Copper-induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels

Wilfried Sanchez, Olivier Palluel, Laurent Meunier, Marina Coquery, Jean-Marc Porcher, Selim Ait-Aissa

► To cite this version:

Wilfried Sanchez, Olivier Palluel, Laurent Meunier, Marina Coquery, Jean-Marc Porcher, et al.. Copper-induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels. Environmental Toxicology and Pharmacology, 2005, 19 (1), pp.177-183. 10.1016/j.etap.2004.07.003 . ineris-00961894

HAL Id: ineris-00961894

<https://hal-ineris.archives-ouvertes.fr/ineris-00961894>

Submitted on 20 Mar 2014

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Copper-induced oxidative stress in three-spined stickleback : relationship with hepatic metal levels

Wilfried Sanchez^a, Olivier Palluel^a, Laurent Meunier^b, Marina Coquery^b, Jean-Marc Porcher^a
and Sélim Aït-Aïssa^{a*}

^a Unité d'évaluation des risques écotoxicologiques, Direction des risques chroniques, Institut National de l'Environnement Industriel et des Risques (INERIS), BP 2 , F-60550 Verneuil en Halatte, France

^b Unité de chimie analytique et environnementale, Direction des risques chroniques, Institut National de l'Environnement Industriel et des Risques (INERIS), BP 2 , F-60550 Verneuil en Halatte, France

* Corresponding author. Fax: +33 (0)3 44 55 67 67

E-mail address: Selim.Ait-Aïssa@ineris.fr

ABSTRACT

The aim of this study was to characterise biomarker responses in three-spined sticklebacks exposed to copper. For this purpose, adult sticklebacks were exposed for 3 weeks to copper sulphate at 0, 25, 100 and 200 $\mu\text{g.L}^{-1}$ as Cu. At days 4, 8, 12 and 21, several parameters were measured including liver, gonad and spleen somatic indexes, hepatic biomarkers (catalase, superoxide dismutase, glutathione peroxidase, glutathione, glutathione-S-transferase and 7-ethoxyresorufin-O-deethylase) and hepatic copper and zinc concentrations. Copper induced a rapid and transient increase of antioxidant enzymes and a depletion of glutathione content during the first 8 days of exposure. Significant copper and zinc accumulation in fish liver were observed for the two higher exposure concentrations after 8 and 12 days respectively. This study showed that copper induced an oxidative stress in fish liver before significant metal accumulation in the liver could be detected, suggesting the involvement of differential mechanisms in copper uptake and metabolism. Three-spined stickleback appears to be a sensitive model to study oxidative stress induced by metals.

Key-words : bioaccumulation, biomarkers, copper, liver, oxidative stress, three-spined stickleback

1. Introduction

Mining activity, domestic waste emission or application of fertilizers and pesticides induce contamination of aquatic ecosystems by heavy metals. Among them, copper is a widespread pollutant found in surface waters at concentrations up to 100 µg/L (Roy, 1997). It is also a particular pollutant because it is an essential trace element for living organisms, used as a cofactor for structural and catalytic properties of a variety of enzymes involved in the biological processes of growth, development and maintenance (Turnlund, 1999). Although this metal is a required element, high concentrations appear to be toxic to freshwater organisms with no observed effect concentrations comprised between 4 and 120 µg.L⁻¹ as reported for 14 fish species (Grosell et al., 2002). The freshwater predicted no effect concentration (PNEC) was recently evaluated for copper and the value of 1.6 µg.L⁻¹ was proposed (GRNC, 2002). Copper toxicity varies with water chemistry, temperature and fish species and induces various damages that can lead to the death of the organisms.

At the cellular level, copper can interfere with several metabolic pathways and thereby induce different cellular responses. This metal has been well described as a promoter of oxidative stress by catalysing the formation of highly reactive oxygen species (ROS), such as HO[°] radical through the Haber-Weiss reaction (Matés, 2000) and generating peroxidation of membrane lipids (Chan et al., 1982) and DNA alterations (Ozawa et al., 1993). Copper also binds thiol containing molecules such as glutathione (GSH) or metallothioneins (MT) where it is trapped (Roesijadi, 1996). Direct interaction of copper with proteins can be the source of enzyme inhibition such as cytochrome P450 associated monooxygenase activities (Kim et al., 2002).

This study was conducted to characterise the response of several hepatic biomarkers, including oxidative stress and xenobiotic transformation biomarkers, in three-spined stickleback (*Gasterosteus aculeatus* L.) exposed to sublethal copper concentrations and to

determine whether these responses are related to copper accumulation in the target organ. Three-spined stickleback is present in all aquatic ecosystems in Northern hemisphere and is an abundant fish species in both unpolluted and polluted areas. Recently, this fish has been used as a model organism to study bioaccumulation of organic compounds (Andersson et al., 2001; Falandysz et al., 1998) and metals (Bervoets et al., 2001), as well as biochemical biomarker responses to various xenobiotics such as pesticides (Sturm et al., 2000), organics compounds (Holm et al., 1993) or environmental estrogens and androgens (Katsiadaki et al., 2002; Pottinger et al., 2002). To our knowledge, no study has investigated the effect of metals on the biomarker responses in three-spined stickleback. Hence, as its uses as a sentinel species for aquatic pollution biomonitoring has been suggested (Handy et al., 2002; Pottinger et al., 2002), there is still a need to characterise biomarker responses to widespread environmental contaminants in this fish species.

The biomarkers that we measured were enzymatic [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] and non enzymatic (GSH) antioxidants, as well as two biotransformation enzymes [7-ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST)] involved in oxidative metabolism of xenobiotics. Furthermore, copper measurement in liver was used to link metal accumulation with biochemical responses. In addition, hepatic zinc content was measured in order to evaluate whether MT induction occurred during our experiment.

2. Materials and methods

2.1. Exposure protocol

Male and female adult sticklebacks (3.4-6.8 cm) were collected in outdoor artificial streams during spring 2003 and maintained in denitrated water (ionic composition : K, 3.0 mg.L⁻¹; Mg, 11.7 mg.L⁻¹; Na, 10.9 mg.L⁻¹; Si, 5.8 mg.L⁻¹; Ca, 55.5 mg.L⁻¹; Al<0.01 mg.L⁻¹; Fe<0.02 mg.L⁻¹; PO₄³⁻, <0.1 mg.L⁻¹; NH₄⁺, 0.3 mg.L⁻¹; Cl⁻, 58 mg.L⁻¹; NO₃⁻, 0.45 mg.L⁻¹; SO₄²⁻, 11.7

mg.L⁻¹; alkalinity, 367 mg.L⁻¹ as CaCO₃; pH, 8.35 ± 0.05) for 3 weeks prior to experimentation. Fish were randomly distributed in four groups of 30 fish and exposed to waterborne copper sulphate in semi-static conditions with complete water renewal and food supply every 3 days. The concentrations of copper were chosen to be representative of environmental concentrations encountered in polluted freshwaters (25 and 100 µg Cu.L⁻¹) with an additional higher concentration (200 µg Cu.L⁻¹) that was expected to exert subtoxic events. The control group received no added copper. Water copper concentrations in each aquarium were controlled by inductively coupled plasma optical emission spectrometry (Ultima 2, Jobin Yvon) and showed that actual copper concentrations were stable between two water changes (data not shown) and throughout the experiment (table 1). The water temperature was 13.5°C ± 0.4°C and the photoperiod was a light/dark cycle of 8/16 hours.

2.2. Sampling, biometrical and biochemical analysis

At day 0, 4, 8, 12 and 21, six fish per concentration were randomly sampled. Fish were sacrificed, measured and weighed. Liver, gonads and spleen were dissected and weighed to calculate the corresponding somatic index according to the following equation : organ somatic index = (organ weight / fish weight) x 100.

Liver was 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 10,000 g, 4°C, for 15 min and the supernatant was used for biochemical assays. Total proteins were determined using the method of Bradford (1976) with bovine serum albumin (Sigma) as a standard. SOD, CAT and GPx were assessed according to the methods of Paoletti et al. (1986), Babo and Vasseur (1992) and Paglia and Valentine (1967) respectively, using purified bovine enzymes (Sigma) as standards. For GST activity determination, chlorodinitrobenzene was used as substrate (Habig et al., 1974) and purified GST from equine liver (Sigma) as a standard. GSH and oxidised

glutathione (GSSG) concentrations were measured by the spectrophotometric method of Vandeputte et al. (1994). All measures were carried out at room temperature in microtiter plates, using a microplate reader (Power Wave_x – Bio-Tek instruments). EROD activity was determined by a fluorimetric method in black microplates (Flammarion et al., 1998) using a microplate spectrofluorimeter reader (Victor² Wallac, Perkin-Elmer).

2.3. Chemical analysis

Hepatic copper and zinc concentrations were determined after digestion of homogenized liver using suprapure nitric acid (Carlo Erba) at 120 °C for at least 2 h (1 mL of nitric acid per 20 µL of sample). The volume was then adjusted to 20 mL with de-ionized water. Metal concentrations were determined by inductively coupled plasma optical emission spectrometry (Ultima 2, Jobin Yvon). Quality assurance controls based on certified reference materials of the National Research Council of Canada (DOLT-2, dogfish liver) were carried out to validate this procedure.

2.4. Statistics

All statistical calculations were performed with SPSS 10.1 software. Since data were not normally distributed (Lilliefors' test) and/or exhibited heterogeneous variances (Levene's test), non-parametric tests (Kruskal-Wallis ANOVA and Mann-Whitney U test) were performed (significant for $p < 0.05$). Data are reported as means $\pm$ standard error.

3. Results

During this experiment, low mortality was observed for fish exposed to 200 µg.L⁻¹. Four out of 30 fish died during the first five days and one died at day 16. This mortality prevented the analysis of hepatic copper concentration and biomarkers at day 21. At this concentration, the surviving fishes appeared stressed as indicated by a dark colour, a lower mobility and a loss of appetite. Those behaviour alterations were not observed in the other groups.

A significant bioaccumulation of copper was observed in liver of fish exposed to 200 $\mu\text{g.L}^{-1}$ for 8 and 12 days and to 100 $\mu\text{g.L}^{-1}$ for 12 days (Fig. 1A). At day 21, a trend is noted for copper accumulation at 100 $\mu\text{g.L}^{-1}$ although this was not statistically significant. An increase of zinc concentration in liver was noticed after 12 days of exposure to 100 and 200 $\mu\text{g.L}^{-1}$, whereas a transient increase of zinc level was noticed after 4 days at 25 $\mu\text{g.L}^{-1}$ (Fig. 1B).

No significant variation of somatic indexes was observed for any copper concentration nor any sampling time (data not shown), whereas several differences were observed at the biochemical level.

As seen in Fig. 2, a rapid and transient induction of antioxidant enzymes was measured within the first week of copper exposure. SOD activity was clearly increased at day 4, although this effect was statistically significant only at 25 $\mu\text{g.L}^{-1}$ and returned to the basal level at day 8 (Fig. 2A). CAT activity was characterised by a general increase for all copper treatments after 4 days (Fig. 2B). This increase was maintained up to 12 days in fish exposed to 200 $\mu\text{g.L}^{-1}$. GPx activity showed a significant increase after 8 days in fish exposed to 200 $\mu\text{g.L}^{-1}$ and returned to the basal level at day 12 (Fig. 2C).

In control fish, total and oxidized GSH were characterised by an important variation of the basal level during the experiment (Fig. 3). Nevertheless, some effects of copper can be seen for this biomarker. A significant decrease of both total and oxidised GSH content was noticed in fish exposed to 200 $\mu\text{g.L}^{-1}$ after 4 and 8 days of exposure (Fig. 3A and 3B). This depletion was not accompanied by an impairment of glutathione redox status as no effect on GSSG/GSH ratio was noticed (not shown). After 12 days of exposure, this effect was reversed and fish exposed to the highest copper concentration presented a total glutathione level significantly higher than control fish.

For EROD activity measured in fish exposed 21 days to 100 $\mu\text{g.L}^{-1}$, a significant inhibition was observed as compared to the control group (Fig. 4A). For the other concentrations and

sampling time, no significant variation of this enzyme was detected as compared to the control group. The hepatic GST activity did not vary significantly with respect to either copper concentration or time of exposure (Fig. 4B).

4. Discussion

Despite the low mortality observed after 4 days at the highest copper concentration, our exposure conditions were clearly sublethal. For three-spined stickleback exposed to copper sulphate, the 50 % lethal concentrations (LC50) were reported to be 2.78, 1.96 and 1.59 mg Cu.L⁻¹ after 24, 48 and 72 hours respectively (Svecevicius and Vosyliene, 1996), which are much higher than the concentrations used in our study. However, comparison is rather difficult since copper toxicity is highly dependent on several abiotic factors, such as pH, water hardness and alkalinity that influence copper speciation. The physico-chemical forms in which the metal is present in the aquatic environment (free ionic Cu²⁺, dissolved inorganic or organic complexes, associated with particular matter) will indeed determine its bioavailability for the organisms. In our study, the slightly basic conditions (pH 8.35) together with the elevated water hardness (367 mg.L⁻¹ as CaCO₃) and very low dissolved organic carbon (DOC) suggest that a major fraction of the copper in the water is present as inorganic complexes (mainly CuCO₃) and only a small fraction as free Cu²⁺. Nevertheless, our results showed that these inorganic copper complexes are also bioavailable to the organisms since significant accumulation and biochemical responses to copper were observed.

At day 12, copper did accumulate at similar levels in the liver of fish exposed either to 100 or 200 µg.L⁻¹ (Fig. 1). Copper concentrations in liver measured during this laboratory exposure are in accordance with the levels observed in three-spined stickleback sampled in the field (Bervoets et al., 2001). This may indicate that saturation for copper accumulation occurred right from 100 µg.L⁻¹ and is in agreement with the fact that, in rainbow trout (*Oncorhynchus mykiss*), the time to half saturation for copper in the liver is 6 days (McGeer et al., 2000). At

day 12, the bioconcentration factor ($BCF = ([Cu]_{\text{exposed}} - [Cu]_{\text{control}}) / [Cu]_{\text{water}}$) reached 401 and 208 in the 100 and 200 $\mu\text{g.L}^{-1}$ groups-exposed fish respectively, which are in the same order of magnitude to those previously reported for zebrafish (*Brachydanio rerio*) exposed for 14 days to copper (Paris-Palacios et al., 2000). These relatively low BCF, as compared to those reported for other metals such as Cd, may reflect the existence of homeostatic regulation of copper levels in the liver (McGeer et al., 2000).

The biomarker responses in stickleback were in accordance with the known capacity of copper to promote oxidative stress and to modulate enzymatic and non enzymatic antioxidant expressions in fish tissues, as shown by a rapid and transient increase of SOD, CAT and GPx enzymes and by a decrease of total GSH. In fish, antioxidant enzymes have been shown to be either induced or inhibited by copper, depending on the dose, the species and/or the route of exposure. Zebrafish submitted to 40 and 140 $\mu\text{g Cu.L}^{-1}$ as CuSO_4 presented a clear-cut induction of CAT and GST within two weeks of waterborne copper exposure (Paris-Palacios et al., 2000). Conversely, in carp, CAT and GST were inhibited after 96 hours of exposure to 100 and 250 $\mu\text{g Cu.L}^{-1}$ as CuSO_4 (Dautremepuits et al., 2002). Using intra-peritoneal injection of copper, SOD inhibition was reported in carp after 48 hours (Varanka et al., 2001), while this enzyme was rapidly induced in gilthead seabream (*Sparus aurata*; Pedrajas et al., 1995). In stickleback, the rapid response of both enzymatic and non enzymatic antioxidants is in line with the view that copper is able to act through different oxidative mechanisms, including generation of ROS and direct interaction with intracellular thiols.

SOD are metalloenzymes that play a key role in the defence against ROS by transforming superoxide anions into hydrogen peroxide (Yim et al., 1993). The total SOD activity that we measured in the post-mitochondrial fraction corresponds mainly to the Cu,Zn-SOD activity, the major isoform in the cytosol, nucleus and peroxisomes. Thus, the rapid and marked drop in SOD activity at day 4 may result from direct binding of the metal to the enzyme, as

previously suggested in fish (Pedrajas et al., 1995). In addition, induction of CAT and GPx indicated that copper induce (hydro)peroxides species in the liver. It is likely that the resulting H_2O_2 produced from increased SOD activity was the source for subsequent increase of CAT activity after the fourth day of copper exposure (Fig. 2B). Moreover, the known capacity of copper to oxidize membrane lipids in fish (Pedrajas et al., 1995), together with the role of GPx in the detoxification of lipoperoxidation products (Matés, 2000) may also account for the observed increase of this enzyme at the day 8 (Fig. 2C). Unfortunately, the induction of lipid damages by copper was not measured in this study to confirm this hypothesis.

Reduced glutathione is considered as a first line of cellular defence against metals by chelating and detoxifying them, scavenging oxyradicals and participating in detoxification reactions catalysed by glutathione peroxidases (Sies, 1999). The effects that we observed on this parameter, i.e. inhibition of total GSH (both reduced and oxidised GSH) without any impairment of GSSG/GSH ratio, is in line with the known ability of copper to interact with GSH forming stable GS-Cu(I) binding complexes. Besides, as proposed by Canesi et al. (1999), it is likely an inhibitory effect on GSH regenerating systems (e.g. GSH reductase or GSH synthetase), which was not assessed in our study, that could have contributed to the observed depletion of total GSH. The fact that we did not observe any impairment of GSSG/GSH ratio by copper does not reach previous report in gilthead seabream fish exposed by copper intra peritoneal injection (Rodriguez-Ariza et al., 1994). Finally, although the mechanism by which copper inhibited GSH in fish is not fully understood and remains complex, this rapid depletion may have contributed to the early toxicity observed at the higher concentrations as suggested by Connors and Ringwood (2000).

Interestingly, biochemical responses were more rapid than copper accumulation, suggesting the involvement of differential mechanisms in copper metabolism and accumulation. Cellular copper metabolism involves copper intake by GSH and subsequent formation of GS-Cu(I)

complex, from which the metal is further transferred to metallothionein (MT) apoproteins where it is stored (Freedman et al., 1989; Connors and Ringwood, 2000). The GS-Cu(I) pool is the source for the synthesis of other metalloenzymes such as SOD or cytochrome oxidase, as well as for ROS production through GS-Cu(I) oxidation (Freedman et al., 1989). In our study, antioxidant responses occurred within the first week of exposure and then recovered concomitantly with copper accumulation in the liver (Fig. 1 and 3). This indicates that at day 8 other detoxification systems, such as MTs, have been induced to replace GSH in the uptake and sequestration of copper (Freedman et al., 1989). Moreover, the role of MTs in cellular protection against oxidative stress (Viarengo et al., 2000) argues in the way that these proteins could have been involved in response to copper in three-spined stickleback. This hypothesis is strengthened by the finding that copper accumulation was accompanied by increased hepatic zinc concentrations. In fact, a significant positive correlation was observed between these two metals ($r^2 = 0.596$, $p < 0.001$, Spearman correlation). The sequestration of metals such as cadmium and copper occurs by displacement of zinc from their binding sites, leading to a release of free zinc that could induce *de novo* MT synthesis (Hollis et al., 2001; Roesijadi, 1996). Relationships between liver metal content and MT expression have been described in aquatic organisms (Hollis et al., 2001) but further studies on MT response to copper in stickleback will be necessary to confirm these hypotheses for this fish species.

EROD inhibition by metals has been reported in several *in vitro* and *in vivo* studies on fish (Ghosh et al., 2001; Stien et al., 1997). In our study, we detected an inhibition of EROD activity in fish exposed to $100 \mu\text{g.L}^{-1}$ for 21 days (Fig. 4). This effect, which involves direct interaction of copper with the enzyme leading to a conformational change of the protein (Kim et al., 2002), may reflect cellular toxicity induced by an increased content of metal in the cell (Stien et al., 1997). Although this effect could be considered as minor regarding the responses of the other biomarkers to copper, EROD inhibition should be taken into account when using

it as a biomarker of exposure in fish exposed to a mixture of chemicals, i.e. containing both organic and metallic compounds, which mostly occurs in environmental pollution.

In summary, this dynamic study showed that waterborne copper induced several biochemical responses in three-spined stickleback, in a transient manner, suggesting the occurrence of adaptive processes. Rapidly after the beginning of the exposure, antioxidant biomarkers were induced in order to compensate the oxidative stress generated by the metal. In a second phase, copper accumulation was correlated to a recovery of GSH and a return of antioxidant enzymes to the basal level, suggesting that other detoxification mechanisms, such as MTs, have been involved to allow fish adaptation to copper. This study shows the suitability of three-spined stickleback as a model fish species to study oxidative stress biomarker responses to waterborne metal exposure. Field studies that evaluate several biomarkers, including oxidative stress biomarkers, EROD and vitellogenin, are under progress to determine the potential of stickleback as an indicator species of sublethal stress in multipollution contexts.

Acknowledgements

We wish to thank Jean-Pierre Blanquet for the measure of physico-chemical parameters of the water and Dominique Hervin for technical assistance in fish maintenance. This work was supported by the French Budget Civil de la Recherche et du Développement (BCRD 00-102).

References

- Andersson, P.L., Berg, A.H., Bjerselius, R., Olsén, H., Orm, P.E., Tysklind, M., 2001. Bioaccumulation of selected PCBs in zebrafish, three-spined stickleback and arctic char after three different routes of exposure. *Arch. Environ. Contam. Toxicol.* 40, 519-530.
- Babo, S., Vasseur, P., 1992. In vitro effects of thiram on liver antioxidant enzyme activities of rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 22, 61-68.

- Bervoets, L., Blust, R., Verheyen, R., 2001. Accumulation of metals in the tissues of three spined stickleback (*Gasterosteus aculeatus*) from natural fresh waters. *Ecotoxicol. Environ. Saf.* 48 117-127.
- Bradford, M.M., 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.
- Canesi, L., Viarengo, A., Leonzio, C., Filippelli, M., Gallo, G., 1999. Heavy metals and glutathione metabolism in mussel tissues. *Aquat. Toxicol.* 46, 67-76.
- Chan, P.C., Peller, O.G., Kesner, L., 1982. Copper (II)-catalyzed lipid peroxidation in liposomes and erythrocyte membrane. *Lipids* 17, 331-337.
- Connors, D.E., Ringwood, A.H., 2000. Effects of glutathione depletion on copper cytotoxicity in oysters (*Crassostrea virginica*). *Aquat. Toxicol.* 50, 341-349.
- Dautremepuits, C., Betoulle, S., Vernet, G., 2002. Antioxidant response modulated by copper in healthy or parasitized carp (*Cyprinus carpio* L.) by *Ptychobothrium* sp. (Cestoda). *Biochim. Biophys. Acta* 1573, 4-8.
- Falandysz, J., Strandberg, L., Strandberg, B., Rappe, C., 1998. Polychlorinated naphthalenes in three-spined stickleback *Gasterosteus aculeatus* from the Gulf of Gdansk. *Chemosphere* 37, 2473-2487.
- Flammarion, P., Migeon, B., Garric, J., 1998. Statistical analysis of cyprinid ethoxyresorufin-O-deethylase data in a large french watershed. *Ecotoxicol. Environ. Saf.* 40, 144-153.
- Freedman, J.H., Ciriolo, M.R., Peisach, J., 1989. The role of glutathione in copper metabolism and toxicity. *J. Biol. Chem.* 264, 5598-5605.
- Ghosh, M.C., Ghosh, R., Ray, A.K., 2001. Impact of copper on biomonitoring enzyme ethoxyresorufin-o-deethylase in cultured catfish hepatocytes. *Environ. Res.* 86, 167-173.

- GRNC, 2002. Risque pour l'environnement - Evaluation des risques associés aux rejets chimiques des installations nucléaires du Nord-Cotentin. Groupe de Radioécologie du Nord Cotentin, pp. 210.
- Grosell, M., Nielsen, C., Bianchini, A., 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. *Comp. Biochem. Physiol. C* 133, 287-303.
- Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-Transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130-7139.
- Handy, R.D., Runnalls, T., Russel, P.M., 2002. Histopathologic biomarkers in three spined sticklebacks, *Gasterosteus aculeatus*, from several rivers in Southern England that meet the freshwater fisheries directive. *Ecotoxicology* 11, 467-479.
- Hollis, L., Hogstrand, C., Wood, C.M., 2001. Tissue-specific cadmium accumulation, metallothionein induction, and tissue zinc and copper levels during chronic sublethal exposure in juvenile rainbow trout. *Arch. Environ. Contam. Toxicol.* 41, 468-474.
- Holm, G., Norrgren, L., Andersson, T., Thuren, A., 1993. Effects of exposure to food contaminated with PBDE, PCN or PCB on reproduction, liver morphology and cytochrome P450 activity in the three-spined stickleback, *Gasterosteus aculeatus*. *Aquat. Toxicol.* 27, 33-50.
- Katsiadaki, I., Scott, A.P., Hurst, M.R., Matthiessen, P., Mayer, I., 2002. Detection of environmental androgens: a novel method based on enzyme-linked immunosorbent assay of spiggin, the stickleback (*Gasterostus aculeatus*) glue protein. *Environ. Toxicol. Chem.* 21, 1946-1954.
- Kim, J.S., Ahn, T., Yim, S.K., Yun, C.H., 2002. Differential effect of copper II on the cytochrome P450 enzymes and NADPH-cytochrome P450 reductase : inhibition of cytochrome P450-catalysed reactions by copper II ion. *Biochemistry* 41, 9438-9447.

- Matés, J.M., 2000. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology* 153, 83-104.
- McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout 2: tissue specific metal accumulation. *Aquat. Toxicol.* 50, 245-256.
- Ozawa, T., Ueda, J., Shimazu, Y., 1993. DNA single strand breakage by copper (II) complexes and hydrogen peroxide at physiological conditions. *Biochem. Mol. Biol. Int.* 31, 455-461.
- Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clinic. Med.* 70, 158-169.
- Paoletti, F., Aldinucci, D., Mocali, A., Caparrini, A., 1986. A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. *Anal. Biochem.* 154, 536-541.
- Paris-Palacios, S., Biagianti-Risbourg, S., Vernet, G., 2000. Biochemical and (ultra)structural hepatic perturbations of *Brachydanio rerio* (Teleostei, Cyprinidae) exposed to two sublethal concentrations of copper sulfate. *Aquat. Toxicol.* 50, 109-124.
- Pedrajas, J.R., Peinado, J., Lopez-Barea, J., 1995. Oxidative stress in fish exposed to model xenobiotics. Oxidatively modified forms of Cu,Zn-superoxide dismutase as potential biomarkers. *Chem.-Biol. Interact.* 98, 267-282.
- Pottinger, T.G., Carrick, T.R., Yeomans, W.E., 2002. The three-spined stickleback as an environmental sentinel: effects of stressors on whole-body physiological indices. *J. Fish Biol.* 61, 207-229.

- Rodriguez-Ariza, A., Toribio, F., Lopez-Barea, J., 1994. Rapid determination of glutathione status in fish liver using high-performance liquid chromatography and electrochemical detection. *J. Chromatogr. B* 656, 311-318.
- Roesijadi, G., 1996. Metallothionein and its role in toxic metal regulation. *Comp. Biochem. Physiol. C* 113, 117-123.
- Roy, J., 1997. Environmental contaminants encyclopedia : copper entry. National Park Service, Water Resources Divisions, pp. 99.
- Sies, H., 1999. Glutathione and its role in cellular functions. *Free Rad. Biol. Med.* 27, 916-921.
- Stien, X., Risso, C., Gnassia-Barelli, M., Romeo, M., Lafaurie, M., 1997. Effect of copper chloride in vitro and in vivo on the hepatic EROD activity in the fish *Dicentrarchus labrax*. *Environ. Toxicol. Chem.* 16, 214-219.
- Sturm, A., Wogram, J., Segner, H., Liess, M., 2000. Different sensitivity to organophosphates of acetylcholinesterase and butyrylcholinesterase from three-spined stickleback (*Gasterosteus aculeatus*): application in biomonitoring. *Environ. Toxicol. Chem.* 19, 1607-1615.
- Svecevicius, G., Vosyliene, M.Z., 1996. Acute toxicity of copper to common freshwater fishes of Lithuania. *Ekologija* 2, 17-21.
- Turnlund, J.R., 1999. Copper. In: Shils, M.E., Olson, J.A., Shike, M., Ross, A.C. (Eds.), *Modern nutrition in health and disease*. Lippincott Williams and Wilkins, Baltimore, M.D.
- Van der Oost, R., Beyerb, J., Vermeulen, N., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* 13, 57-149.
- Vandeputte, C., Guizon, I., Genestie-Denis, I., Vannier, B., Lorenzon, G., 1994. A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated

- cells: performance study of a new miniaturized protocol. *Cell Biol. Toxicol.* 10, 415-421.
- Varanka, Z., Rojik, I., Varanka, I., Nemcsok, J., Abraham, M., 2001. Biochemical and morphological changes in carp (*Cyprinus carpio L.*) liver following exposure to copper sulfate and tannic acid. *Comp. Biochem. Physiol. C* 128, 467-478.
- Viarengo, A., Burlando, B., Ceratto, N., Panfoli, I., 2000. Antioxidant role of metallothioneins: A comparative overview. *Cell. Mol. Biol.* 46, 407-417.
- Yim, M., Chock, P., Stadtman, E., 1993. Enzyme function of copper, zinc superoxide dismutase as a free radical generator. *J. Biol. Chem.* 268, 4099-4105.

Fig. 1. Effect of copper treatment on hepatic copper (A) and zinc (B) concentration. Data represent the means $\pm$ S.E. of measurements on six fish per group except for the 100 $\mu\text{g.L}^{-1}$ on day 21 (n=5). *, statistically different from t_0 control ($P<0.05$).

Fig. 2. Effect of copper treatment on hepatic SOD (A), catalase (B) and GPx (C) activities. Data represent the means $\pm$ S.E. of measurements on six fish per group except for the 100 $\mu\text{g.L}^{-1}$ on day 21 (n=5). *, statistically different from t_0 control ($P<0.05$).

Fig. 3. Effect of copper treatment on activities of total glutathione (A) and oxidized glutathione (B) content. Data represent the means $\pm$ S.E. of measurements on six fish per group except for the 100 $\mu\text{g.L}^{-1}$ on day 21 (n=5). *, statistically different from 0 $\mu\text{g.L}^{-1}$ control ($P<0.05$).

Fig. 4. Effect of copper treatment on biotransformation enzyme activities : EROD (A) and GST (B). Data represent the means $\pm$ S.E. of measurements on six fish per group except for the 100 $\mu\text{g.L}^{-1}$ on day 21 (n=5). *, statistically different from t_0 control ($P<0.05$).

Fig. 1.

Fig.2.

Fig. 3.

Fig. 4.

Table 1 Actual copper concentrations measured in the water throughout the experiment^a.

Nominal copper concentration ($\mu\text{g.L}^{-1}$)	Measured copper concentration ($\mu\text{g.L}^{-1}$)
0	<0.05
25	24.6 ± 1.5
100	91.6 ± 2.8
200	176.8 ± 2.3

a : measurements were performed in water sampled 2 hours after each water renewal. Data are mean $\pm$ SE (n=7).