

Effect of copper exposure on growth, condition indices and biomarker response in juvenile sole *Solea senegalensis*

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SUMMARY: Juvenile *Solea senegalensis* were exposed to different concentrations of waterborne copper (Cu) for 15 days in static conditions with continuous aerated artificial salt water at 20°C ($\pm 0.8^\circ\text{C}$) with a normal photoperiod (10 h/14 h light/dark) and daily feeding. Several measures of exposure and effects were determined: 1) biomarkers - metallothioneins and lipid peroxidation level; 2) mass indices - growth rate and morphometric condition indices; and 3) biochemical condition indices - RNA:DNA ratio and lipid and protein content in fish tissues. Copper exposure triggered the response of the biomarkers and resulted in reduced growth and condition (RNA:DNA and lipid content), but the morphometric indices did not vary. The physiological costs of Cu contamination on condition suggested that lipid reserves were allocated as an energy source to enable exposed fish to respond to Cu toxicity as well as to maintain positive growth rates and protein synthesis throughout the experiment, although with lower growth rates than the control fish. This study showed the importance of selecting suitable biomarkers according to contaminant source, fish species and their life-history stage. In addition, the use of several biomarkers of exposure, growth and specific condition indices can improve fish health determination and should be considered in evaluations of the effects of environmental contaminants on fish.

Keywords: fish growth, fish condition, metallothioneins, lipid peroxidation, juvenile sole, biomarkers.

RESUMEN: EFECTO DE LA EXPOSICIÓN AL COBRE SOBRE EL CRECIMIENTO, ÍNDICES DE CONDICIÓN Y RESPUESTA EN BIOMARCADORES EN JUVENILES DE LENGUADO *SOLEA SENEGALENSIS*. — Se expusieron juveniles de *Solea senegalensis* a diferentes concentraciones de cobre (Cu) en el agua durante 15 días en condiciones estáticas, con agua salada artificial continuamente aireada, a 20°C ($\pm 0.8^\circ\text{C}$), fotoperiodo normal (10 h/14 h luz/oscuridad) y alimentación diaria. Se determinaron varias medidas de exposición y sus efectos: 1) biomarcadores - metalotioneínas y nivel de peroxidación de lípidos; 2) índices de masa - tasa de crecimiento e índices de condición morfométricos; y 3) índices de condición bioquímicos - RNA:DNA y contenido en lípidos y proteínas en tejidos de peces. La exposición al cobre disparó la respuesta de los biomarcadores y resultó en una reducción del crecimiento y condición (RNA:DNA y contenido lipídico), en cambio los índices morfométricos no variaron. El coste fisiológico de la contaminación por Cu en la condición sugiere que las reservas lipídicas se destinaron como fuente de energía para conseguir que los peces expuestos respondieran a la toxicidad del Cu así como a mantener tasas de crecimiento positivas y síntesis proteica a lo largo del experimento, aunque con menores tasas de crecimiento que los peces control. Este estudio evidencia la importancia de seleccionar adecuadamente los biomarcadores de acuerdo con la fuente de contaminación, la especie y su estadio de desarrollo. Además la utilización de varios biomarcadores de exposición, crecimiento e índices de condición específicos puede mejorar la determinación del estado de salud de los peces y debería ser considerado al evaluar los efectos de contaminantes ambientales en peces.

Palabras clave: crecimiento en peces, condición en peces, metalotioneínas, peroxidación lipídica, lenguados juveniles, biomarcadores.

INTRODUCTION

Aquatic ecosystems are increasingly threatened by the presence of organic and inorganic anthropogenic pollutants (Daskalakis and O'Connor, 1995). Fish behaviour and physiological responses to specific and multiple stressors have been extensively used to determine individual health and population status, and to assess habitat quality (e.g. Lloret and Planes, 2003; Marchand *et al.*, 2003; Fonseca *et al.*, 2006). Few studies have integrated indicators of exposure to contamination and effects on fish's health and condition, and most have reported unclear or limited responses of individual growth and condition to contaminant exposure (De Boeck *et al.*, 1997; Wu *et al.*, 2002; Humphrey *et al.*, 2007). However, recent work has outlined the importance of applying a multibiomarker approach to assess the causes and effects of stressors on marine systems (e.g. Adams, 2005; Broeg and Lehtonen, 2006).

Biomarkers are considered, in a broad sense, as measurements of changes in biological responses linked to a potential hazard, which may be chemical, physical or biological (van der Oost *et al.*, 2003). When assessing the adverse effects of environmental contamination, exposure biomarkers are commonly used as early signs or functional measures of toxicity of specific contaminants or suites of contaminants (Shugart *et al.*, 1992; Adams, 2002). However, a direct relationship between biomarkers' response to pollutants and further ecological consequences at the individual or higher level is difficult to establish, and still needs further integration of both individual adaptability and the influence of natural attributes (van der Oost *et al.*, 2003).

Trace metals are widespread pollutants in estuaries and coastal areas. Common biomarkers of exposure induced by trace metal toxicity are metallothioneins (MT), thiol-rich proteins that bind to the toxic metals and enable their excretion (Roesijadi, 1996), as well as antioxidant enzymes (e.g. superoxide dismutase [SOD], catalase [CAT]). These defence mechanisms have energetic and physiological costs for the individual and often result in trade-offs with other biological processes, namely reproduction and growth (Eastwood and Couture, 2002; Marchand *et al.*, 2004). Fish growth and condition are linked to the ecological status of the environment (Beyers *et al.*, 1999; Buckley *et al.*, 1999), yet they may vary with non-pollutant stresses, masking the effects of pollution on the ecosystem, which empha-

sises the importance of experimental investigation and background information. It is also imperative to understand how the biochemical mechanisms that induce the biomarkers' responses influence individual growth and condition. A wider ecological scope of the stressors' cost to the individual may be attained by the simultaneous determination of several biological endpoints that correspond to different response levels (Den Besten, 1998; Adams, 2002), from the molecular to the whole individual level.

In the present study, juvenile Senegalese sole, *Solea senegalensis* Kaup, 1858, were exposed to naturally occurring copper (Cu) concentrations observed in Portuguese estuaries. This essential trace metal (toxic at higher concentrations) is very common in estuaries and coastal waters, and several studies have demonstrated its deleterious effects on growth rates, condition and ion balance of different fish species (Marr *et al.*, 1996; De Boeck *et al.*, 1997; Blanchard and Grosell, 2006). Thus, Cu was selected as an example of trace-metal toxicity in fish, and the different biomarkers determined were chosen based on: (1) their sensitivity to Cu exposure (biomarkers of exposure – levels of MT and lipid peroxidation); (2) the information on general condition and growth (morphometric indices and growth rates); (3) the information on physiological condition related to growth and to the energy pathways for juvenile fish. The aims of this study were to determine how sublethal copper exposure simultaneously influences growth, condition indices and biomarker response in juvenile sole, and to determine the usefulness of these indicators for future habitat quality assessment studies.

MATERIAL AND METHODS

Experimental procedures

Cultured juvenile *Solea senegalensis* ($2.3\text{--}6.7\text{ g} \pm 0.01\text{ g}$ wet weight, $5.7\text{--}8.0\text{ cm} \pm 0.1\text{ cm}$ total length) from the same spawning batch were exposed to three sublethal copper treatments at the concentrations of 5, 25 and $100\text{ }\mu\text{g Cu L}^{-1}$, plus a clean water control group, over a 15-day experiment period. The lower copper concentration was chosen based on naturally occurring values determined by water quality control measurements in Portuguese estuaries (source INAG - National Water Institute, 2005) and also reported for other European estuaries (Hall and Anderson,

1999). The other two copper concentrations were chosen in order to simulate episodic contamination events. A total of 51 fish were analysed, 3 at the beginning of the experiment and 48 fish divided into four groups of 12 individuals and subjected to the Cu treatments, plus the control trial. Two separate 14 L tanks were used per treatment, each containing 6 fish individually tagged, measured and weighed at the beginning of the experiment, following a two-week acclimation period. During the acclimation and the experimental period fish were fed with a very common natural prey, the polychaete *Hedistes diversicolor*. The experiment was carried out in static exposure conditions, with continuous aerated artificial salt water at 20°C ($\pm 0.8^\circ\text{C}$), normal photoperiod (10 h/14 h light/dark) and daily feeding. Salt water (18 g L^{-1}) was prepared with tap water ($7.3 \pm 0.5 \text{ pH}$) and artificial salt (Instant Ocean) throughout the 15-day trial and for the two-week acclimation period, to ensure daily water renewal. Copper exposure was carried out by the dilution in distilled water of copper sulphate hydrate (Sigma) and Cu concentrations were monitored during the experimental period by atomic absorption spectrophotometry. Fish were collected on days 3, 5, 10 and 15 (3 fish per treatment per day) and were immediately sacrificed with a cut on the anterior spine. Fish length and body and liver weight were determined. Dissected liver and muscle samples were initially frozen in liquid nitrogen, and then stored at -80°C for subsequent analysis.

Analytical procedures for determining growth, condition indices and biomarker response

Instantaneous growth rates (G) were determined as $G = (\ln W_2 - \ln W_1) / (t_2 - t_1)$, where W_1 and W_2 are fish total body weight at times t_1 (beginning of the experiment) and t_2 (time of collection).

The morphometric condition indices determined for juvenile sole were the condition factor, $K = W / L^3$, where W is the wet weight (in grams) and L is the total length (in centimetres) (Ricker, 1975), and the hepatosomatic index, $\text{HSI} = W_{\text{Liver}} / W$, where W_{Liver} is the liver weight.

Biochemical condition indices, namely RNA:DNA ratio and protein and lipid content, were determined in duplicate for individual muscle samples. RNA, DNA and protein measurements were determined from two replicates of ca. 150 mg wet weight each from the anterior-dorsal portion of the muscle, whereas the remainder of the muscle was

used to determine lipid content (average 400 mg wet weight). Nucleic acid quantification was carried out by the fluorometric method described in Caldarone *et al.* (2001), adapted to a cuvette assay (Fonseca *et al.*, 2006). Muscle samples were homogenised in N-lauroylsarcosine Tris-EDTA buffer (0.1%, pH 7.5), centrifuged, and aliquots of the supernatant were used for the quantification of RNA and DNA fluorescence with ethidium bromide (emission wavelength 590 nm, excitation wavelength 360 nm). RNA fluorescence was determined as the difference between total nucleic acid fluorescence and DNA fluorescence following treatment with RNase A (Sigma). Standard curves were previously determined using pure calf-thymus DNA (Calbiochem) and 18S- and 28S-rRNA (Sigma), and the ratio between the two slopes from each standard curve was 4.04.

Protein content determination was based on the Lowry procedure (Lowry *et al.*, 1951). Aliquots of the samples' supernatant used for RNA:DNA quantification reacted with Folin's and Copper reagent, and the complex's absorbance was read at 750 nm and compared with a standard curve previously constructed with a dilution series of bovine serum albumin (Calbiochem).

Lipid muscle content was determined with an adaptation of the sulphophosphovanilin method described by Knight *et al.* (1972). Prior to lipid analyses, a gravimetric assay ($n = 3$) was used to determine the ratio of saturated and unsaturated fatty acids in sole muscle, in order to construct a representative calibration curve. The standards used were oleic and palmitic acid (Sigma) in a 75:25 ratio. Lipid extraction followed the homogenisation of the remaining muscle tissue with methanol:ethanol:water (2:2:1.8 ratio).

Metallothionein concentration (MT) was determined on individual liver samples, after the cytosol had been separated from the residual fraction (30000 g, 45 min, 4°C), heat-treated at 80°C for 10 min, and subsequently centrifuged (30000 g, 45 min, 4°C). Aliquots of the heat-treated cytosol (25 μl) were used to quantify the MT level by differential pulse polarography (DPP) according to the method described by Bebianno and Langston (1989).

Lipid peroxidation was determined in liver samples according to the method described by Erdelmeier *et al.* (1998) which measures the amount of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) produced during the decomposition of polyunsaturated fatty acid peroxides of membrane

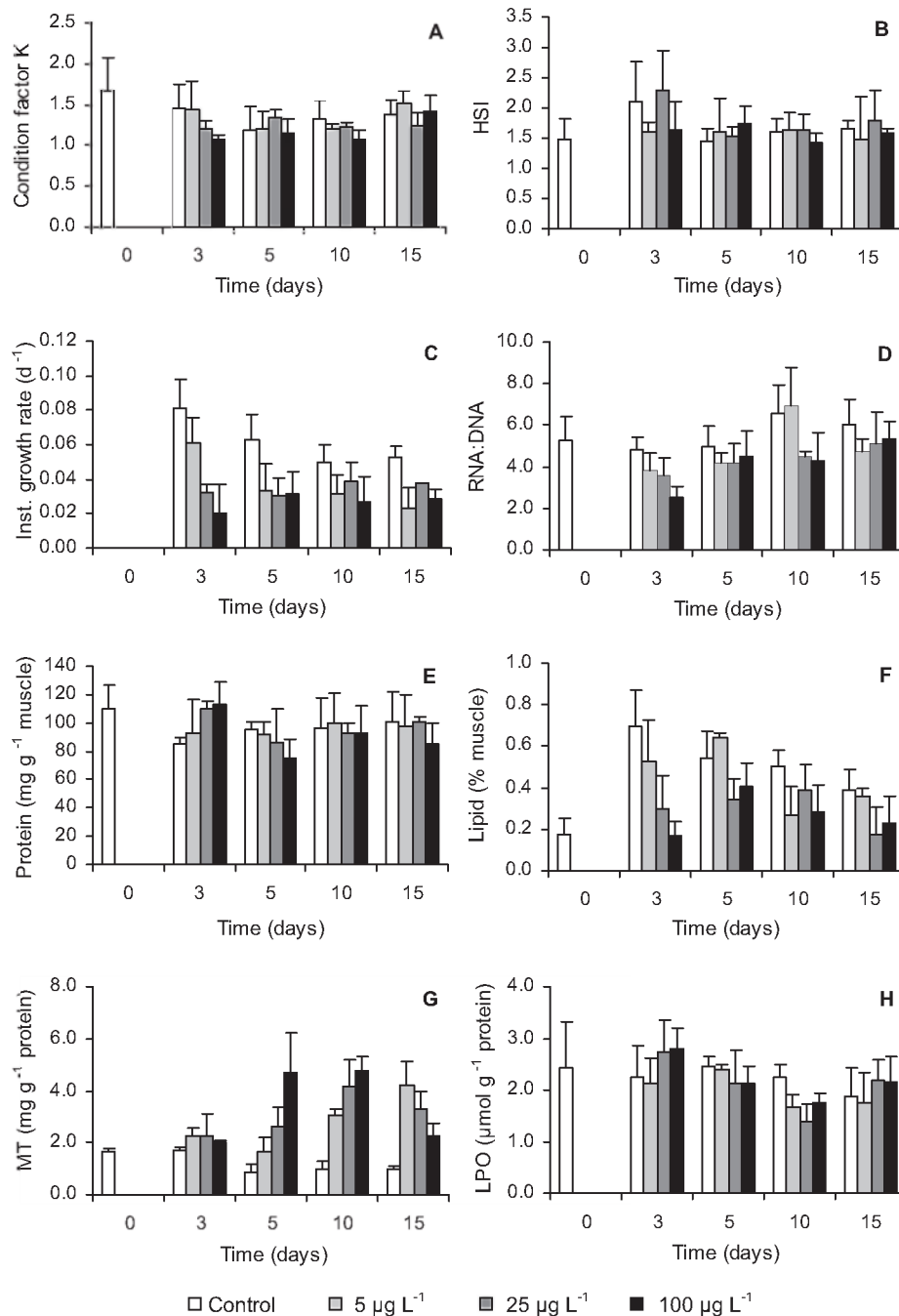


FIG. 1. – Biomarker variation with Cu treatments (5, 25 and 100 µg L⁻¹) and control group, over the 15-days exposure period (mean values and standard error, n = 51): (A) condition factor K; (B) hepatosomatic index HSI; (C) instantaneous growth rate in mass (d⁻¹); (D) RNA:DNA ratio; (E) protein content (mg g⁻¹ muscle); (F) lipid percentage (% muscle); (G) metallothionein level (MT, mg g⁻¹ protein) and (H) lipid peroxidation (LPO, µmol g⁻¹ protein).

lipids. The concentration of lipid peroxidation was expressed as µmoles of MDA and 4-HNE g⁻¹ total protein.

Statistical analyses

Differences in fish growth rates, condition indices and biomarker response between Cu treatments

and periods were tested by two-way analysis of variance (ANOVA), followed by post-hoc Tukey tests whenever null hypotheses were refuted. Correlation analyses were used to test associations between all variables: instantaneous growth rates, condition indices and biomarkers. All analyses were performed using Statistica software and a 0.05 (or lower) significance level was considered in all test procedures.

RESULTS

Morphometric indices, condition factor K and HSI did not show significant differences between copper treatments over the 15 days of the experiment (Fig. 1A and 1B, respectively; Table 1, $p > 0.05$). Mean instantaneous growth rates in mass were affected by Cu exposure with significant differences observed between the control and the 25 $\mu\text{g L}^{-1}$ and 100 $\mu\text{g L}^{-1}$ Cu concentrations (Fig. 1C, Table 1, $F_{3,48} = 5.44$, $p < 0.01$). Lower growth rates were first observed for higher Cu concentrations, but by day 15 all Cu treatments had limited mean instantaneous growth rates.

Biochemical condition indices also reflected Cu contamination, as mean RNA:DNA ratios and lipid content were significantly lower in fish exposed to Cu (Fig. 1D and 1F), although lipid content differed only between the control and the highest Cu concentration, whereas mean RNA:DNA was significantly lower than the control for all Cu treatments (Table 1, $F_{3,48} = 3.09$ and $F_{3,48} = 4.46$, $p < 0.05$, respectively). RNA:DNA values also varied significantly with time, since higher values were observed towards the end of the experiment, by days 10 and 15, relatively to day 3 (Table 1, $F_{3,48} = 5.92$, $p < 0.05$). Muscle DNA concentration remained relatively constant over the experimental period, with no significant differences between treatments ($p > 0.05$). Total protein content did not vary significantly with Cu exposure during the 15-day trial (Fig. 1E, Table 1, $p > 0.05$).

Exposure biomarkers, metallothioneins (MT) and lipid peroxidation level (LPO) indicated Cu-mediated damage, although with different response levels. MT response to Cu exposure was more extensive, since MT values varied significantly with both Cu concentration and time, with higher MT levels generally being observed at the highest Cu concentrations and for the longest exposure times, until day 10 (Fig. 1G,

Table 1, $F_{3,48} = 28.80$, $F_{3,48} = 6.58$, $F_{9,48} = 7.65$, respectively, $p < 0.001$ for all tests). Interaction effects of Cu concentration and exposure time on MT level indicated the most significant differences by day 10, when MT levels from all Cu treatments were significantly higher than those from previous days, as well as by day 15, when MT levels from all Cu treatments differed from all controls (Tukey tests, $p < 0.05$). Lipid peroxidation only showed early time effects, as LPO levels by day 3 were significantly higher than by day 10 (Table 1, $F_{3,48} = 6.33$, $p = 0.01$), although no significant differences were observed between treatments. MT and LPO levels also showed inverse response patterns, with an increased MT level up to day 10 followed by a decrease until day 15, whereas LPO decreased from day 3 to day 10 and increased up to day 15 (Fig. 1G and 1H).

Cu concentrations were negatively correlated with RNA:DNA and instantaneous growth ($r = -0.30$ and $r = -0.32$; respectively, $p < 0.05$ for both), and were positively correlated with MT level ($r = 0.44$; $p < 0.05$). Other significant correlations were observed between instantaneous growth rates and RNA:DNA ratios ($r = 0.51$; $p < 0.05$) and between instantaneous growth and lipid content ($r = 0.41$; $p < 0.05$).

DISCUSSION

The present multibiomarker approach identified deleterious effects of sublethal copper exposure on growth and physiological condition of juvenile Senegalese sole. The biochemical indices and the biomarkers measured responded to Cu-induced stress during the 15-day trial, but the morphometric indices did not, indicating that a wider time frame would be necessary to account for any variations following

TABLE 1. – Results of the two-way ANOVA statistical analysis considering the effects of exposure time, Cu concentration and the interaction effects on the biomarkers levels.

	Time (days)				Cu concentration ($\mu\text{g L}^{-1}$)				Interaction Time x [Cu]
	3	5	10	15	0	5	25	100	
K	-	-	-	-	-	-	-	-	-
HSI	-	-	-	-	-	-	-	-	-
Growth	-	-	-	-	a	-	b	b	-
RNA:DNA	a	-	b	b	a	-	-	b	-
Protein	-	-	-	-	-	-	-	-	-
Lipid	-	-	-	-	a	-	-	b	-
MT	a	a	b	-	a	b	b	b	***
LPO	a	-	b	-	-	-	-	-	-

Different letters indicate significant differences, from *post hoc* comparison Tukey tests (a,b); *** - significant interaction effects ($p < 0.001$);

‘-’ no significant differences observed.

trace metal exposure, or even that these indices may not be suitable. However, Levesque *et al.* (2002) observed differences in seasonal patterns of the condition factor (K) and the hepatosomatic index (HSI) in yellow perch (*Perca flavescens*) chronically exposed to field metals. Eastwood and Couture (2002) also found a seasonal negative correlation between liver Cu content and the scaling coefficient (slope of the linear regression between fish body weight and length) in yellow perch, but observed no variation in the HSI.

On the other hand, changes in mass, measured as instantaneous growth rates, showed the adverse effects of Cu exposure, with growth rates being negatively correlated with Cu concentrations. Control group growth rates were in agreement with values observed by other authors in cultured juvenile Senegalese sole (Rueda-Jasso *et al.*, 2004). The initial decrease in instantaneous growth rates in fish exposed to higher Cu concentrations, compared to the control group growth rates, was maintained towards the end of the experiment, and the control values were never reached. Similar results have been found in other studies (e.g. Marr *et al.*, 1996; Lundebye *et al.*, 1999), whereas yet others have found that an initial reduction in growth of exposed fish was followed by a recovery period (e.g. Seim *et al.*, 1984; De Boeck *et al.*, 1997). Growth is a direct measure of individual fish health, but it is not specific to fish stress responses to pollutants, as several other biotic and abiotic stressors can affect it. Environmental conditions must be considered when one is integrating growth responses and pollution stress. Also, measuring growth in field studies is labour-intensive and time-consuming, so it is necessary to use proxy measures when direct growth measures are difficult to attain.

RNA:DNA ratio showed negative dose and time-response effects due to Cu exposure. It is well known that Cu contamination may lead to DNA oxidative damage through the increase in cellular reactive oxygen species (ROS), which influences the RNA:DNA ratio assumption of DNA concentration constancy (Bullock, 1970). Humphrey *et al.* (2007) described lower RNA:DNA values in barramundi (*Lates calcarifer*) from a highly contaminated estuary in North Queensland, but their study estimated liver nucleic acid, which undermines the use of this biomarker as a proxy for somatic growth. De Boeck *et al.* (1997) questioned the use of RNA:DNA as a biomarker in toxicological studies since RNA:DNA

and growth were poorly correlated in juvenile common carp exposed to copper. However, in both these studies DNA concentration (liver and muscle) also varied significantly with the degree of contamination, which was not the case in the present study. Additionally, our study shows a positive correlation between growth rates and RNA:DNA, suggesting that RNA:DNA ratio indicates growth impairment in juvenile Senegalese sole subjected to sublethal Cu concentrations. Nye *et al.* (2007) also found that RNA:DNA was a good indicator of growth and condition in 14-day post-hatch larvae of *Fundulus heteroclitus* exposed to polycyclic aromatic hydrocarbons (PAHs), but also described the influence of other factors on RNA:DNA, namely life-stage and maternal influence.

Another assumption regarding the use of the RNA:DNA ratio is that the RNA concentration increases with protein synthesis involved in somatic growth (Bullock, 1970). However, in cases of exposure to contaminants, an increase in RNA might be related to the induction of protein detoxification systems but also to an increase in protein turnover rate, which together would lead to high RNA:DNA ratios but decreased growth rates. The question is how significant is the increase in muscle RNA that is directly linked to cell response to contaminants, especially taking into account that the liver is the main organ for the detoxification of xenobiotics, and whether this increase overshadows the potential decrease in RNA production due to lower growth investment in fish facing toxic stressors. One way to look at this problem is to effectively measure growth and protein synthesis. As mentioned above, growth was positively correlated to RNA:DNA, indicating that RNA variability is in any case linked to growth, although it could also be linked to other protein defence mechanisms not measured in this study. However, protein content was not significantly different between copper treatments and control, nor was it associated with growth or RNA:DNA. This result may reflect a possible technical fault, such as inaccurate detection sensitivity in protein determination, or protein synthesis could have been similar for control and exposed fish due to different requirements, namely for somatic growth that was observed in both cases and additionally for toxicity defence mechanisms in Cu exposed fish. Therefore, RNA:DNA ratio may be a valuable condition indicator in toxicology studies, if the initial assumptions are met throughout the study, such as fish in

comparable early life-stages, preservation of the DNA concentration throughout the contamination period, and the relation between RNA variability and growth previously established. Ultimately it should be complemented with another growth indicator, as suggested by several authors (e.g. Ferron and Leggett, 1994; Suthers, 1998).

The variation in muscle lipid content between the highest Cu concentration treatment and control suggests differential energy allocation strategies, since lipid reserves of control fish were generally higher than fish subjected to Cu treatments. A general decrease with time was also observed in lipid content of fish exposed to Cu. Both observations could be linked to oxidation processes derived from Cu exposure, or on the other hand imply that lipid reserves were mobilised to respond to the toxicity stress, but also to maintain positive growth rates and thus protein synthesis even under Cu contamination. Previous studies have reported similar depletion in lipid reserves of fish subjected to metal trace exposure (Rowe, 2003). Additionally, higher lipid percentages were also observed for all treatments and the control group when compared to the initial reference percentage at day 0, except for the higher Cu concentration. This may be due to the change in diet from pre-experimental conditions, as trial fish were fed daily with a richer lipid diet, and despite the Cu exposure their lipid storage was higher than at the beginning of the experiment.

The biomarkers of exposure response, metallothioneins (MT) and lipid peroxidation (LPO) indicated fish Cu contamination, although only the MT level showed significant responses to different Cu treatments and periods of exposure. MT levels were within the range of previous studies on metal exposure, including Cu, in *Solea senegalensis* (Riba *et al.*, 2004). LPO only showed differences between days of exposure. Given that lipid peroxidation was quite low for Cu-treated fish and showed a general pattern of decrease, it does not seem to be the main factor responsible for the above-mentioned decrease in lipid content of exposed fish, thus reinforcing the idea of lipid expenditure to sustain the toxicity defence mechanisms and the positive growth rates observed, although at slower rates than those observed for control fish. Moreover, these biomarkers showed inverse response patterns, such that the decrease in MT induction towards the end of the experimental period may be due to several factors: i) exceeded Cu toxicity in the liver resulting in a diminished MT

induction; ii) a biphasic pattern of MT induction with continued Cu exposure; iii) precipitation of Cu-MT complex in granules; or iv) an increase in other antioxidant response mechanisms (Sanchez *et al.*, 2005). However, the inverse LPO response suggests an increase in oxidative stress concomitant with MT decrease.

The lowest Cu concentration tested in the current work matches environmental values commonly observed in many European estuarine systems (Hall and Anderson, 1999). Although our results indicate that for this level of copper exposure no significant effects on fish condition were observed (only MT level indicated Cu contamination) in a short time frame, the ecological implications due to a permanent exposure were not tested, and future work should explore this issue.

The results emphasised the need for a multi-biomarker approach for habitat quality assessment and toxicology studies, since common indicators of fish health, such as growth and condition, reflected the adverse effects of pollutants (Cu in this case). This interaction may influence conclusions drawn from these fitness indicators in a long-term data analysis, if the alteration of physiological processes due to pollution is not taken into account.

In conclusion, exposure to high concentrations of copper in the water had direct physiological costs for juvenile Senegalese sole, expressed as decreased growth and lower physiological condition (RNA:DNA values), even under optimal feeding conditions. Nonetheless, lack of variation in protein content could suggest that protein requirements were both for somatic growth and protein detoxification systems. Lipid reserve decreases seemed to sustain the toxicity defence mechanisms and the positive growth rates of exposed fish, although at slower rates than those observed for the control fish.

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