water for nearly 400,000 inhabitants (Monza et al., 2013). Sampled sediments from 17 stations along the Neuquén River, including areas impacted by oil and gas production, agriculture and urban discharges between 2007 and 08 showed low levels of aliphatic hydrocarbons at several stations and almost no polycyclic aromatic hydrocarbons (PAHs) with the exception of naphthalene and pyrene (40 ng/g dw and 50 ng/g dw, respectively) at one site only. However, potentially contaminating activities related to hydrocarbon extraction, transport and processing have been greatly increased since 2010, when a large non-conventional oil and gas reserve (Vaca muerta) was discovered.

PAHs have been extensively studied as contaminants which can affect human and environmental health. These compounds can be present in the environment as a result of oil pollution, petroleum refining, organic material combustion, sewage and industrial discharges,

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vehicle exhaust and also from natural sources, such as forest fires, natural petroleum seepage and volcanism (reviewed by Abdel-Shafy and Mansour, 2016). PAHs can cause a variety of effects including interference with cell membrane functions, teratogenesis, carcinogenesis, mutagenesis and immunosuppression (Davila et al., 1996; Pelkonen and Nebert, 1982; Peluso et al., 2008; Uno et al., 2004). Among other chemicals, some PAHs induce the expression of cytochrome P450 oxidases, particularly those of the cytochrome P450 1A subfamily (CYP1A), which play an important role in the Phase I oxidative biotransformation of xenobiotics. These enzymes metabolize PAHs to epoxides which are highly toxic intermediates but can be detoxified by phase II enzymes such as Glutathione S-transferase (GST) and other transferases, and then excreted by phase III transporters (Baird et al., 2005). In fish ecotoxicology, the subfamily CYP1A is by far the most studied CYP isoform and one of the most studied detoxifying enzymes. CYP1A expression is normally low but is highly induced in fish exposed to several PAHs (Goksøyr et al., 1991; Di Giulio and Clark, 2015). Induction of CYP1A is mediated by the aryl hydrocarbon receptor (AhR) which resides in the cytoplasm linked to chaperone proteins. When AhR is activated by a ligand, it is released from the chaperones and translocated into the nucleus, where it dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR-ARNT heterodimer binds to xenobiotic response elements in the promoter of *Cyp1a* (and many other genes), inducing transcription and protein expression (Denison and Nagy, 2003). Increased levels of CYP1A induce phase I biotransformation of PAHs and other xenobiotics (Whyte et al., 2000). In relation to the effects of the exposure to crude oil hydrocarbons on the AhR pathway, a transcriptomic study by Whitehead et al. (2011) reported up-regulated transcription of *Ahr* and several Ahr targets (*e.g. Cyp1a*, UDP-glucuronosyltransferase; *UGT*) in Gulf killifish (*Fundulus grandis*) from a site impacted by the Deep Water Horizon oil spill.

Besides PAHs, CYP1A can transform xenobiotics, such as organophosphate insecticides (OPs) into more toxic derivatives. For example, in OPs with a thione group (P═S) like Chlorpyrifos (O, O-diethyl-O-3, 5, 6-trichlor-2-pyridyl phosphorothioate; CPF), CYP1A oxidizes the P═S group to the corresponding oxon (P═O); a derivative which is more active and less stable than the parent OP (Fukuto, 1990). Created CPF-oxons inhibit CYP1A activity (Neal, 1980), possibly affecting the metabolism of PAHs and other CYP1A substrates. CPF is a broad spectrum OP, which has caused unintended effects on aquatic organisms by aerial overspray or run-off (Somnuek et al., 2009) and is one of the most widely used insecticides in Argentina (Salgado Costa et al., 2018). CPF is more persistent than other Ops, with a half-life in water ranging from 29 to 74 days (Rivadeneira et al., 2013), which increases the risk of prolonged exposure of aquatic animals. CPF-oxon inhibits β-esterase enzymes, such as acetylcholinesterase and carboxylesterases (AChE, CEs) mostly by stoichiometric binding. AChE is the main target of OPs and other insecticides since its inhibition results in the accumulation of the neurotransmitter acetylcholine in the synaptic space, leading to severe neurotoxicity (Fulton and Key, 2001; Kwong, 2002; Sanchez-Hernandez, 2007; Sogorb and Vilanova, 2002). In general, CEs are more sensitive to OPs than AChE, and protect the organism from anticholinesterase effects by removing OPs through the hydrolysis of ester bonds and by binding to the OP with higher affinity than AChE (Jokanovic, 2001; Maxwell, 1992; Sanchez-Hernandez, 2007; Tang and Chambers, 1999; Wheelock et al., 2005). In addition, CPF and other Ops have been reported to increase oxidative stress and antioxidant responses in various fish tissues (*e.g.* Faria et al., 2015; Ferrari et al., 2007; Guerreño et al., 2016).

There is little information about how previous exposures to PAHs may affect the toxicity of CPF on fish. Clark and Di Giulio (2012) have reported that populations of the Atlantic killifish (*Fundulus heteroclitus*) chronically exposed to PAH down-regulate the AhR pathway and have lower susceptibility to CPF toxicity than reference populations. These authors suggest that, in resistant fish populations, the lack of induction of CYP1A expression reduces the CPF activation exerted by AhR agonists, as observed in the control population. In addition, Clark and Di Giulio (2012) have observed that PAH adapted killifish are more resistant to CPF in the absence of AhR agonists and to other chemicals which are detoxified by CYP1A. Related works reviewed by Di Giulio and Clark (2015) show that, in addition to a recalcitrant AhR, these fish have increased antioxidant capacity and phase II enzyme activity (including GST), and higher expression of the multixenobiotic resistance transporter P glycoprotein (Pgp, ABCB1), which may explain in part the resistance to many unrelated xenobiotics.

The rainbow trout, *Oncorhynchus mykiss*, has been introduced worldwide and is abundant in North Patagonian rivers and lakes where it can be exposed both simultaneously or subsequently to PAHs, inducing CYP1A expression, and to pesticides (such as CPF) which may be activated by CYP1A. The objective of this study is to investigate whether previous exposures to WAFs of crude oil affect *O. mykiss* liver *Cyp1a*, *AhR*, and *ARNT* mRNA expression, and CYP1A protein expression and activity. We also analyze whether the expected augment in CYP1A activity enhances the effects of a subsequent short-term exposure to CPF on CE, AChE, and GST activities in *ex vivo* liver preparations.

2. Materials and methods

2.1. Water-accommodated fraction of crude oil

The WAF was prepared immediately before each experiment, according to Singer et al. (2000), using 4.75 g of crude oil per L of Chimehuin River water (alkalinity 34 mg L^{-1}, conductivity 36 μS cm^{-1}, pH 7.6, 8.37 mg L^{-1} dissolved oxygen at a temperature of 10–12 °C). Crude oil was obtained from the oil spill of an abandoned exploitation, which has not been stopped and continues flowing at present into the La Mina stream, Río Negro Province, Argentina (41°17′21″ S - 71°10′58″ W). The oil sampled from surface seepage was characterized as immature heavy crude oil (Ro = 0.44–0.53%, American Petroleum Institute (API) = 18° and sulfur = 0.45%; Cazau et al., 2005), composed of 33.7% saturated hydrocarbons, 17.8% aromatic hydrocarbons, 5.9% asphaltenes and 42.6% NSOs (compounds with nitrogen, sulfur, oxygen and heavy metals; data provided by YPF S.A. Argentina). Samples were transported on ice to the laboratory of Aquatic Ecotoxicology, Centro de Ecología Aplicada del Neuquén (CEAN, Junín de Los Andes, Argentina) and kept at 4 °C in glass bottles, as recommended for PAH samples before analysis. The obtained WAF was analyzed by the method of the Environmental Protection Agency (USEPA) 3510C- 8015D GC-FID. After liquid-liquid extraction from 1 L, total petroleum hydrocarbons (TPH, C6 to C36, including 16 priority PAHs USEPA, without discrimination, (Supplementary File 1). were determined with a detection limit of 0.002 mg L^{-1}, and a quantitation limit of 0.010 mg L^{-1}. TPH (C6-C36) in WAF were 1.24 mg L^{-1} (CV < 7%). The experimental aquarium water was prepared by diluting WAF at 5% in Chimehuin river water, in order to obtain a nominal TPH concentration of 62 μg L^{-1}. This concentration is similar to the concentrations recorded by Leggieri et al. (2017) from 0 to 1600 m downstream from the oil spill in the La Mina stream, where abundant juvenile rainbow trout can be observed.

2.2. Chlorpyrifos

A standard CPF solution of 20 mg L^{-1} in acetone was prepared by dissolving 1 mg of CPF (O,O-diethyl O-[3,5,6-trichloro-2-pyridyl phosphorothioate], 99% purity, Chem Service, West Chester, Pennsylvania, USA) in 50 mL of chromatographic quality grade acetone (Cicarrelli Reagents S.A, Argentina). The exact concentration of the standard solution was verified by gas chromatography (Agilent 6890 series, Wilmington, USA).