RESEARCH/REVIEW ARTICLE

Heavy metal contamination and hepatic toxicological responses in brown trout (*Salmo trutta*) from the Kerguelen Islands

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Abstract

The Kerguelen Islands include various species of freshwater fish such as brown trout (*Salmo trutta*). These trout are among the most isolated from direct anthropogenic impact worldwide. This study was designed to analyse cadmium (Cd) and copper (Cu) concentrations in the liver of Kerguelen brown trout, and to assess the possible impacts of these metals on hepatic histopathology and oxidative stress parameters (superoxide dismutase and catalase activity and glutathione levels). Trout were caught in the Château River, the Studer Lakes and the Ferme Pond, close to the scientific station of the Kerguelen Islands, corresponding to three morphotypes (river, lake and station). Kerguelen trouts' hepatic concentrations of Cd and Cur were similar to those reported in previous studies in salmonids populations from areas under anthropological impacts. Clear hepatic disturbances (fibrosis, nuclear alteration, increased immune response, melanomacrophage centres [MMCs]) were observed in all tested trout. A similar histo-pathological trend was observed among the trout from the three morphotypes but anti-oxidative responses were higher in the trout from the "station" morphotype. Hepatic alterations and the presence of MMCs in the livers of Kerguelen brown trout may be related to the high levels of Cd and Cu measured in this fish at all sampling sites.

Pollution of aquatic ecosystems by different chemical pollutants of natural and anthropogenic origins such as persistent organic pollutants or heavy metals, such as cadmium (Cd), copper (Cu) and zinc (Zn), has an impact on individuals and populations that live in those ecosystems. At sub-lethal concentrations, these pollutants cause a general stress syndrome associated with several physiological disturbances in fish (Sanchez et al. 2005; Paris-Palacios & Biagianti-Risbourg 2006). Fish concentrate and integrate all chemical pollutants from their living environment, either by direct uptake from water or suspended materials, or by biotransfer from trophic input (Van der Oost et al. 2003). The interactions between these chemicals and biological systems give rise to biochemical disturbances (Gül et al. 2004). Fish health status is therefore usually used in ecotoxicological studies as an integrative indicator to describe the adverse effects of the mixtures of chemicals that exist in aquatic ecosystems (Van der Oost et al. 2003; Fernández et al. 2006). For example, some biochemical parameters in fish liver tissue, such as antioxidant enzyme activation, are considered to be sensitive biomarkers that can indicate the presence of toxicants before hazardous effects occur in aquatic organisms (Gül et al. 2004; Cazenave et al. 2014). Biomarkers are defined as observable and/or measurable modifications at the molecular, biochemical, cellular, physiological or behavioural levels that reveal the present or past exposure of an organism to at least one

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xenobiotic (Lagadic et al. 1997). At a low organization level, biomarkers are used for the early detection of pollutant effects on aquatic organisms. Among the various biomarkers, hepatic ones (liver histopathology, oxidative stress, etc.) have been used to evidence physiological stress induced by different contaminants (Schwaiger et al. 1997; Paris-Palacios et al. 2000; Schwaiger 2001). Indeed, the liver is a key organ involved at different levels of fish normal physiological functions, and it plays a crucial role in bioaccumulation and detoxification processes (Biagianti-Risbourg 1990; Carrola et al. 2009). It can be structurally and functionally disturbed in response to chemical stressors (Schmidt et al. 1999). However, fish hepatic physiology is also affected by several natural or human-induced environmental changes, such as temperature, salinity and so on. The interaction of multiple stress sources is associated with acclimation or the appearance of degenerative syndromes (Bernet et al. 2004). Additionally, the anthropogenic degradation of ecosystems most frequently results in the modifications of physical (habitat degradation) and chemical (e.g., pH, O_2, pollutants) parameters. The real impact of chemicals on fish in field studies is therefore difficult to estimate (Bernet et al. 2004). For this reason, the use of a multiparametric approach based on a set of complementary biomarkers is commonly applied in environmental biomonitoring programmes (Mayon et al. 2006; Sanchez et al. 2007).

The Kerguelen Islands (49°S, 70°E) are situated in the northern part of the Southern Ocean. The human population on this island averages around 60–100 non-permanent scientists who live exclusively in the French scientific station of Port-aux-Français. From 1955 to 1992, salmonid species, mainly brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*), were introduced into the Kerguelen freshwater systems, and now constitute well-acclimated populations (Davaine et al. 1997). These two species are now probably among the most isolated freshwater fish species worldwide, and less subject to direct human influence. But despite geographical isolation, previous studies showed high Cd and Cu concentrations in liver and muscle tissues of river brook trout (Jaffal, Paris-Palacios et al. 2011). Additionally, other studies have shown that some marine organisms, octopuses and fish, living around the Kerguelen's Islands are also contaminated with Cd (Bustamante et al. 1998; Bustamante et al. 2003).

This study investigated the consequences of Cd and Cu bioaccumulation on some selected hepatic biomarkers in Kerguelen brown trout. In addition to histological studies, heavy metal contents and enzymatic and non-enzymatic oxidative stress responses—superoxide dismutase (SOD) activity, catalase (CAT) activity and glutathione (GSH) levels—were analysed in brown trout (*Salmo trutta*). These biomarkers were selected because they are part of the most relevant markers of stress in fish experimentally exposed to chemicals (e.g., pesticides and heavy metals; Paris-Palacios et al. 1998, 2000; Sanchez et al. 2007).

Earlier analyses of the salmonid's otolith shape had demonstrated that lakes, rivers and ponds close to the scientific station of Kerguelen contain salmonid populations belonging to three distinct morphotypes (Morat et al. 2008). The three morphotypes are associated with geographic isolation and behavioural variability in closed or running freshwater and to subsequent genetic evolution from the same "French" genetic pool (Bruslé & Quignard 2001; Morat et al. 2008). Three different brown trout morphotypes ("river," "lake" and "station") were taken into account in this study.

Materials and methods

Study area

The Kerguelen Islands are an archipelago of approximately 7000 km^2, located in the Southern Ocean (49°S, 70°E). These sub-Antarctic islands are more than 3300 km from the nearest inhabited country (Fig. 1a). The cold sub-Antarctic climate is characterized by a mean annual temperature of +4.5°C. The freshwater hydrographic system consists of rivers, ponds and lakes (Fig. 1b) containing simple trophic webs with only a few species of micro- and macro-invertebrates and no endemic ichthyofauna. Human activities are exclusively localized around the scientific station.

Sites and sampling procedure

Brown trout (*Salmo trutta*, $n = 112$) were caught by angling in three different sites localized in Courbet Peninsula: the Château River, the Studer Lakes (considered as one site) and, close to the scientific station Port-aux-Français, Ferme Pond, (Fig. 1c). Each site was characterized by one trout morphotype (river, lake or station morphotype, respectively) according to Morat et al. (2008). During the year 2006, monthly sampling campaigns were achieved to collect fish from each site.

The fish were immediately killed after angling by the dislocation of their neural axis, and the morphological parameters of each of them were determined in the field. The liver tissues were dissected and samples were immersed in a fixative medium for histology, whereas other portions of the liver were immediately stored at −20°C for chemical and biochemical analyses, considering the

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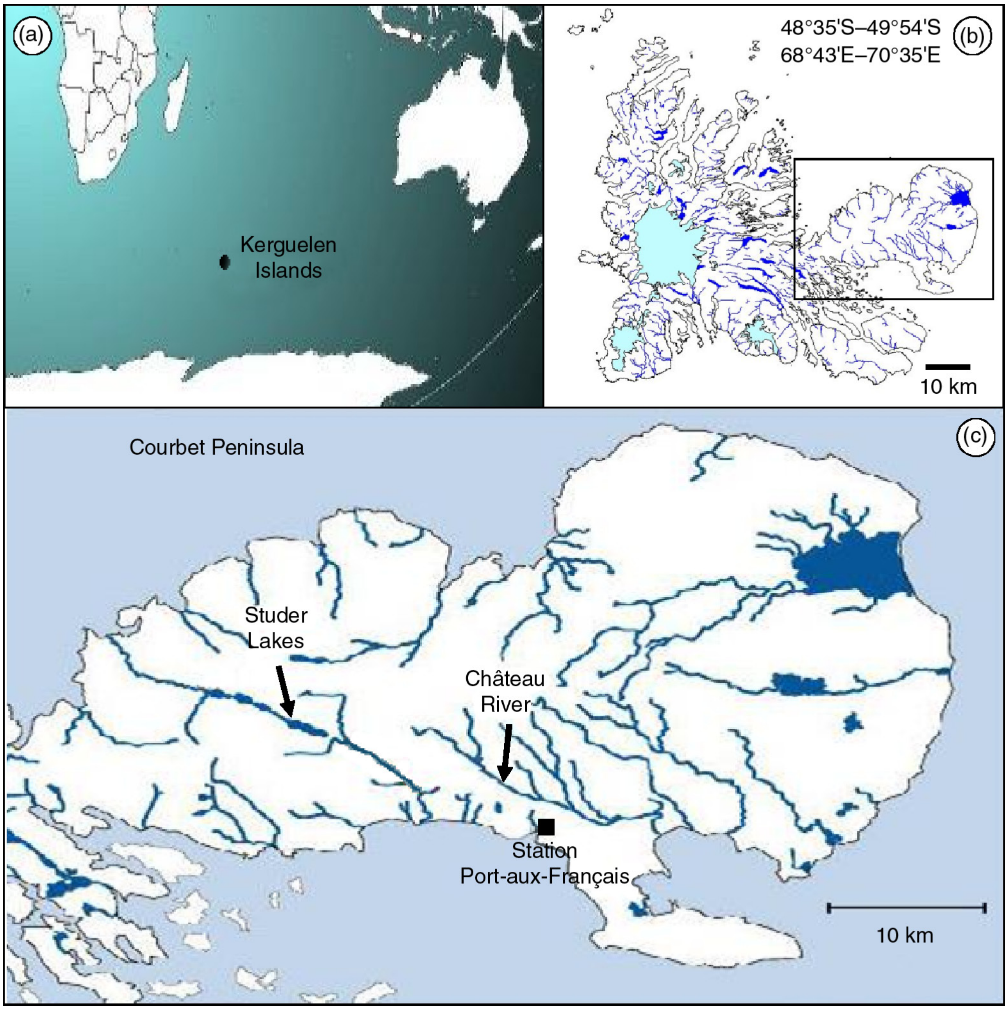

Fig. 1 The sub-Antarctic Kerguelen Islands and the locations of the study sites.

logistic transit condition from Kerguelen Islands to France. During the dissection procedure, fish sex was determined by the morphological observation of the gonads.

The physico-chemical characteristics of water in different sites were recorded monthly, between January and December 2006, according to the Standard Methods of the American Public Health Association (Table 1).

Histo-pathological analysis

Small pieces of the liver fixed in Bouin's fluid for 24 h were dehydrated in successive ethanol baths of increasing concentrations (from 70° to 100°). After 24 h in ethanol 100°, tissues were stored in butanol and sent to our laboratory in France for the rest of the histological treatment. Dehydrated tissues were then embedded in paraffin wax. Liver sections (5 µm) were stained with nuclear fast red and picro-indigo-carmin and mounted on glass slides for photon microscopy. Microphotographs were taken with a digital camera (Imaging Source, Bremen, Germany) coupled to a Laborlux photon microscope (Leitz, Wetzlar, Germany) connected to a computer using Archimed software version 5.6.0.

Sample sections from 40 trout ($n = 12$, $n = 14$ and $n = 14$ from river, lake and station morphotypes, respectively) were examined for the quantitative assessment of melanomacrophage centres (MMCs) and semi-quantitative assessment of liver pathologies.

Six liver sections separated by more than 100 µm were selected for the quantitative assessment of each individual's MMCs. The total surface of the selected sections was calculated (4–10 mm^2 by individual) using computerized image analysis (Archimed software version 5.6.0). The surfaces and the numbers of MMCs were calculated within the total observed areas and expressed in µm^2 of MMCs/mm^2 or in number of MMCs/mm^2.

Table 1 Water physico-chemical characteristics of sampling sites. Values are expressed as mean and extreme values (minimum–maximum). Some values were not determined (nd) or are not available (NA).

Site/morphotype	Château River	Studer Lake	Ferme Pond
Temperature (°C)	4.1 (−0.1–11.9)	5.2 (0.3–8.6)	5.7 (−0.1–19.4)
pH	7.5 (7–8.7)	7.4 (5.6–8.1)	7.6 (5–9)
Dissolved oxygen (mg/l)	12.3 (10.9–14.2)	11.6 (9.2–12.6)	11.9 (8.6–18.4)
Conductivity (μS/cm)	70 (62–97)	71.9 (46–83)	146.4 (96–182)
Cl^- (mg/l)	11.6 (9.6–16.2)	10.8 (9.7–11.8)	21.9 (7.4–26.4)
SO_4^{2-} (mg/l)	0.9 (0–4)	0.9 (0–2)	1.5 (0–5)
NO_3^- (mg/l)	0.021 (0.01–0.06)	0.016 (0–0.03)	0.012 (0–0.09)
NO_2^- (mg/l)	0.004 (0.003–0.007)	0.004 (0.001–0.006)	0.004 (0.0005–0.009)
PO_4^{3-} (mg/l)	0.87 (0.75–1.06)	0.94 (0.74–1.3)	0.72 (0.39–1.44)
NH_3–N (mg/l)	0.031 (0–0.13)	0.026 (0–0.06)	0.153 (0–0.36)
K^+ (mg/l)	0.27 (0.2–0.4)	0.26 (0.2–0.4)	0.58 (0.2–0.95)
Total iron (mg/l)	0.1 (0.03–0.47)	0.16 (0.02–0.58)	0.34 (0.03–0.81)
Chemical oxygen demand (mg/l)	37 (0–120)	39.4 (3–103)	42.2 (1–147)
Phenolic compounds (mg/l)	0.022 (0.007–0.072)	0.011 (0.001–0.034)	0.055 (0–0.185)
Cadmium (μg/l)	0.03 (nd–0.09)	0.05 (nd–0.34)	NA[a]
Copper (μg/l)	1.11 (nd–2.65)	1.19 (nd–6.84)	NA[a]

[a]Water sampled in Ferme Pond in 2005 presented metal concentration < 2 μg/L for copper and < 0.1 μg/L for cadmium, but such results were not recorded on a monthly basis (unpublished results).

Additionally, 18 separate zones of hepatic parenchyma (separated by more than 50 μm from each other) were observed in each individual slide in order to assess the extent of nuclear alteration (total observed surface = 300000 μm^2 of hepatic parenchyma).

Each histological abnormality was scored between 0 and 5 to estimate nuclear alterations, immune cell infiltration into parenchyma, fibrosis and vacuolization in liver sections. "Zero" corresponded to a normal structure and "5" to severe alterations. For example, severe fibrosis alterations (score of 5), nuclear alterations, vacuolization and immune cell infiltration are illustrated in Fig. 4a, d, e & f, respectively. A global score, taking into account all the different observed alterations, was calculated for each individual:

$$\text{global score} = (\text{Score}_{\text{MMCS}} + \text{Score}_{\text{Immune cell infiltration}} + \text{Score}_{\text{Altered nucleus}} + \text{Score}_{\text{Fibrosis}} + \text{Score}_{\text{Vacuolization}})/5$$

Quantification of Cd and Cu

Heavy metal quantification was performed on a total of 88 trout liver samples (10, 24 and 54 from river, lake and station morphotypes, respectively) dried at 70°C for 15 days, weighed (dry weight) and then digested in nitric acid (65%, Normatom) at 70°C. The resulting clear solutions were diluted with high-quality deionized (Milli-Q) water.

Hepatic heavy metal concentrations were determined using a Zeeman 220 graphite furnace atomic absorption spectrophotometer (Varian, Palo Alto, CA, USA) for Cd quantification and an AA 240 FS fast sequential atomic absorption spectrometer (Varian) for Cu. Metal concentrations were calculated using calibration curves and the results were expressed in μg/g dry weight (d.w.). Standard stock solutions (DOLT-3) corresponding to certified standard liver material from the dogfish *Squalus acanthias* were obtained from the National Research Council of Canada. Instrumental quantification limits were 0.023 μg/l and 6 μg/l for Cd and Cu, respectively.

Water metallic analyses were performed using the Zeeman 220 graphite furnace atomic absorption spectrometry (Varian) with an instrumental quantification limit of 0.023 μg/l for Cd and 0.3 μg/l for Cu.

Biochemical analyses

The liver samples from 58 trout ($n = 17$, 20, 21 for river, lake and station morphotypes, respectively) were homogenized in 200 μl of ice-cold phosphate buffer (100 mM, pH 7.8) supplemented with 20% (v/v) glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a protease inhibitor. The homogenate was centrifuged at $10000 \times g$ at 4°C, for 15 min and the supernatant was collected for biochemical assays. Total proteins were determined using the Bradford method (Bradford 1976) with bovine serum albumin (Sigma) as a standard. SOD and CAT activity were assessed according to the methods of Paoletti et al. (1986) and Babo & Vasseur (1992), respectively, and used by Sanchez et al. (2005). Assays were calibrated using purified bovine enzymes (Sigma) as standards.

Total GSH concentrations were measured in liver samples by the spectrophotometric method of Vandeputte et al. (1994). All biochemical assays were optimized for brown trout and adapted to micro-titre plates. Time-course measurements were performed using a PowerWave HT microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Statistical analyses

Statistical analyses were performed with XLSTAT package of Microsoft Excel (Addinsoft SARL 2010). Values were expressed as mean ± standard deviations. Gaussian distribution and homogeneity of variance were not demonstrated among all the studied groups. Therefore, the non-parametric Kruskal–Wallis test was used to compare data from different morphotypes. For all data, differences among mean were detected by multiple comparisons using the Conover–Inman test. The Mann–Whitney *U* test was used to compare the mean of females and males. Associations among hepatic metal concentrations and biomarkers were evaluated by Pearson correlation analyses. All tests were considered statistically significant at $p < 0.05$.

The scientific protocol was assessed regarding animal welfare and approved by the Ethics Committee of the Midi-Pyrénées Region, France.

Results

Physico-chemical characteristics of the sampling sites

The physico-chemical characteristics of the sampling sites' water corresponded to good quality water favourable to salmonid life (EU 2006), because of a low level of trophy and a good rate of oxygenation. Moreover, the low levels of Cd and Cu detected in waters were often below the instrumental quantification limit (Table 1). Additionally, it can be noted that the three sites had comparable physico-chemical characteristics, except for the chlorine ions enrichment at Ferme Pond site associated with high levels of conductivity due to marine influence (Gay 1981).

Liver metal concentrations

Kerguelen brown trout had a mean hepatic Cu concentration of 577 ± 403 µg/g d.w. and a Cd concentration of 1.4 ± 2.6 µg/g d.w. Hepatic Cu levels were significantly different ($p < 0.0001$) between morphotypes (204 ± 127, 821 ± 301 and 543 ± 413 µg/g d.w. for river, lake and station morphotype, respectively; Fig. 2a). Cu levels were the lowest in river trout and the highest in lake trout (Fig. 2a). High inter-individual variations in hepatic Cd contents were observed and were related to the small number (5.6%) of "hyper-accumulator" individuals.

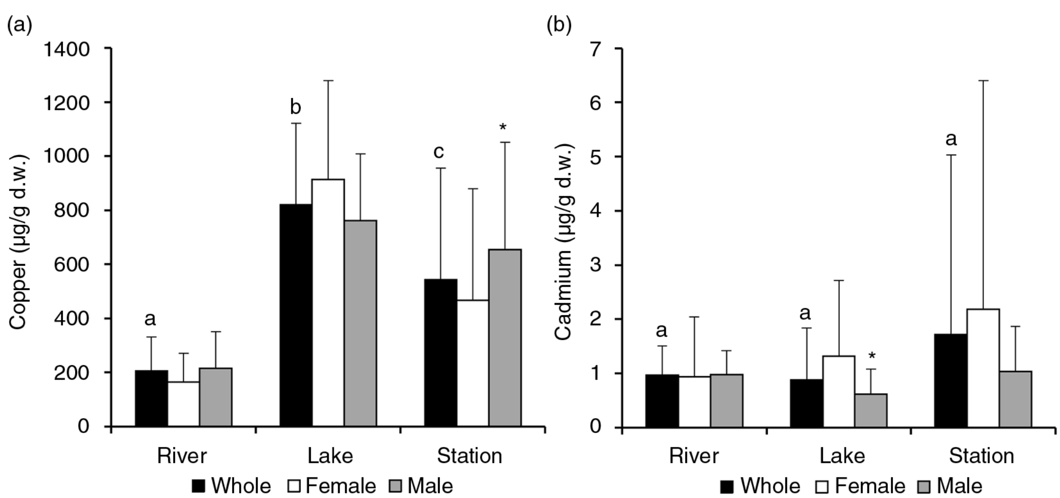

Fig. 2 (a) Copper and (b) cadmium concentrations in livers of brown trout from the Kerguelen Islands from three Kerguelen morphotypes: river ($n = 10$), lake ($n = 24$) and station ($n = 54$). Bars represent mean ± SD of metal concentrations measured in the whole sample, in the livers of females and males in each morphotype. The different letters indicate significant differences in hepatic metal mean measured in the whole sample between the three morphotypes (Kruskal–Wallis test). Asterisks indicate significant differences in mean between females and males in each morphotype (Mann–Whitney *U* test).

We considered fish presenting a Cd accumulation three times higher than the standard deviation to be "hyper-accumulator" organisms. No significant difference in Cd levels was observed between morphotypes (0.97 ± 0.54, 0.88 ± 0.96 and 1.72 ± 3.32 µg/g d.w. for river, lake and station morphotype, respectively; Fig. 2b). It can be noticed that "hyper-accumulator" trout frequencies were higher at the station morphotype (7.4%), with no significant difference in the global Cd concentration.

No significant influence of sex was observed for the hepatic Cd or Cu levels of Kerguelen brown trout as a whole. However, when taking morphotypes into account, a slight difference appeared between males and females for Cd, but only for the livers of lake trout, with males having somewhat lower hepatic Cd levels than females ($p = 0.053$; Fig. 2b). For Cu, the only inter-sex difference was observed in the livers of station trout: males displayed significantly higher Cu contents than females ($p = 0.022$; Fig. 2a).

Liver histological structures

Kerguelen trout displayed variations in hepatic histological patterns. Whereas some trout had few structural alterations and mainly normal liver histology (Fig. 3a, b), others showed clear hepatic disturbances (22 out of a total of 40 individuals). These individuals showed an increase in the number and size of MMCs, which corresponded to zones of macrophagic cell agglomeration (Fig. 3c–f, 4b), a high frequency of immune cell infiltrations into the hepatic parenchyma (Fig. 3e, 4d, f), and obvious peri-vascular and peri-ductular fibrosis (Fig. 4a, b). Additionally, hepatocytic alterations related to necrotic processes were notable (nuclear pyknosis, important amounts of residual bodies) (Fig. 3e, 4c, d, f). An increase in lipidic vacuolization in quantity and in size was observed (Fig. 3c, 4e,). These alterations observed in some fish livers cannot, however, be considered as lipoid degeneration.

In order to improve the characterization of hepatic alterations, total MMC surfaces and numbers were assessed (Table 2). No correlation was found between the frequency or the surface of MMCs and age, condition factor or morphotype individuals. However, a significant correlation was observed between the two sexes. Males displayed a higher frequency ($p = 0.003$) and size ($p = 0.002$) of hepatic MMCs than females (Table 2).

Scoring marks for nuclear alterations, immune cell infiltration into parenchyma, fibrosis and vacuolization are reported in Table 2. No significant inter-sex or inter-site relationship was observed for any of the tested histopathological marks. The same results were reported with global scores taking into account all the observed different pathologies (Table 2). They showed that trout from different morphotypes presented similar histopathological trends.

However, a significant positive correlation exists between hepatic Cu concentration and numbers of MMCs; as well as between Cd and fibrosis scores (Table 3).

Liver functional anti-oxidative activities

The oxidative stress biomarker responses measured in this work (SOD, CAT and GSH) were characterized by the absence of a gender-related difference. Station trout exhibited higher CAT and GSH values compared to the responses recorded in the other two investigated sites (Fig. 5). More accurately, CAT activity values were between 292 and 1881 U/g of protein for the station site, which was significantly higher than the river and lake ($p = 0.015$; Fig. 5a). Similar response profiles were observed for GSH, with values between 0 and 16 µmol/g of protein (Fig. 5b). SOD activity was between 1.2 and 7.3 U/mg of protein. Contrary to CAT and GSH, SOD values were statistically significant lower in the station trout compared to the river ($p = 0.01$; Fig. 5c). No correlation links were demonstrated between oxidative stress biomarkers and hepatic metal concentrations (Table 3).

Discussion

Liver metal concentrations

According to data available in the literature, heavy metal bioaccumulation in trout is more related to the degree of anthropogenic impact than to the geographic location of the study sites. For instance, hepatic Cu levels in brown trout caught in four Spanish sites differently polluted by Cu ranged from 75 to 435 µg/g d.w. (Lamas et al. 2007). In Norwegian and Russian lakes situated in important mining and metallurgic regions, brown trout had liver tissues contaminated by Cu (11–180 µg/g d.w.) and Cd (0.19–0.6 µg/g d.w.; Amundsen et al. 1997). However, in Blackfoot River, USA, affected by headwater inputs of acid-mine effluent, hepatic Cu concentrations in brown trout (*Salmo trutta*) were much higher than those found in our study (between 494 and 2399 µg/g d.w.), and hepatic Cd concentrations were in the same range as in Kerguelen brown trout (0.07–0.83 µg/g d.w.; Moore et al. 1991). In this study, Kerguelen trout livers presented similar Cd and Cu concentrations compared to the above-cited studies. These results are in accordance with data obtained in a previous study about Cu and Cd concentrations in the livers of Kerguelen brook trout (*Salvelinus*

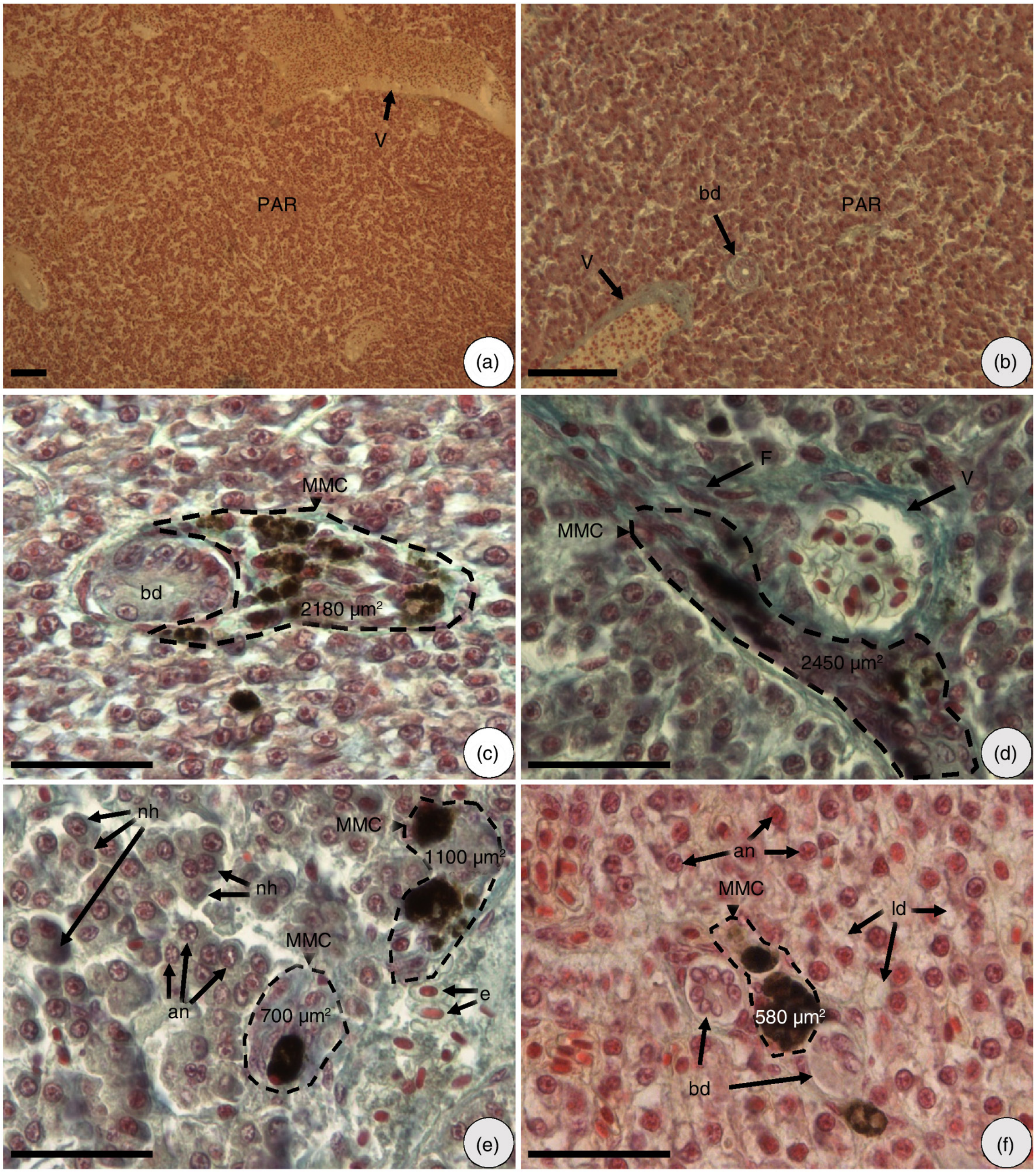

Fig. 3 Photomicrographs of liver sections from brown trout from the Kerguelen Islands illustrating (a, b) normal parenchyma. (c–f) Important numbers of melanomacrophage centres (MMC). The following terms are abbreviated: parenchyma (PAR), vein (V), biliary duct (bd); MMC; fibrosis (F), necrotic hepatocyte (nh); altered nucleus (an); erythrocyte (e), lipidic droplet (ld). The dotted line represents the MMC boundary. The scale bars are 40 µm in length.

fontinalis) caught in 2005, in which hepatic Cd and Cu levels were shown to be close to those detected in fish from regions under human activities' impact (Jaffal, Paris-Palacios et al. 2011). In the area around the Kerguelen Islands, benthic and pelagic marine fish species had also high hepatic Cd levels (from 10 to 52.1 µg/g d.w.; Bustamante et al. 2003). Cu concentrations measured in the same body tissues of these marine fish were lower (0.9–24.7 µg/g d.w.) than those quantified in our freshwater's trout (Bustamante et al. 2003). To explain the high Cd levels observed in sub-Antarctic marine fauna, many authors have evoked a telluric origin related to volcanism (Bustamante et al. 2003; Abollino et al. 2004; Bargagli 2008). However, in our

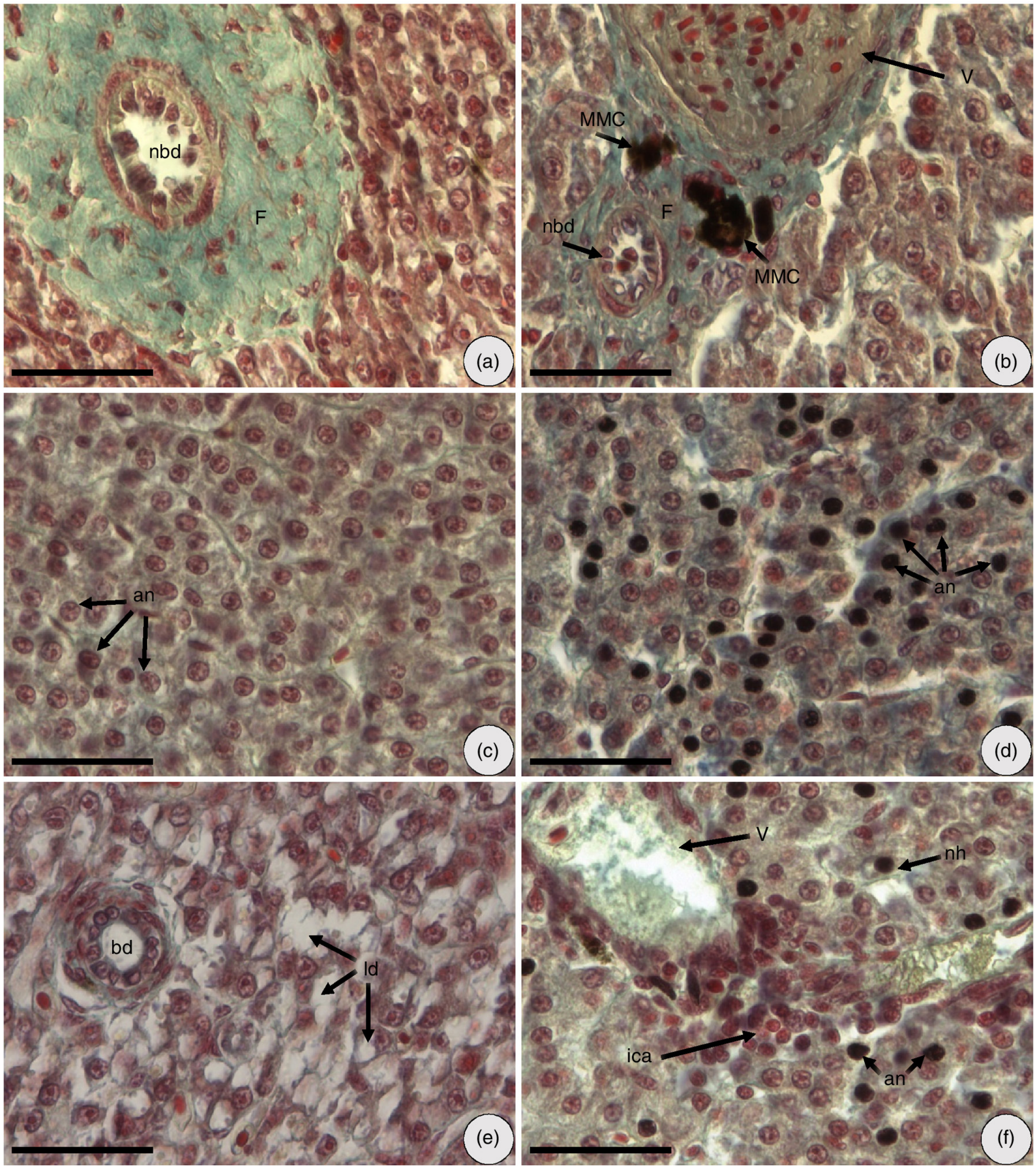

Fig. 4 Photomicrographs of liver sections from brown trout from the Kerguelen Islands showing different alterations: (a) fibrosis, (b) fibrosis with melanomacrophage centres (MMC), (c, d) altered nucleus, (e) parenchyma with lipidic droplet and (f) parenchyma with immune cell infiltrations. The following terms are abbreviated: fibrosis (F), necrotic biliary duct (nbd), vein (V), MMC, altered nucleus (an), biliary duct (bd), lipidic droplet (ld), necrotic hepatocyte (nh), immune cell agglomerate (ica). The scale bars are 40 μm in length.

study it is difficult to find support for this hypothesis because Cd and Cu levels in water were globally as low as those observed in other polar or subpolar regions (Evans et al. 2005; Bargagli 2008). In this context, the major part of bioaccumulation should be coming from the trouts' diets. The elemental composition of sediments and trout prey will be necessary to confirm the origin of heavy metals in Kerguelen aquatic ecosystems. In another way, bioaccumulation of Cu appeared significantly different between the three studied morphotypes. Two explanations could be evoked. First, high levels of Cu were observed in the livers of brown trout living in the Studer Lakes and near the station, in Ferme Pond. These two sites corresponded to lentic hydrosystems, where fish diet

Table 2 Histological alterations observed in male and female Kerguelen brown trout from "river," "lake" and "station" morphotypes. Values are expressed as mean (minimum–maximum). The different letters indicate significant differences for each alteration (Kruskal–Wallis test, $p < 0.05$), with the number of observations in parentheses.

	River		Lake		Station	
	Females (24)	Males (48)	Females (36)	Males (48)	Females (42)	Males (42)
Melanomacrophage centre areas (μm²/mm²)	246 (54–531)^{a,b}	1003 (314–2753)^{c}	349 (25–746)^{a,b,c}	854 (0–2395)^{a,b,c}	184 (47–395)^{a}	1043 (14–2693)^{b,c}
Numbers of melanomacrophage centres (/mm²)	1.6 (0.6–3.5)^{a,b}	3.5 (2.2–4.9)^{b}	1.9 (0.4–3)^{a,b}	4.8 (0–11)^{a,b}	1.3 (0.2–3.4)^{a}	6.5 (0.1–15.5)^{b}
Nuclear alteration scores	4.2 (3.6–5)^{b}	3.2 (1.5–5)^{a,b}	3 (2.1–3.8)^{a,b}	2.8 (1–4.3)^{a}	3.5 (2.5–5)^{a,b}	3.8 (2.1–5)^{a,b}
Vacuolization scores	1.7 (0–4.3)^{a,b}	0.5 (0–3.1)^{a}	2.7 (0.3–5)^{b}	2.7 (1–5)^{b}	1.5 (0–4.5)^{a,b}	1.4 (0–4.6)^{a,b}
Fibrosis scores	2.2 (1.3–3)^{a,b,c}	2.8 (1.7–4)^{c}	2.7 (1.1–4.6)^{b,c}	2.7 (1–3.3)^{c}	1.8 (1.1–2.8)^{a,b}	1.6 (1–2.1)^{a}
Immune cell infiltration scores	1.8 (0.1–2.6)^{a}	2.6 (1.5–3.8)^{a}	2.3 (1–3.1)^{a}	1.7 (0–3.3)^{a}	1.6 (0.1–3)^{a}	2.2 (1–3.8)^{a}
Global scores	2.2 (1.2–3.2)^{a}	2.3 (1.7–3.1)^{a}	2.4 (1.5–3.3)^{a}	2.3 (1.5–3.2)^{a}	1.9 (1–2.7)^{a}	2.3 (1.4–3.5)^{a}

is essentially composed of small planktonic crustaceans (Copepoda, cladocera; Bryère & Charrier 2009). These invertebrates have the respiratory pigment haemocyanin, which contains Cu. The high levels of Cu in both the Studer Lakes and Ferme Pond populations may be explained by their common diet. Second, Kerguelen trout sizes were different between morphotypes at the same age (26.5 ± 7.8, 35.7 ± 5.6 and 43.6 ± 4.4 cm for river, station and lake morphotypes, respectively). A positive relationship can be found between hepatic Cu level and fish size ($r = 0.469$, $p = 0.021$): big fish display higher metal levels than small ones. Many studies show that hepatic Cu concentrations depend on fish age and size (Farkas et al. 2003; Kojadinovic et al. 2007). It seems likely that hepatic Cu concentrations of each morphotype were influenced by both fish size and diet.

Liver histological structures

The alterations observed in the livers of Kerguelen trout belonged to the focal necrosis type. In this study, MMCs, immune cell infiltrations into the hepatic parenchyma, fibrosis and lipidic vacuolization were observed. These liver damages have been observed in fish exposed to poly-contaminated and treated sewage water (Adams et al. 1996; Schmidt et al. 1999; Bernet et al. 2004), to pulp and paper mill effluents (Adams et al. 1996) and to polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), heavy metals and pesticides (Schwaiger et al. 1997). However, such alterations are not specific. They are associated with the general response of hepatocytes to toxicants or/and stressors (Hinton & Laurén 1990; Bernet et al. 2004; Greenfield et al. 2008; Marchand et al. 2009) or to oxidative-stress-related disturbances associated with exposure to xenobiotics (Betoulle et al. 2000).

Table 3 Pearson correlation coefficients among hepatic metal concentrations and biomarkers. Values in boldface indicate significant correlations at $p < 0.05$.

	Cd	Cu
Glutathione levels	−0.029	−0.070
Superoxide dismutase activity	−0.115	0.141
Catalase activity	−0.009	0.038
Melanomacrophage centre areas	0.048	0.112
Numbers of melanomacrophage centre	0.017	**0.406**
Nuclear alteration scores	−0.110	−0.159
Vacuolization scores	0.212	0.054
Fibrosis scores	**0.462**	0.142
Immune cell infiltration scores	0.127	−0.113
Global scores	0.270	0.017

Fibrosis with immune cell infiltration is a prevalent lesion. It commonly indicates irritation of the bile ducts by xenobiotics and is considered a consequence of oxidative burnout (Hinton & Laurén 1990). The relative risk (prevalence ratio) for trout to show this lesion has been found higher with increasing pollution (Bernet et al. 2004). Inflammatory response is one of the most frequently observed reactions in fish and particularly in salmonids worldwide (Schwaiger 2001). The amounts of immune cells and of MMCs in hepatic parenchyma have been used as indicators of a number of natural and anthropogenic stressors (Broeg 2010). MMCs contain melanin, ceroid/lipofuscin and hemosiderin pigments. They are believed to be part of non-specific immune responses and of the immune memory of teleosts (Borucinska et al. 2009). MMCs have often been evidenced in various fish species. However, a relatively small number of studies are available dealing with the effects of environmental stress on this histological parameter in salmonid species (Schwindt et al. 2006). MMCs are produced in various organs in salmonids (kidney, liver, spleen) but MMC quantification is particularly difficult in salmonids. In fact, in salmonids MMC were poorly organized, irregular and smaller than those observed in other teleosteans (Schwindt et al. 2006). MMCs are hypothesized to be

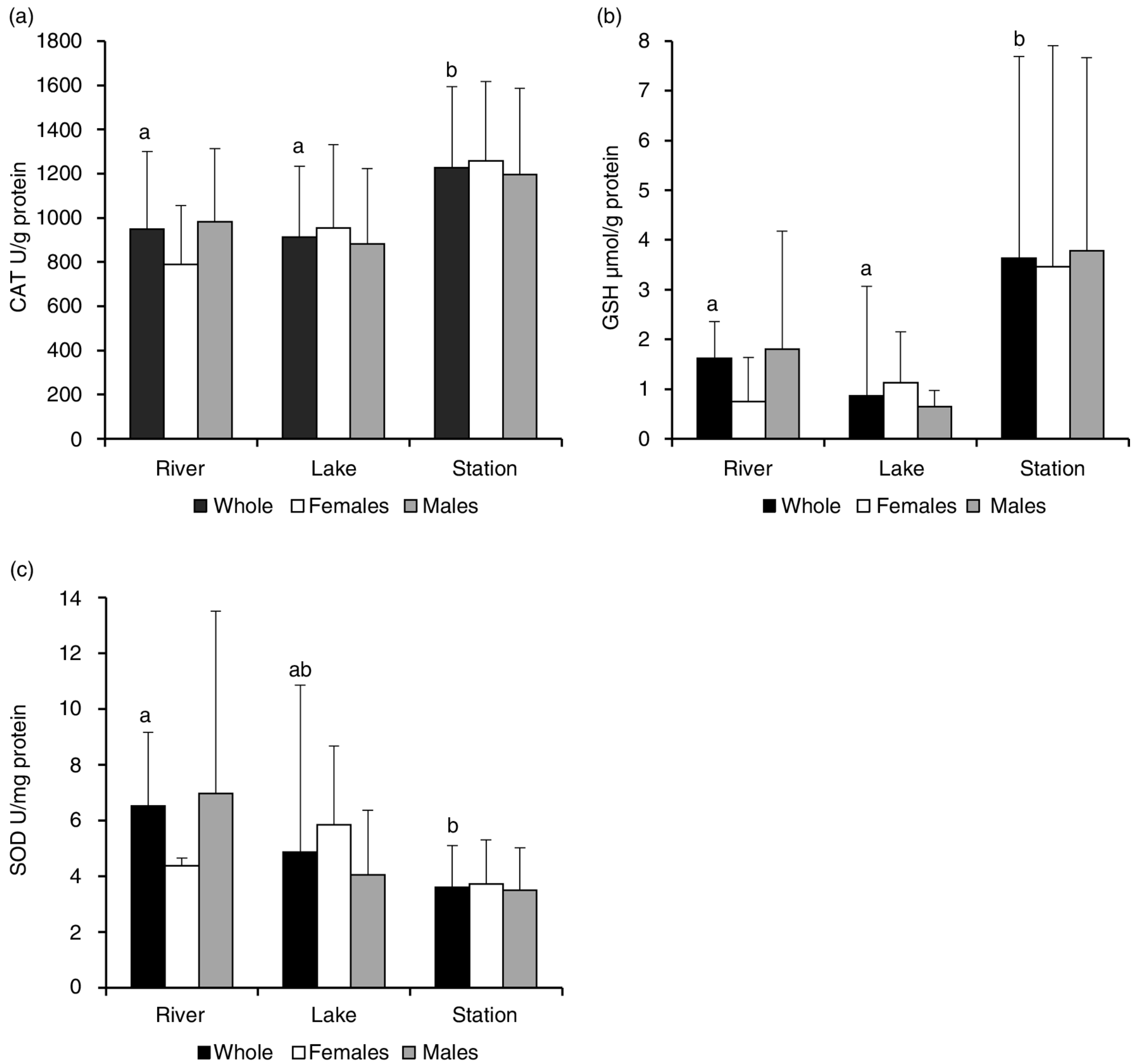

Fig. 5 (a) Catalase activity (CAT), (b) glutathione levels (GSH) and (c) superoxide dismutase activity (SOD) in the livers of brown trout from three Kerguelen Island morphotypes: river ($n = 17$), lake ($n = 20$) and station ($n = 21$). Bars represent mean ± SD of biomarker levels measured in the whole sample, in the liver of females and males in each morphotype. The different letters indicate significant differences in biomarker levels measured in the whole sample between the three morphotypes (Kruskal–Wallis test).

able to form intercellular connections and networks that trap and process antigens and endogenous products of cellular degradation (Johnson et al. 2005). Their increased presence in tissues is probably related to higher rates of degenerative processes or to greater extents of tissue catabolism (Schwindt et al. 2006; Borucinska et al. 2009). Therefore, the numbers and morphological characteristics of MMCs can be influenced by xenobiotics as well as by the natural physiological conditions (Agius 1981; Manera et al. 2000).

The exposure to Cd and Cu has been shown to cause histopathology of the liver, even at low concentrations (Paris-Palacios et al. 2000; Schwaiger 2001; Carrola et al. 2009). This hepatic response to metals includes: fibrosis, necrosis, formation of MMCs and increased immune cell infiltration, changes in the nuclear and nucleolar patterns, modification of hepatocytic energetic storage (increased lipids and decreased glycogen) (Paris-Palacios et al. 2000; Greenfield et al. 2008). That pathology is in agreement with the one we observed in the livers of Kerguelen trout. The high Cu and Cd levels observed in Kerguelen trout could therefore be directly related to the structural pathology revealed in the liver tissue. Indeed, in this study, a positive correlation was observed between hepatic Cu concentration and histological perturbations (numbers of MMCs) and fibrosis scores showed positive correlation with Cd concentration.

In our study, MMC numbers or areas in Kerguelen brown trout were not related to age, condition or morphotype. But males had a higher frequency and size of hepatic MMCs than females. Schwindt et al. (2006) found a positive correlation between macrophage centres and age, and a negative correlation between MMCs and condition factor in brook trout, but no relationship between sex or maturity and MMC levels. No inter-sexual differences appeared concerning rates of fibrosis, nuclear alteration or in the global hepatic alteration score observed in Kerguelen trout. The faint inter-sex difference in hepatic metal concentrations observed in trout from each morphotype suggests that comparable levels of metallic contents between sexes are related to the similar intensities of hepatic structural disturbances shown between sexes (except for MMCs). The hepatic metal levels we measured cannot explain the higher intensity of MMC development in males. Indeed, some studies showed gender-dependent differences in liver tissue sensitivity to pollution, with variations according to chemicals, species, age and reproductive status (Braunbeck 1998). According to Bruslé & Anadon (1996), the sexual dimorphism of fish hepatocyte may be observed in the content of lipid and glycogen stores also in the amount of rough endoplasmic reticulum and Golgi vacuoles particularly higher in females than in males. This cytological observation is correlated with exogenous vitellogenesis. Vitellogenin is synthesized in the female fish liver in response to oestrogen. Ovarian hormones are then responsible for hepatocyte changes, which are indicative of enhanced metabolic activity, as demonstrated by Peute et al. (1985). This change could lead to the lower MMC accumulation in female livers. Furthermore, during the spawning period, females mobilize chemicals and may transfer them to the eggs (Loizeau & Abarnou 1994). Therefore, differences in the hepatic metabolism of females could explain the lower intensity of MMC in females versus males.

The lesions described here were also found in fish exposed to organic contaminants or to pesticides (Paris-Palacios et al. 2000; Schmidt-Posthaus et al. 2001). Previous studies indicated that salmonid populations of the Kerguelen Islands display important levels of contamination by PCBs (ΣPCB [29 congeners] = 28 ng.g^{-1} wet weight in brown trout muscle) similar to those measured in the same species living in other regions of the world less isolated from pollution (Jaffal, Givaudan et al. 2011). No sex-related difference was found in the total muscle concentrations of PCBs in Kerguelen trout or in the class distribution of PCB indicators or PCB dioxin-like congeners (Jaffal, Givaudan et al. 2011). This organic contamination, probably resulting from the global atmospheric dispersion of pollutants, may be part of the development of the structural disturbances observed in Kerguelen trout livers.

Liver functional anti-oxidative activities

Hepatic biomarker responses linked to protection against oxidative stress showed that station trout were characterized by a response profile significantly different from the responses observed in the trout from the other two morphotypes we investigated. This result confirms the previous observations about trout from the Kerguelen Islands. Indeed, Morat et al. (2008) studied the crystallization form of otoliths from brown trout in the Kerguelen Islands. They revealed the presence of vaterite otoliths with the highest frequencies observed in fish living in the aquatic system near the station. The statistical analysis of these results revealed that abnormal crystallization forms (vateritic otoliths) can be linked to particular physico-chemical characteristics. As observed in Table 1, water conductivity in aquatic system of Ferme Pond, near the station, was higher than in the other two sites. Such a difference was related to the proximity with the seaside, which led to an enrichment in chlorine ions of the waters by wind and rain in the aquatic systems near the station (Gay 1981). Such physico-chemical characteristics of Ferme Pond may influence the fish physiology by modulating their hydromineral homeostasis and could be responsible for differences in biomarkers responses observed in these brown trout population in the present study. In fact, the environmental salinity could affect the antioxidant enzymes activities of trout (Kolayli & Keha 1999). A comparative study of antioxidant enzyme activities in freshwater and seawater-adapted rainbow trout has demonstrated significant differences between the two groups. SOD activity was found to be lower in the seawater-adapted trout than in the freshwater-cultured trout (Kolayli & Keha 1999). These results appear to be consistent with the lower SOD values obtained from the fish population in the pond near the station. Furthermore, salinity could indirectly affect these biomarkers by modulating metal bioavailability (Baysoy et al. 2012). In fact, metal bioavailability mostly depends on the conductivity of water which is positively correlated to free ion levels in water. According to Baysoy et al. (2012), salinity and metal exposures cause variation in the antioxidant enzyme activities and SOD activity generally increase in fish liver exposed to salinity and metals. Another hypothesis can be the relative high fishing pressure exerted on the salmonids living near the station, where fishing is a popular hobby. Finally, local anthropogenic pollutants related to human activities at the station could also be responsible for the differences in anti-oxidative activities observed between brown trout near the station and those at more distant sites. Biomarkers can provide useful data to trace the origin of the environmental stress in Kerguelen trout. Indeed, several heavy metals and organic pollutants, such as PCBs and PAHs, are able to induce reactive oxygen species production in fish and to induce associated defence mechanisms (Wilhelm Filho et al. 2001; Ait-Aissa et al. 2003; Sanchez et al. 2006). The contamination of aquatic ecosystems of the Kerguelen Islands by heavy metals and PCBs may have contributed to the level of anti-oxidative defences we observed.

Conclusions

The hydrosystems of the Kerguelen Islands are geographically isolated. However, the livers of Kerguelen brown trout showed high levels of Cu and Cd and clear hepatic disturbances (fibrosis, nuclear alteration, increased immune response). In this study, the presence of MMCs was revealed in the livers of brown trout, and they were correlated with hepatic Cu concentration. Similar histopathological trends were observed among trout from river, lake and station morphotypes, but anti-oxidative responses were different in trout from the station morphotype. Some of the hepatic alteration and MMCs in the livers of Kerguelen brown trout may be related to the high levels of Cd and Cu measured in these fish but also to PCB contamination, as revealed in a previous study. Hence, further investigations are needed to assess the effects of global contamination on fish health in the Kerguelen Islands.

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