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Authors: Leonardo Ramón Leggieri, Julieta S De Anna, Juan G. Cárcamo, Gerardo A. Cerón, Luis Arias Darraz, Antonella Panebianco, Carlos M. Luquet

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Gills CYP1A of *Oncorhynchus mykiss* as a sensitive biomarker of crude oil pollution in freshwater environments

Leggieri, Leonardo Ramón[1], Julieta S. De Anna[1], Juan G. Cárcamo[2,3], Gerardo A. Cerón[4], Luis Arias Darraz[3], Antonella Panebianco[1] & Carlos M. Luquet[1]

[1]INIBIOMA – CONICET, Argentina

[2] Centro FONDAP, Interdisciplinary Center for Aquaculture Research (INCAR), Chile.

[3] Instituto de Bioquímica y Microbiología, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile

[4]Laboratorio de Zoología, CRUB (UNCo) – CONICET, Bariloche, Argentina

*Corresponding author at: INBIOMA-CONICET, Ruta Provincial N°61 Km 3,
Junín de los Andes, Casilla 8371, Neuquén, Argentina.Tel: +54 02972 413956 Int. 217.
E-mail address: lrleggieri@gmail.com (L.R. Leggieri).

Highlights:

- Petroleum induces CYP1A protein expression in the gills and livers of rainbow trout.
- Petroleum increases the EROD activity in the gills but not in the liver.
- Gill EROD activity could be good biomarker for crude oil in water.

Abstract

The induction of CYP1A activity (EROD) and protein expression was compared in liver and gills of rainbow trout from a stream polluted with crude oil, and through laboratory exposures to 1% and 5% of water accommodated fraction of the crude oil (WAF) for 1 and 4 days. Gills EROD increased 1.6-2.7-fold in fish from the polluted stream and during experiments, while liver EROD was induced only by 1% WAF at day 1 (1.5-fold). Contrastingly, crude oil pollution strongly induced both liver and gills CYP1A protein expression in the field (14-36-fold) and in experiments (4-25-fold). This highlights that crude oil induced CYP1A activity markedly in gills but only slightly or not

at all in the liver, suggesting that differences between organ EROD activities are related to the modulation of CYP1A enzyme activity but not to the regulation at transcriptional or translational levels.

Keywords: CYP450; petroleum pollution; Rainbow trout

1. Introduction

Petroleum hydrocarbons are commonly found in the environment because of widespread crude oil exploration, exploitation and storage. Exposure to crude oil has been associated with disease, recruitment failure, and mortality of aquatic organisms. Since direct measurement of environmental hydrocarbon concentrations generally does not provide a reliable indication of past pollution events, a wide range of biomarkers in fish have proven to be useful for evaluation of aquatic ecosystems health. Crude oil is a complex mixture of many types of compounds, including polycyclic aromatic hydrocarbons (PAH), saturated hydrocarbons, resins, heteropolycyclic aromatic hydrocarbons and heavy metals, which induce liver CYP1A in several species of fish such as rainbow trout *Oncorhynchus mykiss* (Ramachandran et al., 2004), Atlantic salmon *Salmo salar* (Gagnon and Holdway, 1999) and others (Jung et al., 2009; Danion et al., 2014; Frantzen et al., 2015). The expression of CYP1A is constitutively low and is induced by aryl hydrocarbon receptor (AhR) agonists (Uno et al., 2012). Thus, petroleum pollution is usually detected as an increase of CYP1A activity in several fish species (Bucheli and Fent, 1995; Oris and Roberts, 2007; Whitehead et al., 2012).

O. mykiss, in particular, is a useful model for toxicological studies because of its sensitivity to chemicals and because it has been introduced in all kinds of water courses around the world (Bailey et al., 1996; Buhler and Wang-Buhler, 1998). CYP1A catalyzes 7-ethoxyresorufin O-deethylase (EROD), which has become a well-established biomarker of exposure to AhR agonists such as PAH (Clark et al., 2010; Goksøyr and Förlin, 1992; Stegeman and Hahn, 1994). The PAH exposure is routinely estimated by a standardized laboratory bioassay of CYP1A induction in liver (Hodson et al., 1996), which is the main organ for metabolism of toxic oil constituents (Thomas and Rice, 1981).

The gills, primary routes of PAH uptake (Levine and Oris, 1999), have received less attention although they were proposed as more sensitive than the liver when comparing the induction of EROD activity, CYP1A protein or CYP1A transcripts in response to certain PAH

(Abrahamson et al., 2007; Jönsson et al., 2006, 2010). Most recent findings have shown that gills EROD activity can provide a good indication of exposure to petroleum hydrocarbons in marine fishes, such as Atlantic cod *Gadus morhua* (Abrahamson et al., 2008; Holth et al., 2014) and polar cod *Boreoga dussaida* (Nahrgang et al., 2010), indicating that, probably, gills CYP1A metabolizes part of the absorbed PAH before it reaches the kidney and liver (Jönsson et al., 2006).

The main objective of this study was to compare CYP1A activity and protein expression responses to crude oil in liver and gills of juvenile rainbow trout. First, we compared the wild fish response from two field sites, upstream (unexposed) and downstream (exposed) of a Patagonian stream affected by a permanent crude oil spill, which was recently reported (Leggieri et al., 2017). Second, we compared the response of fish experimentally exposed to water-accommodated fractions (WAF) from the same oil spill at 1 and 4 days, in order to examine time and dose-dependent variations.

2. Materials and methods

2.1. Study area

La Mina stream is located in Río Negro, Argentina. The local climate is cold-temperate. There is a point source of pollution that delivers 2-4 liters of crude oil per day to La Mina stream (Leggieri et al., 2017). The oil sampled from the surface seepage was characterized as immature heavy crude petroleum (Ro = 0.44-0.53 %, API = 18° and sulfur = 0.45 %) (Cazau et al., 2005), composed by 33.7% saturated, 17.8 % aromatics, 5.9 % asphaltenes and 42.6 % NSO's (NSO: compounds with nitrogen, sulfur, oxygen and heavy metals; data provided by YPF S.A. Argentina). The field study was conducted in two 300-m-long reaches: upstream-control (41°17'34'' S - 71°11'14'' W, 1011 masl) and downstream-impacted (41°17'21'' S - 71°10'58'' W, 1001 masl) of the oil spill during summer 2015. Fish were represented by a single species, the exotic rainbow trout *O. mykiss*. The upstream reach is located above a 1.4 m high waterfall, 50 m upstream from the spill. This impairs juvenile fish to migrate from the downstream to the upstream reach, but it might not necessarily prevent migration from upstream to the downstream reach. Total Petroleum Hydrocarbons (TPH) concentration detected in water was null (4.5 ± 15.0 µg L^{-1}) in the upstream-control reach and 106.4 ± 51.1 µg L^{-1}in the downstream-impacted reach (Leggieri et al., 2017). TPH concentration in sediments was zero (0.17 ± 0.20 mg g^{-1}) in the upstream-control reach and 3.16 ± 1.55 mg g^{-1} in the downstream-impacted reach (Leggieri et al., 2017).

2.2. Fish samples and treatments

Twelve wild juvenile rainbow trout were caught by net casting and killed by a blow to the head. Liver and gills from four fish were aseptically dissected out and pooled into one sample of each organ, obtaining a total of three pooled samples per organ and per reach. For the experiments, juvenile rainbow trout (3.9 ± 0.8 g, 75 ± 5 mm) were obtained from the CEAN hatchery (Neuquén, Argentina) and acclimatized for 2 days before exposure. Fish were kept in 1.5 L glass fishbowls (4 fish per fishbowl, ~10 g fish L^{-1}) with continuously aerated fresh water from Chimehuin river, at a temperature of 16-18°C and pH 7.4-7.6 (12 h light–12 h dark photoperiod). Treatments consisted of 1 d and 4 d exposure to 1% and 5% WAF in six individual containers per treatment, with the respective controls. Fish were killed and dissected as described above for field samples. Field and laboratory samples were stored with PBS-Triton X-100 in liquid nitrogen for enzyme activity and Western blot analysis, and samples for mRNA expression analysis were stored in RNA-later® (Life Technologies) at −20 °C.

2.3. CYP1A activity

CYP1A activity was determined using the EROD (7-ethoxyresorufin) substrate, as described in (Jönsson et al., 2006). Briefly, 100 mg of liver tissue and 200 mg of gills tissue were homogenized in CaHBSS (Calcium Hank's Balanced Salt Solution; Gibco®) pH 7.8, sonicated twice for 15-30 s on ice, and then centrifuged at 14,000 x *g* for 5 min at 4°C. The 150 µL of assay solution contained 30 µL of sample, 6.25 µM ethoxyresorufin (Sigma-Aldrich), 10 µM dicoumarol (Sigma-Aldrich) and 1 mM NADPH (Sigma-Aldrich) in CaHBSS pH 7.8. The reaction was performed at 30 °C in a 96-well tissue culture microplate with a black flat bottom. Each sample was analyzed in triplicate. Fluorescence readings were monitored every 42 s for 6 min with a BioTek Synergy™ HT Multi-Mode Microplate reader, at excitation and emission wavelengths of 530/525 and 620/640 nm, respectively. Enzyme activity was expressed as pmol of resorufin mg^{-1} protein min^{-1}.

2.4. CYP1A protein expression

Protein extraction was performed as described in (Hasselberg et al., 2008). Approximately 50 mg of liver tissue and 300 mg of gill tissue were homogenized with a pestle and then sonicated for 5-10 s on ice in 1 mL of RIPA buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA) supplemented with Protease Inhibitor Cocktail Set I (Calbiochem) and 200 µM

PMSF. Samples were frozen in liquid nitrogen, sonicated again, and centrifuged at 30,000 x *g* for 30 min at 4 °C. The supernatants were collected and stored at −20 °C. Protein concentration was measured using a BCA kit (Pierce Biotechnology, Rockford, IL). CYP1A protein expression was studied by Western blot. Protein samples from 50 and 100 µg of liver and gills tissue, respectively, were separated by 8% SDS-PAGE for 30 min at 70 V and 90 min at 120 V. Electrophoresis was followed by transference at 1.5 A for 21 min onto a nitrocellulose membrane, using a Trans-Blot Turbo device (BioRad). Non-specific binding sites were blocked for 3 h with 0.1% Casein in 1x PBS. The anti-CYP1A mouse monoclonal antibody (C10-7, Abcam Inc., Cambridge, USA) was diluted 1:3000 for liver and 1:1000 for gills in antibody dilution buffer (0.1 % Tween 20 and 0.1 % Casein in PBS). Anti-β-actin rabbit antibody (Sigma-Aldrich) was diluted 1:3000 for liver and 1:5000 for gills in the same buffer. The nitrocellulose membrane was incubated with primary antibodies overnight at 4°C with slow agitation. The membrane was washed three times with PBS, 0.1% Tween 20 and incubated in the dark with IR Dye 700DX labeled goat anti-rabbit and IR Dye 800CW labeled donkey anti-mouse secondary antibodies (Rockland immunochemicals, Gilbertsville, Pennsylvania) diluted 1:10000 in antibody buffer, for 40 min at room temperature. Membranes were rinsed with washing buffer and the signal was detected by infrared emission at 700 and 800 nm using ODYSSEY® CLx Imager (LI-COR Biotechnology). Images were quantified with the software Image Studio Lite Ver 5.0 (LI-COR Biotechnology). The AccuRuler RGB pre-stained protein ladder (Maestrogen) was used as the molecular mass marker. β-actin was used as a housekeeping protein and loading control. The presence of a single product of 42 kDa was considered a positive result for β-actin protein and a single product of 55 kDa was considered a positive result for CYP1A protein. The relative CYP1A expression was calculated as the CYP1A signal / β-actin signal ratio.

2.5. Data analysis

All statistical analyses were performed using SPSS 11.5 (Inc., Chicago, Illinois, USA), under the License from National University of Luján, Argentina. All data are expressed as the mean ± standard deviation (*n*). The enzyme activity of CYP1A and the CYP1A protein expression (CYP1A/β-actin) data from field samples were analyzed by Student's T-test while data from the laboratory experiment were analyzed by two-way ANOVA. The explanatory variables in the two-way ANOVA were exposure time (1 and 4 d) and WAF concentration (control, 1 and 5%). We performed post hoc comparisons between control and treatments, for both 1 d and 4 d, of all pairs of groups using two-sided significance levels with a Bonferroni adjustment.

3. Results

3.1. CYP1A activity

Trout gill samples, collected downstream of the oil spill, showed 2.6-fold higher CYP1A activity (28.9 ± 1.3 pmol mg_{prot}^{-1} min^{-1}) than those collected upstream (12.7 ± 2.4 pmol mg_{prot}^{-1} min^{-1}; $P < 0.05$), while liver CYP1A activities did not differ between upstream (54.5 ± 8.1 pmol mg_{prot}^{-1} min^{-1}) and downstream reaches (57.1 ± 1.6 pmol $mg_{prot}^{-1}min^{-1}$; Fig.1). In the laboratory study, liver and gills CYP1A activities were affected by WAF concentration, time of exposure and the interaction of both (two-way ANOVA, $P < 0.05$), indicating different effects at different times (Table 2). Liver CYP1A activity increased 1.5-fold with 1% WAF exposure at 1 d (Bonferroni test, $P < 10^{-4}$) and returned to normal values at 4 d. This variable was not affected by 5% WAF. Gill CYP1A activity of trout exposed to both 1% and 5% WAF, at both times, was significantly higher than the respective control values (1.6-2.7-fold, $P < 10^{-4}$), being the intrinsic activities of control and exposed trout higher at 4 d than at 1 d (2.5-fold; Fig.2; Table 1 and 2).

3.2. CYP1A protein expression

In wild trout liver and gills, CYP1A protein expression was 14 and 36-fold higher in downstream individuals than in upstream ones ($P < 0.001$ and $P < 0.05$, respectively; Fig.3). In the laboratory, liver protein expression increased with WAF concentration (4-6-fold with 1% WAF, 9-25-fold with 5% WAF), being more evident at 4 d than at 1 d, without interaction time * WAF (Fig.4.a; Tables 1 and 2). Gill protein expression increased with WAF concentration (5-13-fold with both WAF concentrations) and with time of exposure (1.2-fold, from 1 d to 4 d; Fig.3.b; Tables 1 and 2). The Bonferroni test showed that liver protein expression from the three WAF treatments (control, 1 and 5% WAF) were in separate subsets ($P < 0.001$), and gill protein expressions under 1% and 5% WAF treatment were separated from the control ($P < 0.05$; Table 2).

4. Discussion

Our results show that crude oil induces gill CYP1A in rainbow trout. The significant gill EROD induction observed in the field and laboratory studies in contrast to the minor or null liver-EROD induction indicates that petroleum compounds are better-sensed or perceived in rainbow trout gill cells and possibly partially metabolized. Similar results have been reported in marine fish under crude oil pollution (Abrahamson et al., 2008; Holth et al., 2014; Nahrgang et al., 2010) and in

rainbow trout affected by certain PAHs, such as benzo-(α)-pyrene and indigo (Jönsson et al., 2006, 2004). Some studies suggest that gill CYP1A is more sensitive to polyaromatic hydrocarbons than liverCYP1A when comparing not only the CYP1A activity but also CYP1A protein and transcript levels (Jönsson et al., 2006, 2010).

From our experiments, protein expression of CYP1A was dose-dependent in liver but not in gills, perhaps due to a saturation in the response, and CYP1A transcription was induced after one and four days of exposure to WAF in liver but after only one day in gills. The differences between organs would be related to the conserved physiological function of each organ and the constitutive and inducible expression of each CYP1 form, as it was previously described in *O. mykiss* (Jönsson et al., 2010) and other fish (Jönsson et al., 2007; Zanette et al., 2009). Leggieri and colleagues (2017) found a low increase of mRNA levels, relative to protein levels, under the same crude oil exposition, which could be a reflection of the speed of gene transcription and the mRNA degradation rate (Zhu et al., 2008), as well as the speed of translation of the existing mRNA or in protein stabilization (e.g. Fu et al., 2013; Xing et al., 2014).

The increase in protein levels is not always consistent with the enzyme activity. Here, the response pattern of CYP1A protein expression and CYP1A activity was different. The CYP1A protein expression was strongly induced in trout from the impacted reach of La Mina stream in both liver (14-fold) and gills (36-fold) and it showed a clear dose-dependent induction upon WAF exposure in the laboratory in liver (4-25-fold and 5-13-fold, for liver and gills, respectively). In contrast, a high proportion of induced liver CYP1A proteins remained with basal enzymatic activity. These differences could be related to the negative modulation of CYP1A enzyme activity rather than regulation at transcriptional or translational levels. Liver CYP1A activity would be inhibited by oxidative stress, an excess of substrate contaminants or components from the complex mixture of crude oil, as heteropolycyclic aromatic hydrocarbons or metals (Goksøyr, 1995; Viarengo et al., 2007). Alternatively, the inconsistency between protein levels and enzyme activity could be related to the CYP1A activity measurement technique, which is typically assessed as microsomal EROD activity, while the CYP1A protein is assessed as total levels (e.g. Cárcamo et al., 2014). Also, the induction of CYP1A by PAHs requires de novo protein synthesis, affecting the enzyme synthesis, but not necessary the enzymatic activity already existent. However, the specific reason why the up-regulation of the expression of CYP1A in our study was not reflected in the enzyme EROD activity of CYP1A needs to be further studied.

Quick gill metabolism of hydrocarbons (Jönsson et al., 2006) or liver damage (Gagnon and Holdway, 2000) would determine relatively low levels of CYP1A protein but, in our study, those levels remained high. Taken together, these results extend the findings of Jönsson and colleagues (Jönsson et al., 2006, 2010) about the positive relationship between gills CYP1A activity and PAH, supporting that CYP1A activity in rainbow trout gills is a more sensitive biomarker for crude oil pollution in water than liver CYP1A activity.

Conclusions

In conclusion, gills CYP1A activity was induced by crude oil in the field rainbow trout and by a water-accommodated fraction at one and four days. The results point out that petroleum compounds could be partially metabolized in gill cells of rainbow trout, and that even though a high proportion of CYP1A liver proteins are induced, the liver enzymatic activity remained at basal levels. Finally, the results also show that gill CYP1A activity could be a sensitive biomarker for crude oil in water.

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Figure captions:

Fig.1. CYP1A activity (pmol min^{-1} per mg of protein) in liver and gills of rainbow trout, using EROD substrate, upstream and downstream of crude oil point discharge. Values are expressed as mean ± SD (*n* = 3; *** P < 0.05).

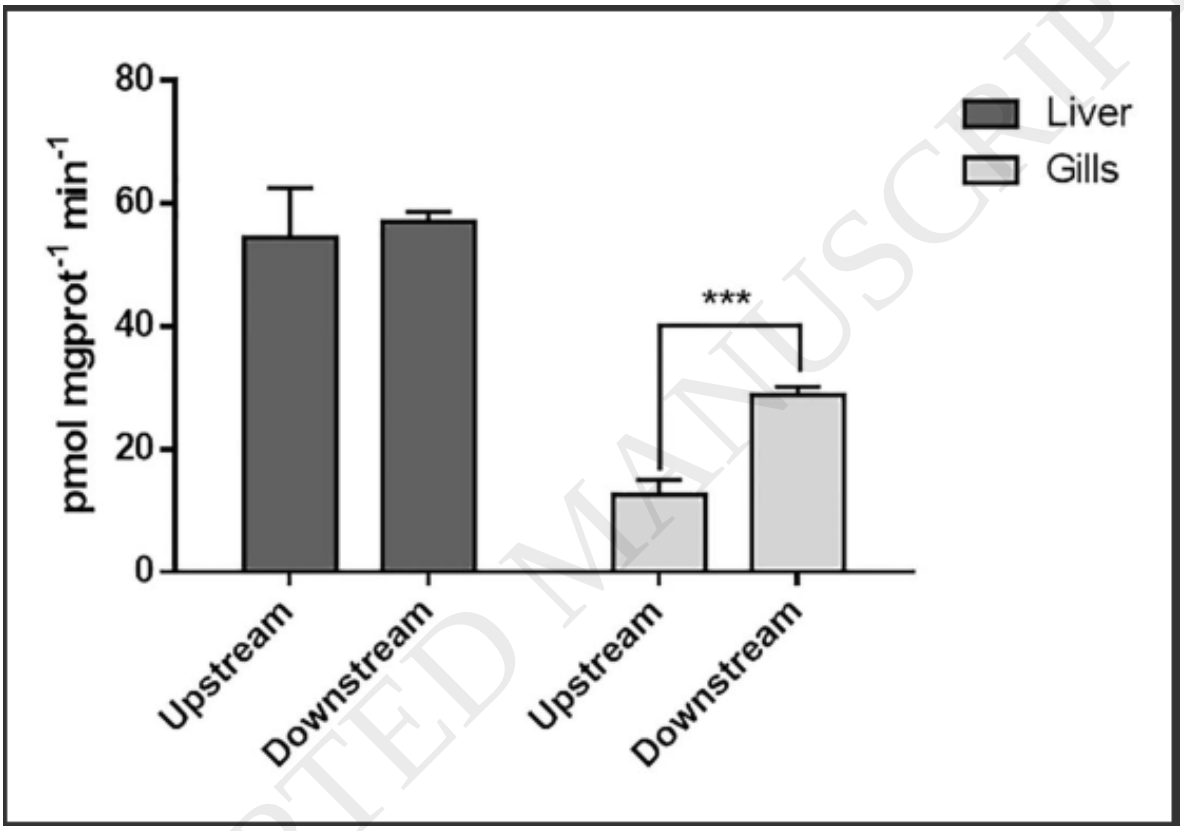

Fig.2. CYP1A activity (pmol min^{-1} per mg of protein) in liver and gills of rainbow trout, using EROD substrate, after 1 day and 4 days of exposure to 1% WAF and 5% WAF. Values are expressed as mean ± SD (*n* = 6).

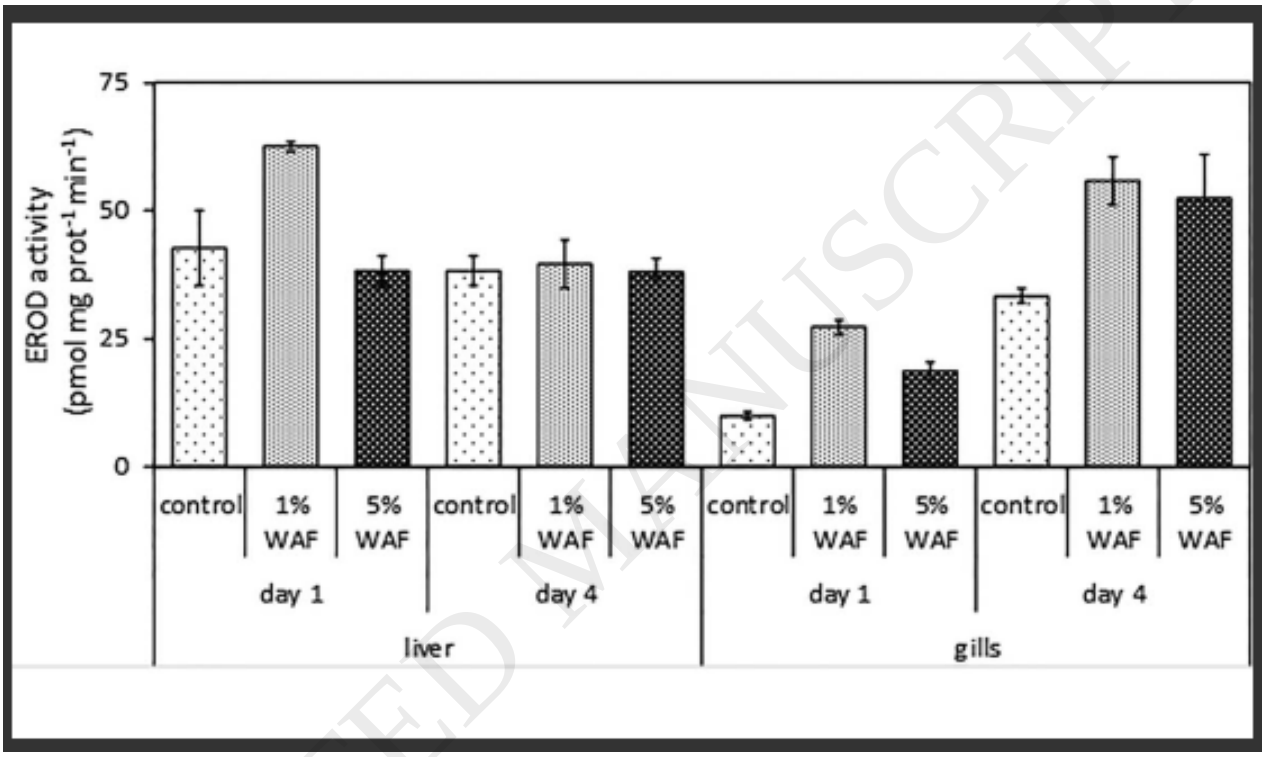

Fig.3. Representative polyacrylamide gels showing the up-regulation of the expression of protein CYP1A (relative to β-actin) in liver and gills of wild rainbow trout from downstream reach to the oil spill in La Mina stream, relative to trout from upstream samples (*Student's t-test*, ** $P < 0.001$, * $P < 0.05$).

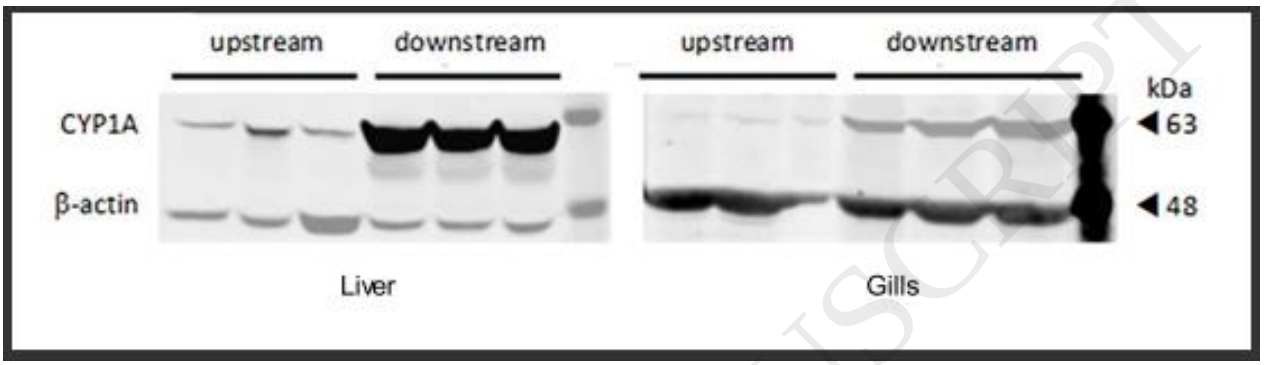

Fig.4. Expression of protein CYP1A in liver (a) and gills (b) of rainbow trout *O. mykiss* after 1 and 4 days of exposure to 1% WAF and 5% WAF (water accommodated fraction prepared from crude oil obtained from La Mina spill). Fold-change values, relative to control, are shown above the polyacrylamide gels (3 out of 6 replicates are shown here).

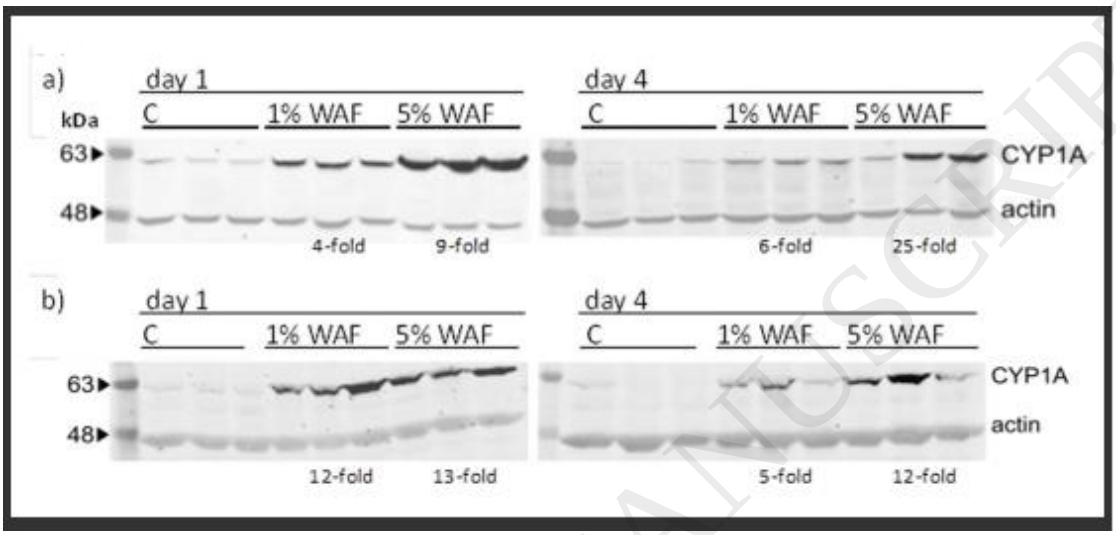

Table 1. Fold-change in activity (CYP1A) and protein expression (CYP1A) of rainbow trout *O. mykiss* liver and gills after 1 and 4 days of the exposuresto 1% WAF and 5% WAF (prepared from the crude oil obtained in La Mina spill). Values are mean ± SD (*n* = 6). Asterisks denote significant differences among treatments within each time relative to control (*P < 0.05 and **P < 0.001), and lowercase letters denote the difference between times of exposure from Bonferroni test (P< 0.05).

		Liver								**Gills**							
Parameter		1% WAF				5% WAF				1% WAF				5% WAF			
CYP1A activity	1 d	1.46	±	0.02	*	1.03	±	0.12		2.71	±	0.15	**	1.67	±	0.14	*
	4 d	0.89	±	0.07		1.00	±	0.06		1.87	±	0.17	*	1.57	±	0.26	*
CYP1A protein	1 d	3.67	±	0.06	**[a]	8.82	±	1.20	**[b]	11.98	±	6.56	*	12.79	±	3.96	*
	4 d	5.53	±	0.81	**[a]	25.31	±	8.15	**[b]	5.25	±	1.89	*	11.88	±	6.64	*

Table 2. WAF concentration, time, and interaction effect on trout CYP1A activity and CYP1A protein expression after crude oil WAF treatment in liver and gills. Asterisks indicate * P < 0.05 and ** P < 0.001 (two-way ANOVA *test*) of significant components of each model.

		Liver			**Gills**		
Trait	**Source**	***F***		**Relative contribution**	***F***		**Relative contribution**
CYP1A activity	WAF	6.7	*	15%	105.9	**	79%
	time	19.9	**	22%	18.5	*	7%
	Interaction	21.7	**	49%	13.4	**	10%
CYP1A protein	WAF	154.7	**	69%	17.6	**	59%
	time	123.8	**	28%	10.0	*	17%
	Interaction	1.7		1%	1.0		3%