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Bioaccumulation of trace metal elements and biomarker responses in caged juvenile flounder at a polluted site: effects of fish density and time exposure.

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Abstract

This study investigates the effect of fish density and exposure duration on trace metal elements (TME) bioaccumulation and several biomarkers response. Juvenile flounders were caged at low, medium and high densities and exposed during 15 or 30 days in the Seine estuary. The concentrations of the TME measured in the muscle of the caged fish were all in agreement with their bioavailability percentage in the sediments. Higher concentrations of TME were found in flounders' muscle exposed for 15 days compared with those caged for 30 days. For the same exposure time, the density of fish had no effect on the accumulation of the TME in the flounders' muscle. Biomarkers responses varied according to density and duration of exposure. Special care should be taken in their interpretation. We underline that for an optimal assessment of TME pollution in the field, 15 days with low densities of fish per cage are sufficient.

Key words: flounder density, trace metal element, caging duration, biomonitoring, fitness indices, biomarkers of damage, innate immune response,

In recent decades, many legislative tools have been put in place to limit pollution and improve the ecological and chemical homeostasis of water bodies. In parallel, many tools have been proposed to monitor the quality of aquatic ecosystems. Often, the ecological quality has been assessed through studies of the biological communities (i.e. benthic invertebrate fauna, fish fauna), while chemical quality has been defined by monitoring the 45 "priority chemicals" in the Water Framework Directive (WFD). However, the first of these approaches doesn't addressed the origin of the pollution, while the second is not exhaustive enough. These shortcomings have prompted increasing interest in multi-biomarker approaches for environmental monitoring programs (Catteau et al., 2022; Sanchez et al., 2007). In particular, both passive and active biomonitoring methods have been proposed. The first approach concerned the determination of pollutants and their effect in native individuals with their own life history traits and specific spatial distributions in the wild. Many authors have proposed an alternative, active biomonitoring approach which is based on the caging of selected sentinel species in the area to be studied. The reduction of inter-individual variability and capacity to precisely control the location and duration of exposure could give more realistic results in studies of bioavailability, bioaccumulation and biological effects of contaminants on fish (Oikari, 2006).

One of the major constraints of caging that can affect growth and development of caged fish is the limitation of access to food (Jørgensen et al., 1999), which can also influence exposure to chemical contaminants through food compared with fish *in natura*. In addition to having effects upon fish's exposure to contaminants, caging can lead to a decrease in fitness indices (Kerambrun et al., 2012) which even result in the death of caged organisms. Fitness indices of larvae and juvenile fish such as specific growth rate, otolith increments; morphometric indices (Fulton's K); lipid and protein reserves have therefore been used to assess the health status of individuals and populations, as well as the habitat quality (Amara et al., 2007). But a drop in these fitness indices linked to a lack of food can confound interpretation of the effects of the metal trace elements bioaccumulation, for example, as well as other biomarkers which are considered to be responses to the concentration of these elements in the caged organisms.

To overcome the lack of food for caged fish, we tested for the first time, cages fitted with a device that could allow the fixation and development of prey.

While high fish densities in cages can have adverse effects on fitness and immunological biomarkers (Le Guernic et al., 2016), the effect of fish density and exposure duration on the accumulation of metal trace elements in fish are not fully understood.

For caging, a large number of fish species can be captured in a weakly contaminated natural habitat before being caged at the study site. The European flounder *(Platichthys flesus*) is one of

the most-used sentinel species for the assessment of chemical contamination in aquatic environments (Dupuy et al., 2014), and is recommended by OSPAR for the biomonitoring of marine environment (Burgeot et al., 2017).

The aims of this study, was to analyse the effect of fish density and exposure duration (15 and 30 days) on (i) the accumulation of TME (Ag, Ba, Cd, Cu, Co, Cr, Hg, Pb, Ni, V, Fe, and Sr) in juvenile flounder muscles, ii) the response of fitness indices (condition index, lipids and protein reserves), and iii) health status biomarkers such as the rate of DNA damage, the detoxification enzyme (EROD), the responses of the nervous system (acetylcholinesterase activity – AChE) and the immune system (leucocyte distribution, apoptosis, necrosis, phagocytose activity, respiratory burst and lysosomal presence).

The 70 juvenile flounders (size = 10.1±1.0cm; weight = 14.2±4.8g) were caught in the Canche estuary (France), a small estuary where anthropogenic pressures are relatively low (Amara et al., 2009). Before being deployed in the caged site, they were acclimatized for 15 days in a 500-L aquarium supplied with an open seawater circuit (16±0,15°C) in the Mareis aquarium center (http://www.mareis.fr/). Temperature and salinity of the acclimation tank were similar to those of the study site where the cages were to be deployed. During acclimatization, fish were fed daily (3% of their body weight) with live prey (Mysidacea, shrimp and artemia). One day before the caging experiment, 10 fish were dissected to obtain control values (T0).

The study site was located in the Seine estuary, chronically polluted, near Rouen (49°22.995'N; 01°00.676'E). Water quality in Rouen is impacted by pollutant inputs to the river system and reflects the anthropogenic pressures on the watershed. Besides the Parisian metropolis (10 million habitants) which represents an important contribution of upstream contaminants in the Seine River, Rouen (400000 inhabitants) is the second largest metropolis on the Seine River before Le Havre at its mouth (250000 inhabitants) (Romero et al., 2016). The water quality of the Seine in Rouen is also impacted by the two large industrial areas of Elbeuf and Rouen, as well as the intensive agriculture in the river basin (Fisson et al 2014). These activities are all potential sources of contaminants reaching the estuary through precipitation and run-off.

The sediments from the upstream of the Seine River are known to be heavily contaminated by metal trace element (Borcier et al., 2019, Hamzeh et al., 2016). In order to confirm the state of contamination by metal trace elements in the environment, and to assess their bioaccumulation in flounders, samples of sediments were taken at the beginning of the experiment with a grab on the first fifteen centimeters of the caging site. The samples were then stored in plastic bags and frozen at -20 °C until analysis.

Salinity, pH, dissolved oxygen and temperature of the water column were recorded at the beginning of the experiment (day 0), at day 15 and at the end (day 30). Water salinity (0,1 PSU), pH (8.5) and dissolved oxygen (7.8mg/L) were relatively similar at the beginning, at D15 and at D30. However, the water temperature decreased from T0 to D30 (18.5°C, 16.3°C and 15.5°C for D0, D15 and D30, respectively).

The cages used in this study were made of plastic: they were 1m long, 0.6m wide and 0.6m in height (volume = 0.36m^3). Their 15mm mesh size facilitated the circulation of water and allowed the entry of fish prey. To protect the fish against strong movements of water, Plexiglas plates were attached to the base of the four sides of each cage. A PVC (polyvinyl chloride) pipe, bent at both ends and perforated to facilitate the circulation of water tube, was also added to serves as a refuge for fish against strong currents. Finally, in order to facilitate the fixation and the development of potential fish prey, a small artificial reef made up of oyster shells was also attached inside the cage.

The 24th September 2018, 60 fish were randomly distributed in two batches of three cages containing, respectively: 5 (density = 0.173±0.011g/L), 10 (density = 0.373±0.002g/L) and 15 fish (density = 0.617±0.068g/L) corresponding respectively to low, medium and high densities. Then, the cages were fixed to the bottom at about 6 m depth using two 6 kg flat anchors on each side and they were placed separately. In order to study the effect of the exposure duration, the first batch of the three cages were exposed during 15 days and the second batch during 30 days. At the end of each exposure duration, fish were kept alive during transport to the laboratory where they were weighed and measured to calculate their growth and condition indices. Blood samples were taken in heparinized tubes and then quickly frozen in liquid nitrogen before being stored at -80°C for genotoxic analysis. The stomach track was removed and conserve in an Eppendorf tube and stored at – 20°C. Brain, liver and two portions of muscles (an anterior and a posterior, intended respectively for the analysis of protein and for lipid analysis) were frozen at -80 °C. The spleen was recovered for the immune system analyses. The remaining muscles were frozen at -20°C for contaminants analysis.

A total of sixteen trace elements (Ag, Al, Ba, Cd, Cu, Cr, Fe, Hg, Mo, Mn, Ni, Pb, Sr, Ti, V and Zn) were analyzed in sediments and in the muscles of flounders by inductively coupled plasma mass spectrometry (ICP-MS, Varian 820-MS). The bioavailable and total metal trace elements fractions in the sediments and the total metal trace element in fish muscle samples were analyzed according to the protocol described by Ouddane et al. (1999) modified from Huerta-Diaz and Morse (1990) for the determination of the bioavailable fraction.

To see if the fish fed well during caging, the digestive tracts of the fish were taken and the stomach contents analyzed. To estimate the general well-being of the fish, the Fulton morphometric index (K) was calculated with the formula: $K = 100x(weight(mg)/length(mm)^3)$.

Proteins were measured in the muscles of the anterior ventral side of the flounders according to the procedure described by Clemmesen (1989) slightly modified. Total lipids were extracted from the lyophilized dorsal side muscle of flounders according to the method described by Folch et al., (1957). Lipid classes were determined by chromatography following the procedure described by Di Pane et al. (2019).

Genotoxic damage was evaluated using the Comet assay following the methodology specified in (Vandghanooni and Eskandani, 2011; Singh et al., 1988). The results were expressed as a percentage of DNA in the comet tail (%DNA tail) using HCS Studio® software (Thermo Scientific).

The measurement of the EROD activity in the livers of flounders was carried out according to the XP ISO/TS 23893-2 protocol. The calculation of the EROD activity (expressed in picomoles per minute per milligram of protein) was carried out according to Eggens and Galgani (1992).

For acetylcholinesterase (AchE) activity were determined in the brain tissue by proceeding the protocol described by (Bocquené and Galgani, 1998).

Immunological parameters were measured in splenic leucocytes. Leucocyte sub-populations (lymphocyte and granulocyte-macrophage) were identified and characterized by calculating the percentages of each sub-population (Bado-Nilles et al., 2014). The cellular mortality percentages were evaluated by fluorimetry in order to obtain cellular fluorescence parameters indicating respectively apoptotic (FL1, green fluorescence) and necrotic (FL3, red fluorescence) cells (Bado-Nilles et al., 2014). Leucocyte respiratory burst measurement (stimulation index) was based on the technique described by Chilmonczyk and Monge (1999), optimized for the flounder. Intracellular lysosomal presence was determined according to Bado-Nilles et al., (2013) the phagocytosis activity (capacity and efficacity of phagocytosis) was evaluated according to Gagnaire et al., 2004).

Statistic tests were performed by Xlstat software Edinsoft. Normal distribution of the dataset was tested with the Shapiro-Wilks test ($p < 0.05$), while homogeneity of the variances was evaluated with Levene's test ($p < 0.05$). Parametric datasets were analyzed with a one-way ANOVA, followed by Tukey HSD as a post-hoc test. Non-parametric data was further processed with a Kruskal-Wallis test ($p < 0.05$) and a Dunn test for pairwise comparisons. A principal component analysis (PCA) was used to evaluate the relationships between the chemical contaminants in fish depending on their density per cage and time exposure. The Integrative Biological Response as

decribed by Sanchez *et al*. (2013) was used to evaluate the relationships between biomarkers in fish depending on their density per cage and time exposure.

At D15 all cages were recovered, but at D30 the cage containing 10 fish (C5) was not recoverable. The percentage of fish recovery was 100%, 90%, 53.3%, respectively for the low, the medium and the high density exposed during 15 days, and 80% et 93.3% for the low and the high density exposed during 30 days.

The results of the contamination of TME in the fine fraction (<63 µm) of the sediments are shown in table 1. Results of the bioavailable and total fractions of the trace elements expressed in mg/kg dry weight (dw) showed varying contents. Fe was the major element in sediments. It was followed respectively by Ti, Mn, Sr, Zn, and Ba. In contrast, Mo, Cd and Hg showed the lowest concentrations in the sediments from Rouen.

The concentration of Cu, Pb and Zn measured in the sediments in this study were comparable to those previously reported by Hamzeh et al. (2013) at the same site. However, higher concentrations of total Cd and Hg were found in the present study compared with those measured by these authors. This indicates that there is a continuous enrichment of these two trace elements at the Rouen site. When compared with other sites in the Seine River, the concentrations of Cd, Cu, Hg, Pb, Ni and Zn in sediments at the Rouen site were lower than those measured upstream at Poses and Oissel, but higher than those recorded downstream at Duclair and Caudbec (Hamzeh et al., 2016). The concentrations of Ag, Ba, Cd, Co, Cr, Hg, Mo, Ni, V, and Mn recorded in this study were all higher than those recently reported for the sediments at the mouth of the Seine estuary (Borcier et al., 2019). When compared with others rivers in France, the concentration of Cd, Cu, Zn and Pb measured in Rouen's sediments were higher than those recorded in the Loire sediment for Cd, Cu, Zn and Pb (Dhivert et al., 2016) and in the Gironde River (Larrose et al., 2010). Among the eight trace elements identified as priority contaminants in aquatic systems (As, Cd, Cr, Cu, Hg, Ni, Pb and Zn), Fisson et al (2014) underlined that Cd, Cu, Hg, Pb and Zn are the elements found at highest concentrations in the sediments of the Seine. The concentrations of these elements measured in this study were greater, or of the same magnitude, as those found in other polluted Rivers in Europe (Esser et al., 2020). This high TME contamination level in sediments at Rouen were linked with different sources such as industrial and agricultural runoff, and urban waste waters (Fisson, 2014).

The accumulation of TME in living organisms depends strongly upon their bioavailability in the environment. The calculation of the percentage of bioavailable TME in the sediments (table 1) were highest for Sr. It was followed by Mn, Cu, Pb, Ba, Cd, Ni, Co, Zn, Hg and Fe respectively.

In contrast, Al, Mo and Ag were the least bioavailable. Links between metal concentrations in fish and in sediments are also mainly related to the bioavailability of these metal trace elements in sediments. The concentration of TME in the muscle of caged fish compared to the control groups show that regardless of the density and the time of exposure, the average concentrations of Ag(0.13 ± 0.06 mg/kg), Ba(0.53±0.17 mg/kg), Co(0.02 ± 0.0001 mg/kg), Cu(1.95±0.18 mg/kg), Hg (0.55±0.20mg/kg), Mn (2.00±0.42 mg/kg), Ni(0.11±0.02mg/kg), Sr(14.43±1.62mg/kg), Ti(0.10±0.04mg/kg) and Zn(42.3±0.42mg/kg) were all significantly higher than those recorded in the muscles of the control group (Kruskal Wallis test, $p < 0.05$) (table 2). These results indicate that these elements are bioaccumulated by the juvenile fish during the caging exposure. Excepted for Fe and Pb, all the elements with a bioavailability percentage greater than 60% in the sediments were found in the muscles of caged fish at concentrations higher than those in the control group (except Ag, with 34% of bioavailability). After zinc, strontium was the element found at highest concentrations in the caged fishes' muscles. The ratio of concentration of the TME in muscles of caged fish to that of controls shows that Sr, the most bioavailable element in the sediments, was also found at the highest concentrations in caged fish muscles. The concentrations of the TME measured in the muscle of the caged fish were all in agreement with their bioavailability percentage in the sediments (except Fe and Pb). Caged organisms, especially the juvenile flounders, thus seem to be an excellent model for monitoring TME pollution in the aquatic environment.

If the effectiveness of caging on the biomonitoring of aquatic environments has been demonstrated and confirmed in this present study, the effect of the caging duration and the fish density per cage on the accumulation of contaminants remains to be fully characterized. The same is also true of the response of organisms to their environment (as measured by various biomarkers), both as a function of the exposure time and the density of fish per cage.

Results of the accumulation of the TME measured in the muscles of the caged flounders (table 2) show that fish density do not have any effect on TME accumulation (Kruskal Wallis, $p<0.05$). For the same time of exposure, and for all elements, no difference was observed in the accumulation of the TME among the low, medium or high fish densities and this held true for both exposure times (table 2). Unlike density, time exposure seems to affect trace metal elements concentrations by flounders. Figure 1 shows the evolution of the average metal concentrations calculated by considering all the D15 flounders on the one hand, and all the D30 flounders on the other hand, compared with controls. Among the elements accumulated in the flounders (table 2), the highest contents of Ag, Ti, Hg, Mn and Zn were measured in the muscles of the flounders exposed during 15 days. A significant difference (ANOVA, $p < 0.01$) was observed for Ag, Ti,

Hg and Zn measured on D15 compared to those measured on D30. Only Ni was found at greater concentrations in the flounders of D30 compared with those at D15 and no difference was observed for Cu and Mn.

The discriminant function analysis (figure2) of the TME concentration in fish shows that the first axis F1 (explaining 88.24% of the total variance) distinguishes fish according to their time of exposure. On the right side, all the fish exposed for 15 days were grouped, and on the left side all fish exposed for 30 days were grouped with the control fish. Elements like Cu, Mn and Zn are essential for fish metabolism, while non-essential metals such as Hg, Cd and Pb are toxic even at low concentrations. While so-called essential elements can produce toxic effects at high concentrations, their concentrations in fish tend to be auto-regulated by the organism, unlike non-essential metals (Fernandes et al., 2007). The toxicological effects of inorganic compounds depend on the exposure and bioavailability of the elements, absorption, metabolism and their intracellular concentrations (Fent, 2004). The decline in concentrations of Cu, Mn and Zn between D15 and D30, could be due to the fact that at D30, flounders had enough time to integrate their environment and developed mechanisms to regulate these contaminants.

Hg and Ag have no known biological role and are not biodegradable; thus, they tend to accumulate in organisms. Their higher concentrations at D15 compared with D30 is similar to other reports of Hg in fish in several other studies (Schlenk et al., 1995; Palace et al., 2005). Schoyen et al, (2017) found similar variations of this element in caged mussels for 34, 104 and 181 days. Such temporal variation in TME bioaccumulation in fish requires additional studies for a better understanding of the relationships between their rates of accumulation and elimination, which is beyond the scope of this study.

Exposure of fish in polluted environments can have negative consequences on their growth, their physiology and can even cause damage to their DNA.

We found that fish density can have various effects on the responses of physiological and innate immune biomarkers or fish stomach contents. For the same time of exposure, the Fulton K index, protein contents, the TAG/Chol ratio, the average number of prey and the average stomach filling decreased when the density of fish per cage increased (figure 3). A highly significant difference (Kruskal Wallis, $p < 0.01$) was observed between the Fulton condition index, the TAG/Chol ratio of fish at low density compared with those at medium density exposed during 15 days. Similarly, a highly significantly difference (Kruskal Wallis, $p < 0.01$) was observed between the TAG/Chol ratios, the protein contents of fish at low density compared with those at high density exposed during 30 days. This was correlated with the results of stomach contents (figure 3). Further, the

lower the number of fishes per cage, the greater the number of prey in fish stomach which led to a greater availability of food and hence a better nutritional status for organisms. For the same time of exposure, changes observed in fish fitness seem not to be influenced by a response related to TME accumulation but by a variation of the caged fish density. A decrease in physiological biomarkers was observed in *Solea senegalensis* caged at high density (Andrade et al., 2015).

Results of the immune biomarker response (figure 5), showed no difference between flounders caged with low, medium or high density for the same time of exposure. However, when the density increased, the phagocytosis efficiency decreased. In contrary, there was a trend for an increase in the percentage of granulocytes-macrophages, leucocyte necrosis, the oxidative burst and the presence of lysosomes in fish for the same time of exposure when the density increased, though this was not statistically significant.

Unlike the physiological parameters, the rates of DNA damage, the EROD activities and AchE (figure4) did not show, for the same exposure duration, any significant differences between the flounders exposed at different densities (Kruskal Wallis, $p < 0.01$).

Many studies have underlined the induction of stress biomarkers such as lactate and cortisol (Urbinati et al., 2004) or ROS (Le Guernic et al., 2016) when fish are caged at high density. In our study, the absence of such effect were certainly related to the fact that there was no difference on the accumulation of TME between fish exposed at different densities for the same duration. However, like fitness indices, a destabilization of the immune response where observed with increasing density in this study. Other workers have also reported an increase in leucocytes, necrosis and apoptosis when the fish density increased (Le Guernic et al., 2016). Similarly, Vazzana et al. (2002) underlined that a high fish density can suppress immune capacities such as respiratory burst and phagocytosis. In our study, an increase of juvenile' flounders density had negative effects on their immune capacities. Granulocytes-macrophages, leucocyte necrosis, the oxidative burst and the presence of lysosomes in fish for the same time of exposure increased when the density increased. The phagocytosis capacity tends to decrease with increasing density of fish. To maintain an optimal physiological status of the organisms during ecotoxicological evaluations, the number of fishes per cage should be limited even if this means increasing the number of cages for statistical treatments. To maintain animal welfare and to achieve an optimal evaluation of TME pollution, as described in the European Directive 2010/63EU, our result suggests that fish caged at low density (0.173±0.011g/L) would be sufficient to assess pollution by trace elements.

As showed above, the accumulation of the TME is not density dependent but depend of the exposure duration. It was more important in fish caged during 15 days compared to those caged

for 30 days. To investigate the effect of TME accumulation in juvenile' flounders as a function of the caging duration, the Integrative Biological Response (IBR) developed by Beliaeff and Burgeot (2002) and adapted by Sanchez et al. (2013) was calculated. The values of the IBR (figure 6) calculated for all cages with different densities and both durations of exposure compared with controls, was 2.76, 2.88 and 2.77 for fish in low, medium and high density cages exposed for 15 days, and 1.19 and 1.88 for fish in low and high density cages exposed for 30 days. Results of the IBR showed that flounders caged during 15 days were characterised by a decrease in the TAG/Chol ratio, in protein contents and in the capacity of phagocytosis, an induction of AChE, an increase in granulocytes, in cell necrosis and in respiratory burst compared with those of D30. This later were characterized by an increase in the ratio of TAG/Chol and in protein content, an important induction of EROD, an inhibition of AChE, and an elevated presence of lysosomes compared with fish exposed for 15 days. At equal density, the ratio TAG/Chol and the protein contents were significantly higher (Anova, $p<0.05$) in fish caged during 30 days compared to those caged during 15 days (figure3). The results for the lipid index and the amount of protein corroborate those for stomach contents (figure 3). Analysis of the stomach contents performed in caged fish showed that for the same density, the average stomach filling rate and the average number of individual prey was higher in fish exposed for 30 days vs 15 days. The ratio TAG/Chol and the protein were inversely correlated with TME like Ag, Ti, Hg, Mn and Zn (correlation not shown) in D15 flounders. These nutritional indices have been successfully used in numerous studies to assess the nutritional status of fish (Kerambrun et al., 2012; Amara et al., 2007), while a decrease in lipids had been identified as a general metabolic response to stress (Lemly, 1997). The decrease in triacylglycerol compared with controls in the D15 flounders could be the consequence of detoxification and regulation of the metals which were present at higher concentrations at D15 compared with D30. The increase in TAG/Chol ratio and protein levels in flounders after 30 days of caging may be due to a resumption of fish growth after an adaptation and detoxification phase. These fish subjectively seemed to have better wellbeing than the D15 fish, and this was confirmed by the results of the IBR with lower IBR values in D30 fish compared with D15 fish (Sanchez et al., 2013).

The major drawback of the caging of fish was the decline in their fitness related to a lack of access to food. In this study, a gain in fitness indices after 30 days of exposure was observed compared to most of the studies on fish caging which observed a decrease of these indices (Delahaut et al., 2019). It was likely due to the fact that the reefs installed in the cages had permitted the development and growth of their prey. The prey found on the reef attached in each cage were the same as those found in the stomach of the caged fish and was mainly the species

Gammarus zaddachi Sexton. The cages developed and used in this study allow a good survival and better growth of the organisms. They would make it possible to lift the limits on the of fish caging for long durations of exposure.

The increase in the oxidative burst in the D15 flounders could be linked to the presence of TME (Sevcikova et al., 2011) which were more important in these fish. This stimulation index in the D15 flounders is accompanied by a modulation of immune response, especially visible by an increase in phagocytosis efficiency and in the proportion of granulocytes.

However, the induction of EROD and the inhibition of AChE in the D30 flounders demonstrated in this study could be linked to the presence of pollutants which were not assessed during this study, such as pesticides, PAHs, PCBs or other organic compounds (Lushchak, 2011) which are widely found in surface waters and sediments of the Seine River (Minier et al., 2000).

PAHs and PCBs have been demonstrated to have genetoxic effects in flounder's tissues (Dupuy et al., 2014). Similarly, several authors have shown genotoxic effects including damage to the fish DNA by iron (Valko et al., 2005), copper (Alak et al., 2019) and chromium (Ahmad et al., 2006). The rate of DNA damage measured in the fish caged after 15 and 30 days compared with controls suggests that the levels of metal contaminants measured in the caged flounders have fallen below the limit which would cause genotoxicity.

In conclusion, this study demonstrated that juvenile flounder caging is a relevant model for the assessment of the pollution by TME in natural aquatic environment. The accumulation of TME in the flounder muscle is likely dependent of several factors such as their bioavailability and the exposure time. Higher concentrations of metallic elements were found in the muscle of flounders exposed for 15 days compared with those caged for 30 days. For the same exposure time, the density of fish had no effect on the accumulation of the TME in the flounders' muscle. A short time exposure (15 days) seems sufficient to assess the pollution by MTE o. The accumulation of TME in flounders caged for 15 days was linked to a decrease in their condition, lipid reserves and amounts of protein of fish. An increase in leucocyte necrosis associated with leucocyte destabilization, marked by an increase in the granulocytes/macrophages pool, was also observed in the D 15 flounder in comparison with the control and D30 fish. These latter, meanwhile, were characterised by an increased DNA damage, and EROD activity, and an inhibition of acetylcholinesterase activity. These phenomena appear unrelated to the TME contamination in fish, since most were at lower levels compared with the D15 fish. In addition, fish caged for 30 days exhibited better fitness indices, almost certainly due to the fact that their prey had colonized the reefs installed in their cages.

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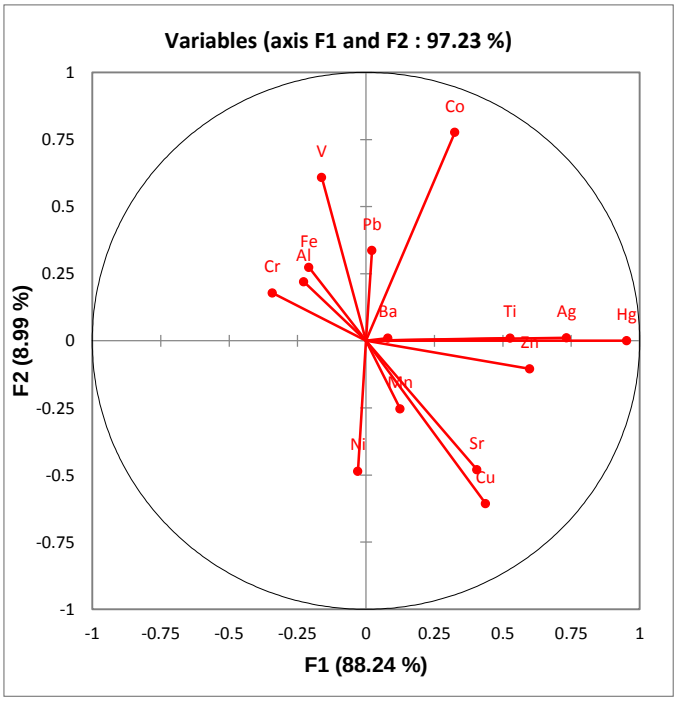
Table 1: Concentration of trace metal elements (bioavailable and total, mg/kg dw) and the percentage of bioavailability calculated in the sediment from Rouen

Elements	Total Fraction	Reactive fraction	**% of Bioavailability**
Ag	7.98 ± 0.62	2.75 ± 0.93	34.4
Ba	107 ± 1	82.9 ± 6.2	77.1
Cd	2.75 ± 0.24	2.05 ± 0.14	74.4
Co	7.13 ± 0.48	4.62 ± 0.36	64.8
Cr	43.8 ± 6.6	21.1 ± 0.8	48.1
Cu	39.1 ± 3.3	32.7 ± 0.2	83.5
Fe	22 529 ± 2,212	13 731 ± 508	60.9
Hg	2.77 ± 3.6	1.63 ± 0.80	61.2
Mn	373 ± 36	336 ± 48	90.1
Mo	1.81 ± 0.31	0.5 ± 0.71	27.8
Ni	16.1 ± 1.5	11.9 ± 0.8	73.8
Pb	48.0 ± 7.6	39.6 ± 4.0	81.5
Sr	230 ± 12	221 ± 10	95.9
Ti	472 ± 95	191 ± 2	40.5
V	33.0 ± 1.6	14.6 ± 0.4	42.8
Zn	224 ± 26	138 ± 2	61.7

Table 2: Concentrations of trace metal elements expressed in mg/kg of dry weight measured in muscles of flounders caged during 0, 15 and 30 days. a, b, c, d, e and f indices indicate significant difference (Kruskal Wallis, $p < 0.05$) with controls, cages C1, C2, C3, C4 and C6 respectively.

Time exposure (day)	Cages	Ag	Co	Ni	Pb	Ti	V	Al	Ba	Hg	Cr	Cu	Mn	Zn	Sr	Fe
0	**Controls**	0.06 ± 0.04	0.02 ± 0.02	0.07 ± 0.04	0.09 ± 0.04	0.06 ± 0.03	0.04 ± 0.03	1.83 ± 0.49	0.48 ± 0.42	0.28 ± 0.04	0.91 ± 0.3	1.29 ± 0.23	1.05 ± 0.08	30.9 ± 10.88	2.98 ± 1.50	10.67 ± 4.92
15	**C1 (Low density)**	0.14 ± 0.02 [a.e.f]	0.01 ± 0.001	0.11 ± 0.06	0.07 ± 0.00	0.12 ± 0.05 [a.e.f]	0.03 ± 0.00	1.24 ± 0.18	0.42 ± 0.12	0.72 ± 0.09 [a.e.f]	0.66 ± 0.07	2.14 ± 0.44 [a]	1.73 ± 0.25 [a]	56.7 ± 13.66 [a.e.f]	13.46 ± 4.28 [a]	7.94 ± 1.58
	C2 (medium density)	0.19 ± 0.10 [a.e.f]	0.02 ± 0.005	0.08 ± 0.09	0.06 ± 0.1	0.13 ± 0.11 [a.e.f]	0.03 ± 0.03	1.03 ± 0.44	0.49 ± 0.25	0.73 ± 0.07 [a.e.f]	0.74 ± 0.21	1.96 ± 0.23 [a]	1.74 ± 0.46 [a]	44.3 ± 11.14 [a.e.f]	15.15 ± 8.83 [a]	8.62 ± 2.88
	C3 (High density)	0.17 ± 0.10 [a.e.f]	0.02 ± 0.005	0.11 ± 0.09	0.12 ± 0.1	0.13 ± 0.11 [a.e.f]	0.04 ± 0.03	0.99 ± 0.44	0.83 ± 0.91	0.63 ± 0.14 [a.e.f]	0.73 ± 0.15	1.67 ± 0.38 [a]	2.67 ± 1.92 [a]	43.7 ± 14.08 [a.e.f]	13.79 ± 5.25 [a]	8.03 ± 2.99
30	**C4 (Low density)**	0.07 ± 0.02	-	0.13 ± 0.07 [a]	0.04 ± 0.01	0.06 ± 0.01	0.01 ± 0.00	1.55 ± 0.93	0.42 ± 0.05	0.33 ± 0.01	0.82 ± 0.20	2.04 ± 0.36 [a]	1.68 ± 0.58 [a]	31.0 ± 0.74	12.86 ± 1.52 [a]	9.01 ± 1.93
	C6 (High density)	0.07 ± 0.03	-	0.14 ± 0.02 [a]	0.05 ± 0.01	0.06 ± 0.03	0.01 ± 0.01	1.03 ± 0.19	0.51 ± 0.32	0.33 ± 0.03	0.80 ± 0.17	1.92 ± 0.25 [a]	2.17 ± 0.93 [a]	35.8 ± 8.66	16.91 ± 9.01 [a]	8.20 ± 1.81



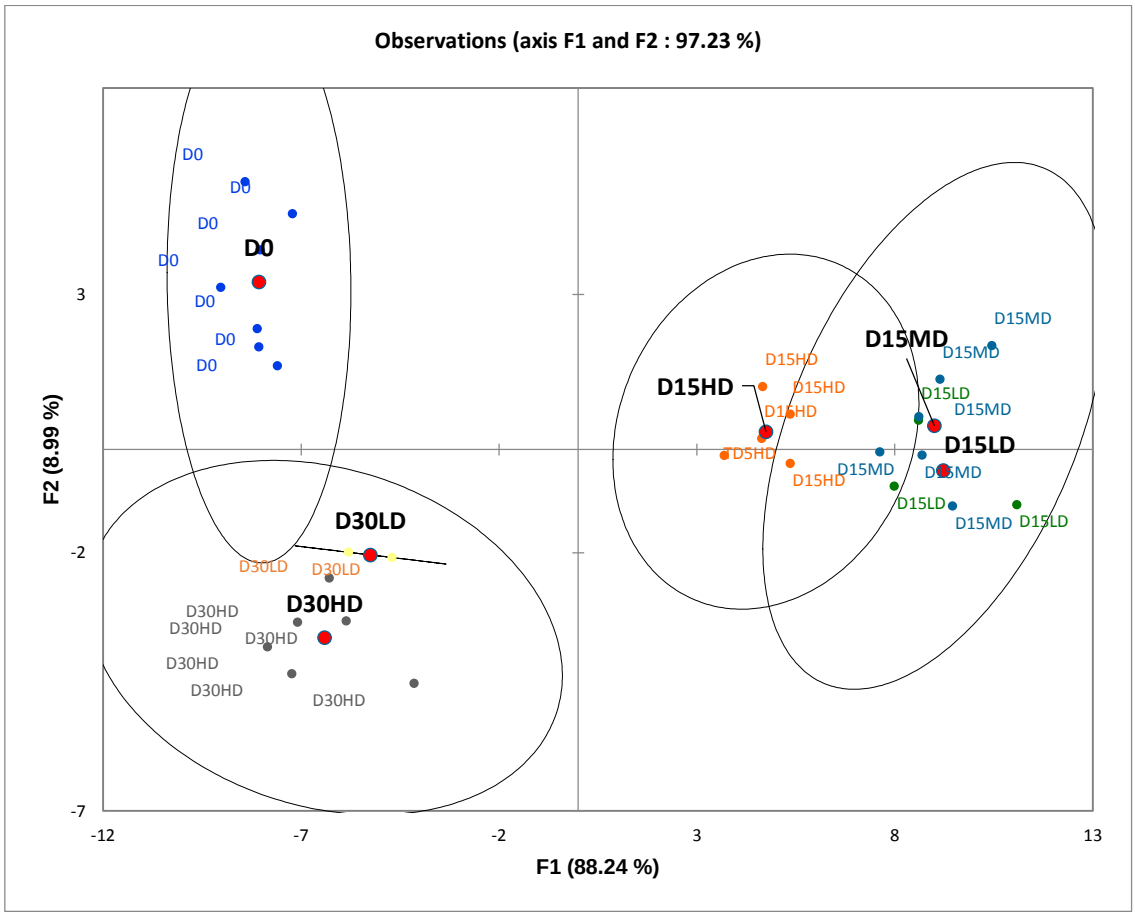


Figure 2: Factorial discriminant analysis showing the distribution of metallic trace elements as a function of caged fish density and exposure duration. D0 Controls, D15LD, D15MD, D15HD fish exposed during 15 days in cage with Low, Medium and high densities of flounders, respectively. D30LD, D30HD fish exposed during 30 days in cage with Low and High densities of flounders, respectively.

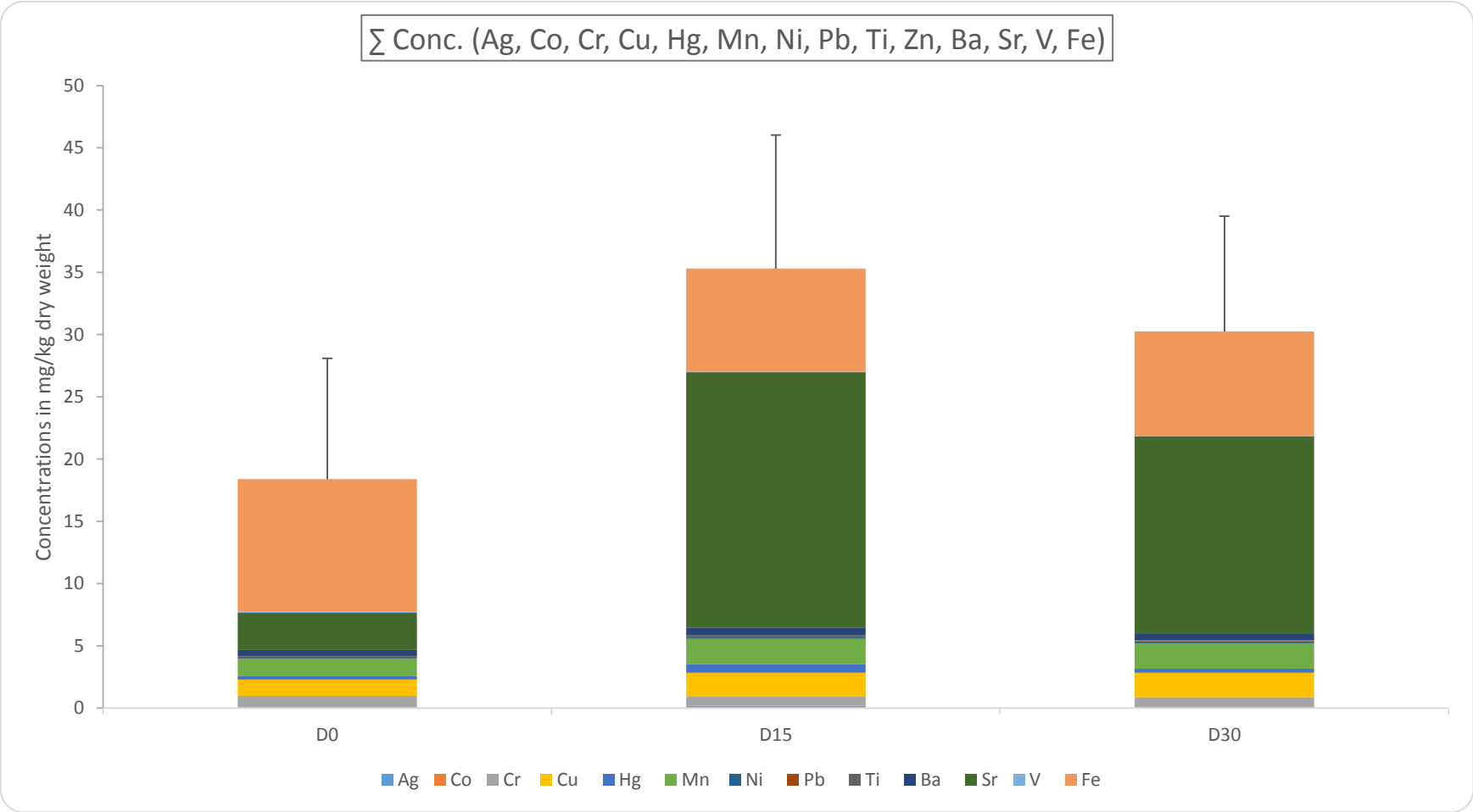


Figure 1: Concentration of trace metallic elements (TEM, mg/kg of dry weight) in the muscles of flounders before caging (Day 0, D0) and after 15 (D15) and 30 days (D30) of active biomonitoring in the Seine estuary at *Rouen*.

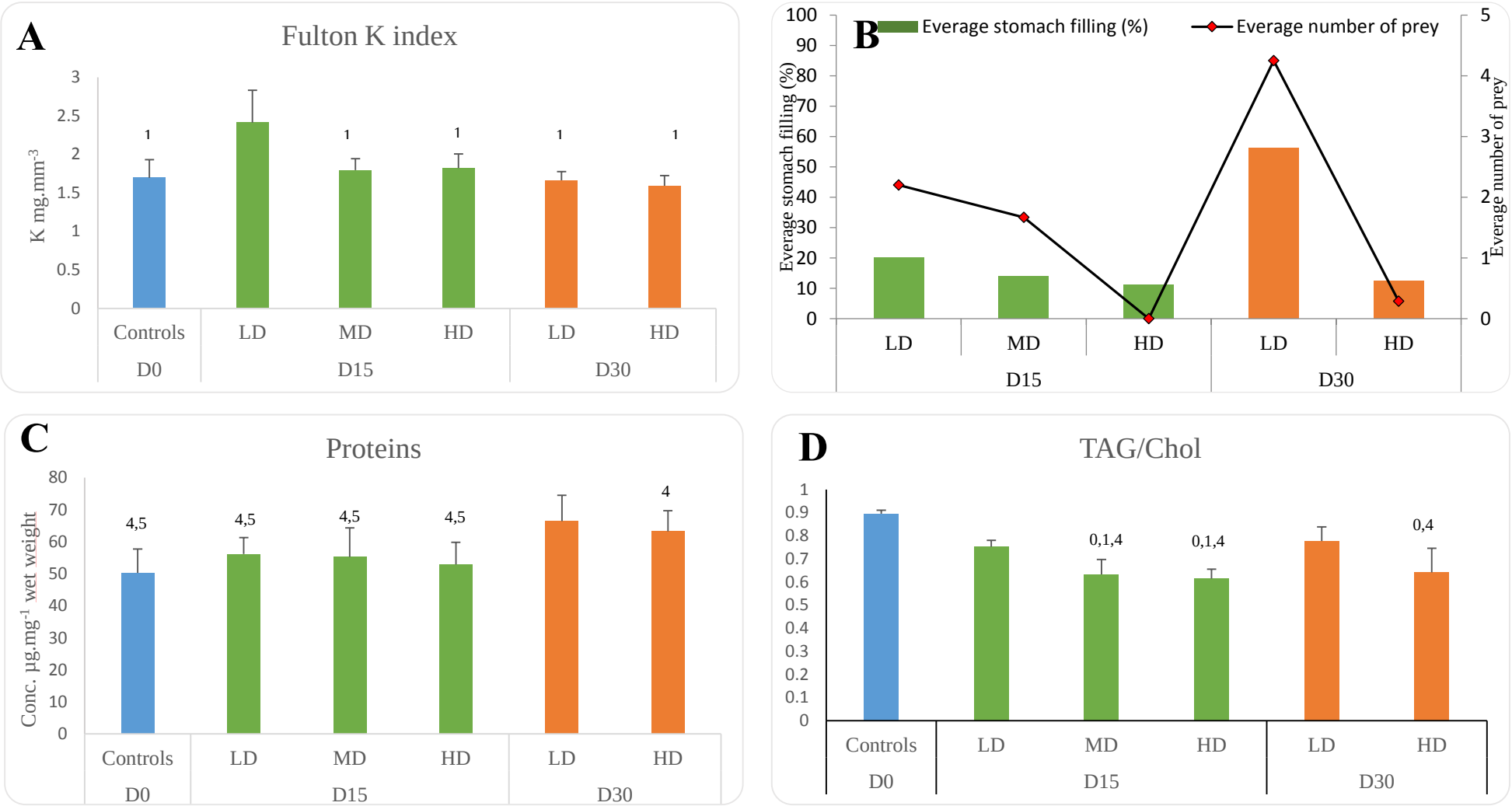


Figure 3: Fulton K index (A), stomach feeling and number of preys (B), protein concentration (C) and TAG/Chol ratio (D) measured in caged flounders depending on their density and exposure duration. The indices 0,1,2,3,4,5 and 6 represent significant differences ($p < 0.05$) compared respectively with Controls, the cages with Low Density (LD), Medium Density (MD) and High Density (HD) exposed during 15 days (D15) and cages with Low Density (LD) and High Density (HD) exposed during 30 days (D30).

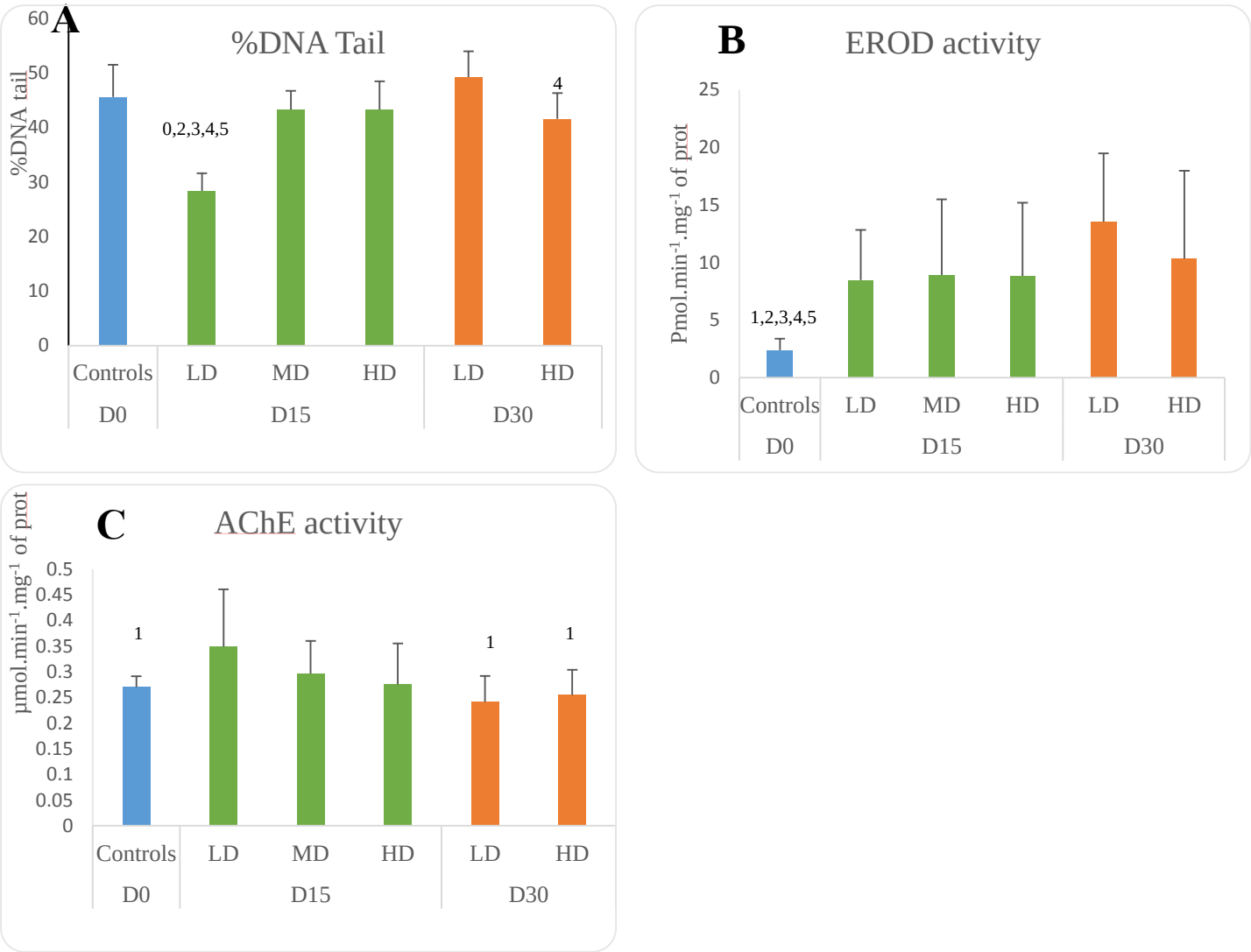


Figure 4: Percentage of DNA tail (A), Etheroxy-O-deethylase activity (B) and Acetylcholinesterase activity (C) measured in caged flounders depending on their density and exposure duration. The indices 0,1,2,3,4 and 6 represent significant differences ($p < 0.05$) compared respectively with Controls, the cages with Low Density (LD), Medium Density (MD) and High Density (HD) exposed during 15 days (D15) and cages with Low Density (LD) and High Density (HD) exposed during 30 days (D30).

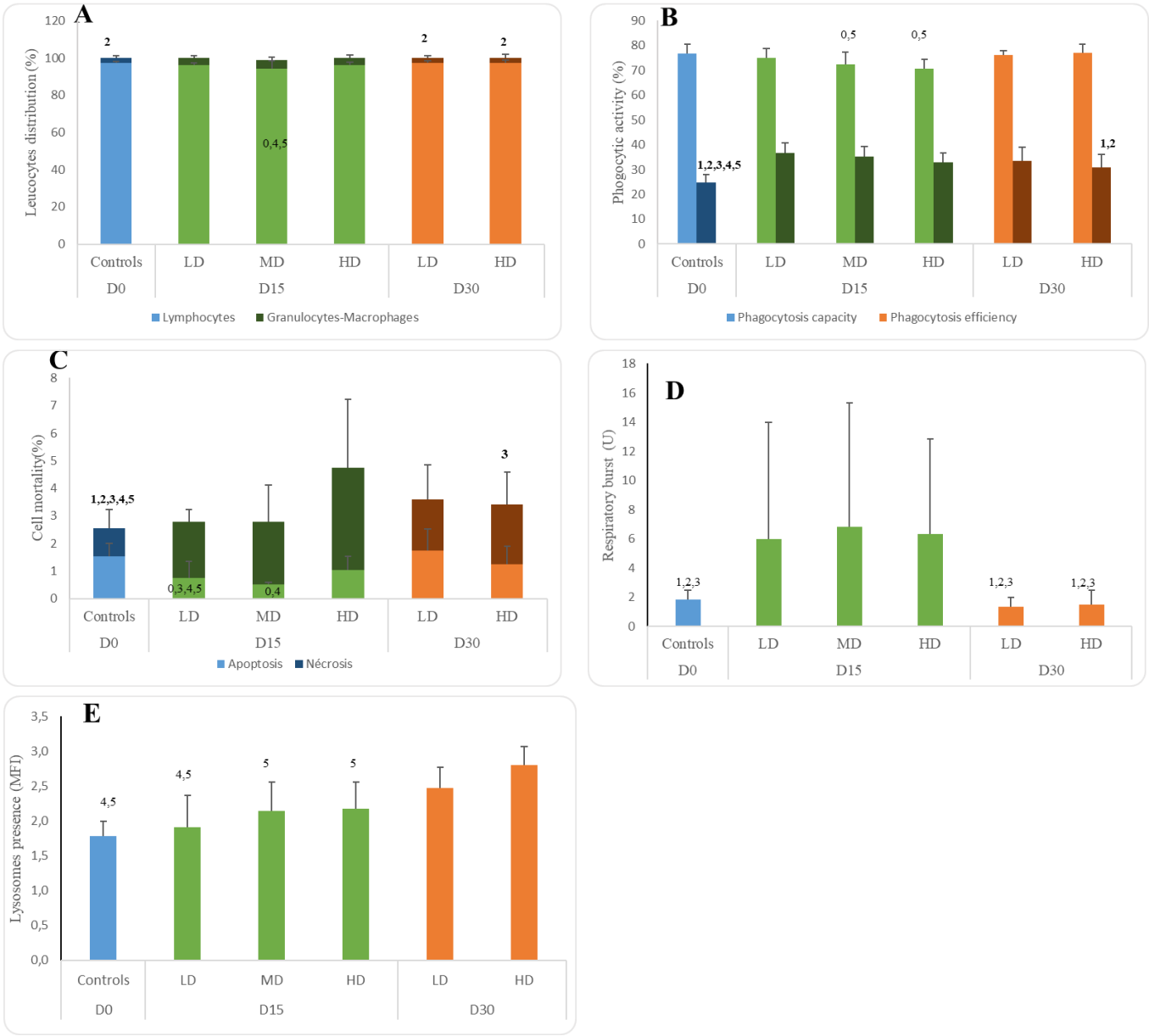


Figure 5: Leucocyte distribution (A), phagocytic activity (B), cell mortality (C), respiratory burst (D) and the presence of lysosomes (E) measured in the spleen of flounders depending on their caging density and exposure duration. The indices 0,1,2,3,4 and 6 represent significant differences ($p < 0.05$) compared respectively with Controls, the cages with Low Density (LD), Medium Density (MD) and High Density (HD) exposed during 15 days (D15) and cages with Low Density (LD) and High Density (HD) exposed during 30 days (D30).

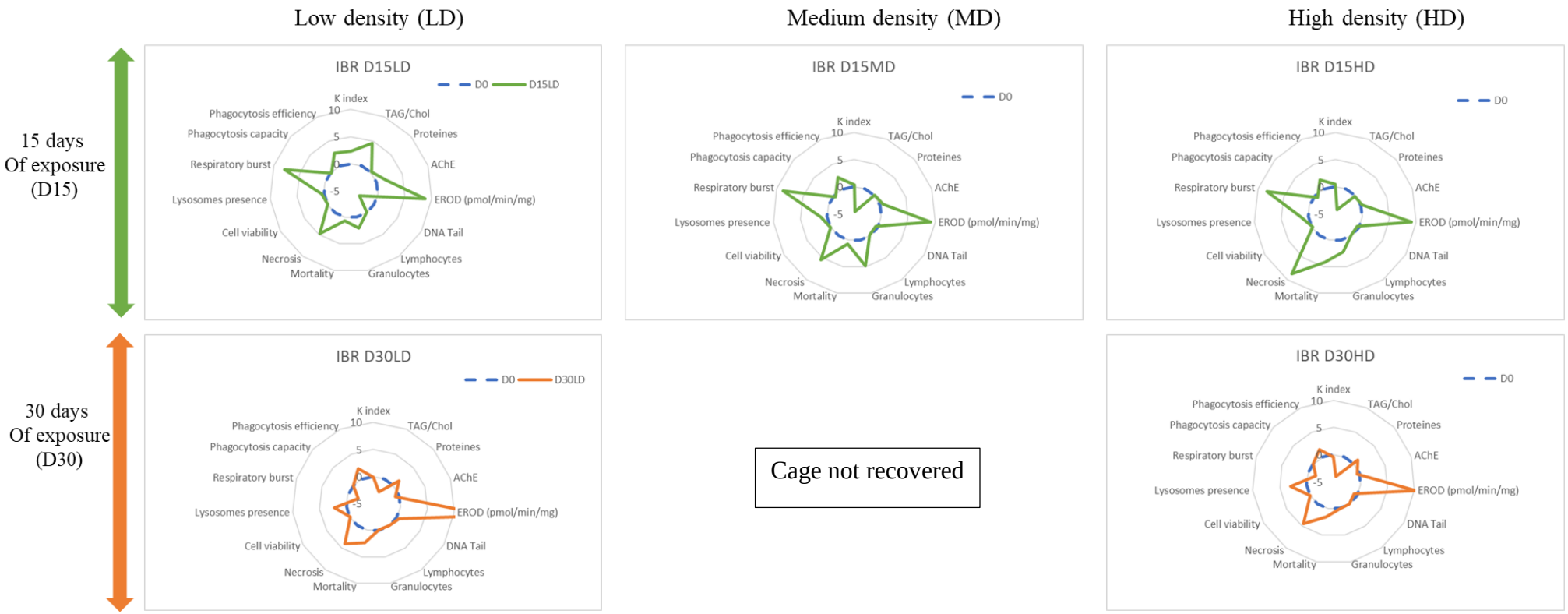


Figure 6 : Integrative Biological Response (IBR) calculated in the caged fish with Low Density (LD), Medium Density (MD) and high Density HD) exposed during 15 days (15D) and 30 days (30D) in comparison with the controls (D0).

Competing financial interest declaration

The authors declare no competing financial interests.

Author contributions

Mamadou DIOP: collected and analysed the sample, conception of the article, collected and analysed the data, developed the text and updating the article as per the suggestions. Jérôme Couteau: sample analysis, revised the text and make substantial contributions towards genesis and design of the article; Anne Bado-Nilles: sample analysis, conception of the article, revised the text and make substantial contributions towards genesis and design of the article; Eric Tavernier: revised the text and make substantial contributions towards genesis and design of the article; Baghdad Ouddane: revised the text and make substantial contributions towards genesis and design of the article; Jeremy Denis: revised the text and make substantial contributions towards genesis and design of the article contribution towards genesis and design of the article; Gwendoline Duong: revised the text and make substantial contributions towards the article; François Gevaert: revised the text and make substantial contributions towards genesis and design of the article. Sebastien Monchy: revised the text and make substantial contributions towards genesis and design of the article. Jean Laroche: revised the text and make substantial contributions towards genesis and design of the article. Rachid Amara: Conceptualization and methodological development of the study, revised the text and make substantial contributions towards genesis and design of the article.