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In vivo effects of the soluble fraction of light cycle oil on immune functions in the European sea bass, *Dicentrarchus labrax* (Linné)

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ABSTRACT

Hydrocarbons are major contaminants that may affect biota at various trophic levels in estuaries and coastal ecosystems. The effects of accidental pollution by light cycle oil (LCO), a refined product of heavy fuel oil, on bioaccumulation, depuration processes and immune-related parameters in the European sea bass, *Dicentrarchus labrax*, were investigated in the laboratory after 7 days of exposure and a 2-week recovery period. Exposure of fish to the soluble fraction of LCO (1600 ng L^{-1}) for 7 days led to the bioaccumulation of some polycyclic aromatic hydrocarbons (PAHs) in muscles: naphthalene, acenaphthene, fluorene, phenanthrene and anthracene. After 7 days of recovery period, half-elimination of naphthalene was reported in fish muscles due to facilitated diffusive loss by the epithelium and a faster elimination rate proven by the presence of a high level of naphthalene biliary metabolites. The other bioaccumulated molecules displayed a slower depuration rate due to their elimination by the formation of hydrophobic metabolites excreted through bile or urine. Three days after the beginning of the recovery period, each contaminated fish showed severe external lesions (tissue necrosis, suppurative exudates, haemorrhagic area). The hypothesis of a possible link with inflammatory phenomenon was supported by (i) an inversion of the leucocyte sub-population percentage, (ii) a significant up-expression in the spleen of the tumour necrosis factor alpha gene, (iii) a significant increase in ACH_{50}. Moreover, the lack of C3 gene regulation in the spleen suggested a non-renewal of this component. The reduction of phagocytic activity and lysozyme concentration reflected immune suppression. Finally, LCO toxicity in this fish was clearly demonstrated to be related to inflammatory reaction and immune depletion.

1. Introduction

Light cycle oil (LCO) corresponds to middle distillates at atmospheric pressure (144–404 °C) of cracked molecules, the lightest products being used to form gasolines (Poveda Jaramillo et al., 2004). LCO refined products are rich in polycyclic aromatic hydrocarbons (PAHs), aromatic sulphurs and nitrogen compounds, and possess a lower cetane number and higher density (Xu et al., 2003; Nylen et al., 2006). Their composition in lower molecular weight PAHs explains their wide usage by some refiners at the end of the fluid catalytic cracking process. In fact, LCO enables the fluidification of residues in order to facilitate their transportation by tankers (Ding et al., 2007), to reduce heavy oil production and to increase value-added products (Xu et al., 2003). All these uses raise the environmental risk of LCO pollution by tanker, home fuel and refinery accidents. So, these different types of accidental pollution could induce the detection of LCO, which is essentially composed of PAHs noted by the United States Environmental Protection Agency (US-EPA) as priority pollutants (US-EPA, 1998), in the aquatic environment and organisms including fishes.

The bioaccumulation process is due to different mechanisms: direct uptake from water by gills, skin or digestive tract and uptake of suspended particles due to ingestion and the consumption of contaminated food (Van der Oost et al., 2003). PAHs are generally bioaccumulated in specific organs, such as lipid-rich tissues (e.g. muscle). After biotransformation and detoxification in liver, these PAH metabolites are found in the gall bladder where

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they are excreted via bile (Van der Oost et al., 2003). These biliary metabolites provide a good assessment of fish contamination by hydrocarbons (Goanvec et al., 2004). Despite their rapid excretion, exposure to PAHs induce physiological disturbances, particularly to the immune system (Ribeiro et al., 2005).

For immunotoxicological risk assessment studies on mammals, innate immune response may be suppressed by xenobiotics (Luster et al., 1988). This innate immune response is part of the first line defence in the immune system of organisms acting against pathogens without prior exposure to any particular microorganism. Phagocytosis, which helps to prevent infectious diseases by non-specific cellular reactions, has been described as an attractive biomarkers of hydrocarbon pollution in fish (Reynaud and Deschaux, 2006). Lysozyme, which is found in many fish tissues and secretions, has been most frequently examined in fish plasma or serum for toxicology assays (Reynaud and Deschaux, 2006). Despite its key role in the defence system and the direct cleavage of the C3 component by diesel exhaust particle extracts in humans (Kanemitsu et al., 1998), the impact of pollutants on the complement system has been barely investigated in fishes (Bado-Nilles et al., 2009a, 2009b). The impacts of pollutant on tumour necrosis factor alpha, a pro-inflammatory cytokine, have been briefly described previously with pesticides (Sato et al., 1998) and hydrocarbons (Ushio et al., 1999).

The aim of the present work was to carry out experimentation in laboratory-controlled conditions with regard to the effects of accidental LCO pollution on bioaccumulation, depuration processes and immune-related parameters in the European sea bass, *Dicentrarchus labrax*. The capacity of adult European sea bass to recover their initial status after 7 days of exposure to a soluble fraction of LCO was monitored by chemical and immunological analysis. Bioaccumulation and depuration processes were performed by gas chromatography coupled with mass spectrometry (GC/MS) and biliary metabolites were quantified by fluorescence analysis. Immune-related parameters were studied by flow cytometry, based on leucocyte mortality, leucocyte sub-population percentages and phagocytic activity, and by spectrophotometry for plasmatic lysozyme concentration and haemolytic activity of the alternative complement pathway (ACH_{50}). For these flow cytometry analysis, peripheral blood leucocyte was used due to (i) their capacities to go in each organ; (ii) their potential direct contact with PAHs after skin penetration; (iii) their concentration which permit analysis of each immune parameter in the same organ. Finally, two immune related genes associated with ACH_{50} regulation (complement component C3) and the inflammatory process (tumour necrosis factor alpha) were monitored by real-time PCR in spleen tissue due to the capacities of this organ to filter plasma, trap blood-borne substances and enrich blood on new immune cells (Press and Evensen, 1999). All parameters were also monitored after a recovery period of 2 weeks. So, this study search for the first time the impact of a refined hydrocarbon product detected during oil spill, the LCO, on fish health. Moreover, some information about toxicity mechanisms on immune system was provided.

2. Materials and methods

All experiments were conducted in accordance with the Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. Cedre is authorised to conduct experimentation on animals in its capacity as a certified establishment, according to the administrative order no. 2006-0429 dated 9 May 2006. Furthermore, the experimentation carried out as part of this study was conducted under the responsibility and supervision of Dr. Stéphane Le Floch, who holds a certificate awarded by the National Veterinary School of Nantes entitling him to direct scientific experimentation on animals.

2.1. Sea bass

The 120 European sea bass, 136 ± 32 g, used for this experiment came from one pond (aquaculture facility: Ecloserie Marine de Gravelines, France) and were raised to juvenile stage in an experimental facility at Anses (French Agency for Food, Environmental and Occupational Health and Safety, Plouzané site, France). Two weeks before the beginning of exposure, the fish were transferred to the facilities at *Cedre* (Centre of Documentation, Research and Experimentation on Accidental Water Pollution in Brest, France) for 2 weeks of acclimation.

2.2. Pollutant

A LCO, commonly used by tankers and given by Total Donges Refinerie, was selected to perform the exposure. This LCO contained 94–97% of light catalytically cracked distillate, 2–4% of polar compounds and 1–2% of sulphurs. The LCO contained 10 lower molecular weight PAHs out of the 16 PAHs listed by the United States Environmental Protection Agency (US-EPA, 1998): naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene and benzo[*a*]anthracene at concentrations ranging from $33 \pm 5\ \mu g\ g^{-1}$ for benzo[*a*]anthracene to $9204 \pm 22\ \mu g\ g^{-1}$ for phenanthrene (Table 1).

2.3. Experimental design

2.3.1. Experimental system

The experimental system was adapted from Anderson et al. (1974) and modified in order to obtain a stable concentration of pollutant throughout experiments (Fig. 1). This system, placed in a thermoregulated greenhouse ($14 \pm 1\ ^{\circ}C$), used six similar, independent units. Each unit (616 L) was composed of one rectangular mixing tank (316 L), one cylindrical exposure tank (300 L) and one degassing column. The mixing tank, which generated the soluble fraction of contaminant, was partitioned to prevent the formation of small oil slicks and suspended droplets during contact between the LCO and the aqueous phase. The exposure tank, in which the organisms were placed, was directly connected to the mixing tank to obtain and maintain a stable LCO concentration. This equipment enable marine organisms to be exposed only to the dissolved fraction of contaminant. A degassing column was installed between the exposure and mixing tanks in order to promote gaseous exchange and to maintain the level of oxygen around 96%. After contamination, the exposure tank was disconnected from the mixing tank. A flow of fresh seawater was supplied at a rate of $0.3\ m^3\ h^{-1}$ to filter the seawater in the exposure tank and the system was connected to a water overflow recovery system (Fig. 1).

2.3.2. Experimental setup

To obtain the water soluble fraction, 3 L of LCO were poured off the water surface of the mixing tanks which were connected to the exposure tanks. Two weeks contact between LCO and seawater on closed circuit were necessary to obtain a stable oil concentration in the exposure tank (Fig. 1). Meanwhile, the fish were acclimated in a 1200 L tank with a flow of $0.3\ m^{3\ -1}$ from Day (-14) to Day 0 (Fig. 2). The sea bass were fed once a day at 1% body weight with dry commercial pellets (Grower Extrude Natura 4 mm, Le Gouessant Aquaculture, France).

To start the contamination period, 90 out of 120 fish were randomly distributed into three control units and three contaminated units on the basis of 15 fish per tank (Fig. 1). Seven days of on closed circuit, from Day 0 to Day 7 (Fig. 2), was chosen due to several reasons: (i) after an oil spill, time before intervention could be very variable, from one day to several months in function of oil spill location and/or of field natural purification; (ii) sea bass, which are subjected to constant contact with pollutants by their skin, have an immune modulation from 5 days of contact with HFO (Bado-Nilles et al., 2009a); (iii) a lapse time of more than 5 days was great to inhibit handling stress (Ortuño et al.,

Table 1
Concentration of 10 priority US-EPA PAHs in light cycle oil (LCO). PAH detection was performed by gas chromatography coupled with mass spectrometry (GC/MS). The results are expressed in $\mu g\ g^{-1}$, $n=3$, values are means $\pm$ standard error.

Name of 10 PAHs US EPA	Molecular weight ($g\ mol^{-1}$)	Concentration ($\mu g\ g^{-1} \pm SE$) in LCO
Naphthalene	128.2	3761 ± 49
Acenaphthylene	152.2	241 ± 4
Acenaphthene	154.2	1017 ± 12
Fluorene	166.2	1732 ± 9
Phenanthrene	178.2	9204 ± 22
Anthracene	178.2	524 ± 8
Fluoranthene	202.3	843 ± 11
Pyrene	202.3	801 ± 35
Chrysene	228.3	100 ± 10
Benzo[*a*]anthracene	228.3	33 ± 5