Multi-metallic contamination around former uranium mines induces adverse effects and acclimation disturbance in three-spined stickleback (*Gasterosteus aculeatus*)

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Introduction

Uranium extraction is a worldwide activity during which thousands of tons of ores can be recovered, but also million tons of tailings and waste rocks are stored on mine sites, also containing uranium and its decay products. These activities result in a uranium contamination of surrounding aquatic ecosystems. In addition to this uranium contamination, the aquatic environment can present significant concentrations of heavy metals, naturally occurring due to geographic location (iron and manganese) or coming from anthropogenic activities (aluminium and barium used to precipitate uranium and its decay products). This multi-metallic contamination impacts all compartments of the ecosystem, including the aquatic environment, last refuge of heavy metals.[1] This is the case in the Haute-Vienne department (Limousin, France), where almost a quarter uranium mines of France were in activity in the twentieth century. In this department, we worked on one former mine area. Several ponds were identified: Pontabrier, a pond which receives wastes and residues from two former uranium mines and two references ponds without uranium [Malessard and Jonchère Saint-Maurice (JSM)]. Heavy metals are known to be toxic for aquatic organisms, including fish. Immune system, antioxidant system and also DNA can be impaired by these pollutants.[2-4] The presence of this multi-metallic contamination shows the importance to evaluate the environmental risk linked to former uranium mines in France.

For this purpose, three-spined sticklebacks were chosen for *in situ* caging experiments. This fish species was selected because of its small size, important criterion for caging, and because it is usually adopted in ecotoxicological studies.[3,5] The aims of this study was: i) to assess several biological responses of three-spined sticklebacks to this multi-metallic contamination; and ii) to observe their acclimation capacities after lipopolysaccharide injections (biological stress).

Materials and Methods

After two weeks of acclimatization in laboratory to environmental conditions, 90 sticklebacks were caged in the Pontabrier, Malessard and Jonchère Saint-Maurice ponds (15 fish per cage; 2 cages per pond). Fourteen days after caging, fish were anesthetized with MS222 (0.1 g/L) and intraperitoneal injections of lipopolysaccharides (LPS; 9 mg LPS/kg fish total weight) or its solvent (phosphate buffer saline) were performed in order to induce a biological stress. Immediately after injections, sticklebacks were returned into cages for four days. Then, sticklebacks were sacrificed in order to recover organs. Spleen and total blood were recovered for analyze immune responses and DNA damage respectively. Total and dissolved water fractions from each pond were collected for assessment of levels of metals, organic and inorganic carbon, major cations and anions, pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), volatile organic compounds (VOCs) and cyanobacteria. Finally, pH and conductivity were recorded at T0, T14 and T18 while temperature was continuously recorded (hourly) with a probe.

Fish weight and size were recorded in order to evaluated the general well-being of fish by the Fulton's K condition index (K=100 x total weight (g)/size $(cm)^3$).[5] Immunomarkers were analyzed by flow cytometry after grinding the spleen through sterilized nylon mesh (40 µm). Necrosis, apoptosis, leucocytes distribution, lysosomal membrane integrity (LMI), ROS (Reactive Oxygen Species) production and phagocytic capacity were recorded using a Guava easyCyte™ cytometer.[6] Finally, DNA damage were assessed using alkaline comet assay on blood erythrocytes, according to protocol used by Santos *et al.*[7]

Results and Discussion

Multi-metallic contamination

Concerning metal concentrations in water, all major metals (U, Al, Mn, Fe, Ba) were more concentrated in Pontabrier pond compared to Malessard and Jonchère Saint-Maurice ponds, especially for uranium (Table 1). Iron, aluminium and manganese concentrations were over the predicted no effect concentrations (PNEC)[8-12] for each pond (Table 1). Others metals were present in lower quantities (under their respective PNEC: Cr, Pb, Cd, Zn, Cu, Ni, Co). PAH, PCB, VOC, pesticides and cyanobacteria in water were under the detection limits (*data not shown*).

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Effects of chemical stress on stickleback

Fulton's K condition index, in laboratory conditions, is around 1. K of sticklebacks caged in the Pontabrier pond was at 0.83±0.08, whereas it was significantly higher (0.97±0.12 and 0.91±0.07) for the two other ponds (*data not shown*). Concerning immune biomarkers, leucocyte necrosis was lower and basal ROS production was higher in fish caged in Pontabrier pond compared to other ponds (*data not shown*). Integrity of lysosomal membrane was lower in the Jonchère Saint-Maurice pond than in the Pontabrier pond and even more than in the Malessard pond (*data not shown*). No significant difference was observed for phagocytosis capacity and apoptosis between ponds for PBS injections. Finally, sticklebacks caged in the Pontabrier pond presented the lowest level of DNA damages (*data not shown*).

DNA damage compared to fish in the other two ponds (*data not shown*), but affected some markers of immune system. Indeed, leucocytes necrosis and apoptosis were enhanced when LPS were injected in fish (*data not shown*). In the same way, LMI decreased after LPS injection compared to PBS injection (*data not shown*). These results show that LPS alter defense system of sticklebacks. Nevertheless, phagocytosis increased after LPS injection, which could demonstrate an enhancement of defense system towards this stress (Figure 1). Analysis of DNA damages showed an increase of DNA alterations with LPS (*data not shown*).

When fish were located in the water with the lowest metal concentrations (Jonchère Saint-Maurice pond), the effects of LPS were more pronounced. Indeed, the immune-stimulation induced by LPS induced greater increases in DNA damage, leukocyte apoptosis and phagocytosis than in the two other ponds (Figure 1). This sug-

Table 1. Concentrations (µg/L) and predicted no effect concentrations (PNEC) of major metals found in the Haute-Vienne ponds on dissolved water fraction. Bold concentration values are higher than values of their respective PNEC.

PONDS	U µg/L	Al µg/L	Fe µg/L	Mn µg/L	Ba µg/L
Po	29.08	79.3	319.8	37.6	53.3
Ma	0.18	59.3	205.8	23.9	41.1
Js	0.03	24.3	274.3	21.6	31.3
PNEC	0.3	0.06	16	15	60

U, uranium; Al, aluminium; Fe, iron; Mn, manganese; Ba, barium; Po, Pontabrier; Ma, Malessard; Js, Jonchère Saint-Maurice ponds.

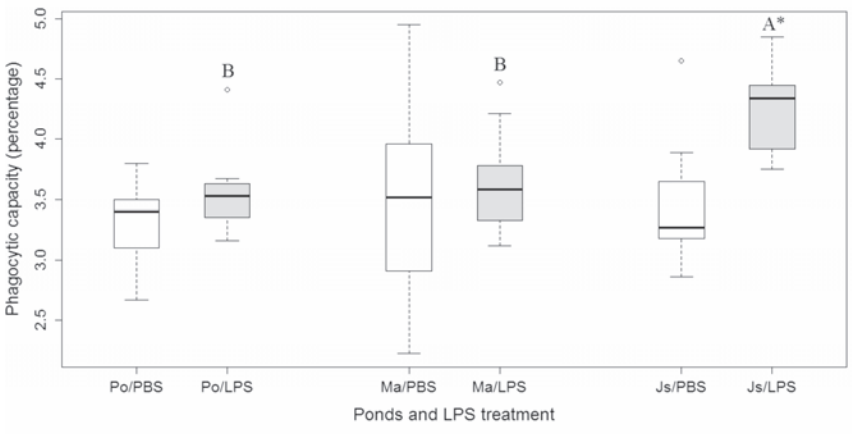

Figure 1. Phagocytic capacity of sticklebacks caged in the Haute-Vienne ponds with (grey) and without (white) injection of LPS. Po, Pontabrier, Ma, Malessard; Js, Jonchère Saint-Maurice ponds. A, B: significant differences between ponds for the same biological stress (LPS), A>B. *Significant difference between PBS and LPS conditions in a same pond.

gests that the higher multi-metallic contaminations in the Pontabrier and Malessard have attenuated LPS-induced effects.

In our study, fish exposed to more polluted sites did not respond to LPS-stimulation, which could indicate that these fish could be susceptible to infections. Other studies revealed the same susceptibility of fish after metal exposure.[13,14] As many authors have identified confounding factors, represented by environmental factors (pH, conductivity and temperature) and/or physiological parameters, can have impacts on biomarkers,[15,16] another experiment will be conducted in the fall (outside breeding season) in order to address the interaction between biological responses and several confounding factors.

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