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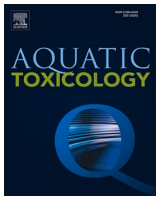

Effects of chronic exposure of metals released from the dissolution of an aluminium galvanic anode on the Pacific oyster Crassostrea gigas

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ABSTRACT

Among the anthropogenic sources releasing metallic species into the marine environment, the galvanic anode cathodic protection system (GACP) is widely used to protect submerged metallic structures from corrosion. Galvanic anodes are an alloy of metals of which the main component is aluminum or zinc. Very few studies were performed to study their potential biological effects. We investigated the chronic toxicity of an aluminum-based galvanic anode on the Pacific oyster, Crassostrea gigas. Oysters were exposed for 84 days to three concentrations of aluminum (50, 100 and 300 μg L^{-1}) obtained with an electrochemical experimental device simulating the dissolution of a galvanic anode. At different exposure times, we studied a battery of biomarkers of the immune system, reproductive parameters and the metabolic state of the oysters. Results demonstrated a sensitivity of oysters at the highest concentration and some biological effects were observed especially for the malondialdehyde content in the digestive gland after 84 days of exposure. In addition to these biomarkers, the bioaccumulation of the different metals composing the anode was measured in oysters' tissues. Bivalves bioaccumulated more zinc than aluminum, even if aluminium was present in greater concentrations during exposures. Moreover, exposure time did not influence the bioaccumulation of aluminum in contrast to zinc.

1. Introduction

Coastal ecosystems are areas subject to significant pressure due to their anthropization, and they have become particularly vulnerable. Indeed, they are impacted by maritime transport, fishing, shellfish farming, sediment dredging, noise pollution, chemical pollution and many others. Among the anthropo-constructions affecting biodiversity, functioning and resilience of aquatic environments, merchant ships, port structures, wind farms or oil platforms are of particular interest. It is common practice to connect galvanic anodes on these steel structures as a corrosion protection system. This type of cathodic protection (GACP for Cathodic Protection by Galvanic Anode) consists in controlling the corrosion of a metallic surface by means of an anode made of a metallic alloy of which electrochemical potential is lower than that of the metal to be protected (Guibert, 2009). The galvanic anode undergoes oxidation in the marine environment and releases various metals in the forms of ions or oxy-hydroxides into the environment. The principal metals composing the anodes are aluminium (Al), zinc (Zn) or magnesium because of their negative corrosion potential. Historically, zinc-based alloys were used first, but the use of aluminum-based galvanic anodes for the cathodic protection of metallic structures has been practiced since the early 1960s (Roy, 1968). The main advantages of Al are its availability, low cost, low density and uniform current distribution, which explains why it is more used in offshore structures (Guibert, 2009). As a metal alloy, galvanic anodes are known to significantly release toxicological elements (Reese et al., 2020). Depending on their physical and chemical behaviours in the solution phase, these elements are present in soluble fraction of the water or associated with suspended particulate matters, resulting in the deposition of these elements in sediments at proximity of the equipped installations when hydrodynamic conditions are low (Caplat et al., 2020). Along the marine coasts, more and more ports are equipping themselves with galvanic anodes to protect their metallic structures such as sheet piles or dolphin (pilings). Private individuals are also equipping the hulls of their pleasure boats for better protection over time. In addition, in the frame of the energy transition, the increasing installation of offshore wind farms also

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involves the installation of galvanic anodes. In an offshore wind farm of about 75 wind turbines, the galvanic anodes composed by Al alloy can represent a mass of about 15 tons per foundation, which corresponds to an estimated transfer of metals into water of 105 kg day^{-1} or approximately annually 45 tons of Al and 2 tons of Zn, contributing to the enrichment of aquatic environments with metals (Kirchgeorg et al., 2018).

It is therefore essential to study the impact that such a metal transfer can have on marine ecosystems. Dissolved aluminium concentrations in open sea are typically below 0.7 μg L^{-1} (Tria et al., 2007). In coastal waters, Al concentrations vary from 0.1 to 39.4 μg L^{-1} and can reach 83 μg L^{-1} in heavily polluted ports (Angel et al., 2016; Golding et al., 2015). In the open ocean, zinc is present at concentrations of the order of 0.01 μg L^{-1} and concentrations are about 10 μg L^{-1} in coastal waters such as the English Channel with measurements about 3 to 12 μg L^{-1} (Sheahan et al., 2007). However, levels reaching 1800 μg L^{-1} have already been recorded in some areas (Barcellos, 1995). Overall, the contribution of anodes to metal concentrations in marine waters appears to be low, except in confined areas with low hydrodynamics and shallow depths where aggregation and precipitation processes are observed in surface sediments (Caplat et al., 2010; Deborde et al., 2015). It has been recently shown that the dissolution of an aluminum-based galvanic anode induces a higher enrichment in Zn than Al in the sediment (Caplat et al., 2020). Few studies have focused on the metals released from galvanic anodes and their potential effects on marine organisms. Recently,Bell et al. (2020) observed slight effects on algal growth. Caplat et al. (2010) showed that Al and Zn from the dissolution of the galvanic anode were less toxic than sulphate salts on the sea urchin, *Paracentrotus lividus*. Further investigations are required to assess the impact of galvanic anodes on the marine environment.

Therefore, the main objective of our study was to investigate the chronic toxicity of aluminum-based galvanic anode on the Pacific oyster, *Crassostrea gigas* (Thunberg, 1793), also known as *Magallana gigas*. Due to their feeding habits, bivalves concentrate environmental contaminants in their tissues by filtering large volumes of water and are therefore bioaccumulative species. This particularity and its economic importance make the pacific oyster a very useful model for the evaluation and monitoring of environments. In this work, oysters were exposed for 84 days to three concentrations of aluminium using an experimental device simulating the dissolution of a galvanic anode. At different exposure times, we analysed a battery of biomarkers of the immune system, reproductive parameters and the metabolic state of the oysters. The bioaccumulation of the different metals composing the anode was also measured in oysters' tissues.

2. Materials and methods

2.1. Organisms

Pacific oysters of 18 months were collected by the experimental center « Synergie mer et littoral » (SMEL) from the shellfish farm "Thalassa" of Blainville-sur-mer (West coast of Normandy, France); oysters were from natural catchment. At the beginning of the experiment, homogeneous (ANOVA, $p > 0.05$) in size and weight (averaged 24.58 ± 0.39 g in weight and 65.83 ± 0.45 mm in length) oysters' batches were constituted. The experiment was realized at Blainville-sur-mer in SMEL center, which dedicated an air-conditioned room to this objective. Oysters were acclimated for 1 month and the room temperature was 16 °C.

2.2. Experimental structure and design

An electrochemical experimental device simulated the dissolution of an aluminum-based galvanic anode (GACP) in controlled conditions to obtain a transfer of metals with the same chemical speciation as in the marine environment (Fig. 1). The device was equipped with reference

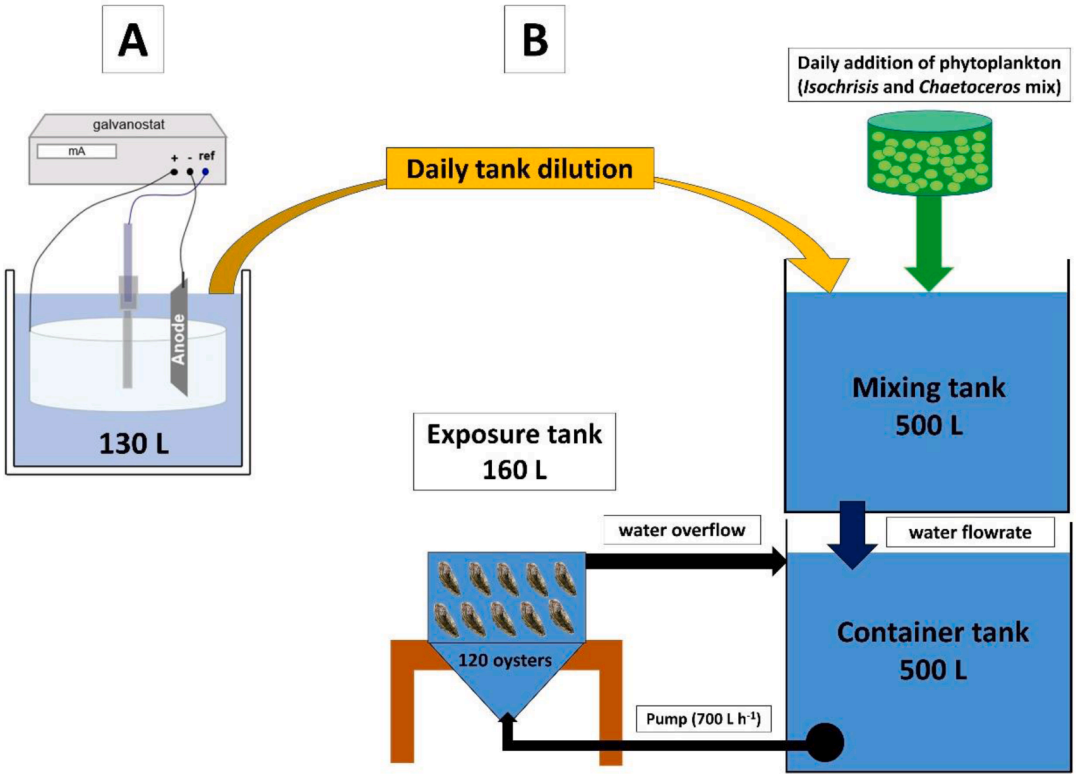

Fig. 1. Scheme of the electrochemical device consisting of a galvanostat and an aluminium galvanic anode simulating its degradation in the marine environment modified according to Caplat et al. (2010) (A). Scheme of the installation of the different tanks for the oysters' exposure to the products resulting from the dissolution of the aluminium galvanic anode. The setup is replicated 4 times for the control and the three aluminum concentrations tested (65, 125 and 296 μg L^{-1}) (B).

electrode (saturated calomel electrode - SCE), a counter electrode (CE) made of a platinize-titanium alloy and an aluminum anode (Al Bera15, Bac Corrosion® Control). The assembly was electrically connected to a galvanostat as already realized in other studies (Caplat et al., 2012, 2010; Mao et al., 2011; Mottin et al., 2012). Aluminum-based galvanic anode is mainly composed of seven elements and the mass percentages of each metal were given by the supplier as following: 93.2% to 97.1% of Al, 2.5% to 6.5% of Zn, 0.13% max of iron, 0.1% max of silicon, 0.01% to 0.03% of indium, 0.01% max of manganese and 0.006% max of copper. This experimental device provided 130 L of contaminated water with a high concentration of metals ([Al] = 11 131 ± 2 764 µg L^{-1}, [Zn]= 500 ± 123 µg L^{-1}) every 24 h. Concentrations in water of the other anode constituents were all lower than the quantification limit.

The exposure was performed during 84 days (from June 2 to August 28, 2020). Regarding the oysters, 23 individuals were collected before the beginning of the experiment and 120 oysters per tank were randomly distributed in 4 experimental systems: 1 control and 3 aluminium nominal concentrations of 50, 100 and 300 µg L^{-1} got after decantation process (for 24 h). Each experimental system was composed of 3 tanks (2 of 500 L and 1 of 160 L) (Fig. 1). The first 500 L tank was used for daily mixing of contaminated water, phytoplankton and natural seawater filtered to 50 µm, as follows: every day, a volume of contaminated water, produced by the experimental device with the galvanic anode, was added to the mixing 500 L tank according to the targeted Al concentration. A mixture of phytoplankton (*Isochrisis galbana* and *Chaetoceros calcitrans* in ratio 2:1) was daily added at a concentration of 1.10^6 cells per oyster. The volume of the mixing tank was completed to 500 L with natural seawater. Then, the mixture of the first tank fell into the container tank located below the first one, which served as a reserve and for the decantation of metals. A pump (700 L h^{-1}) installed in this container tank sent the contaminated water into the exposure tank, in which the oysters were placed on a sieve. Every two days the exposure tank was cleaned to remove all oyster faeces in the aim to limit the water quality degradation. Salinity (34.44 ± 1.04 PSU), temperature (16.27 ± 0.27°C), carbonates (3.18 ± 0.74 meq L^{-1}) and pH (8.06 ± 0.08) were continuously recorded with a multiparameter probe (NKE©, model STYPS+30-SI) and revealed stable under the 4 conditions. Oysters were sampled at five dates: 1 day and then 7, 29, 47 and 84 days. At each sampling date, 23 individuals per condition were randomly picked, 13 for biomarkers analyses and 10 for bioaccumulation assessments.

2.3. Biometric and histological biomarkers

In each experimental condition, the oysters were daily checked to discard dead oysters and calculate mortality rates. On each sampling date, all oysters collected were measured in length, width and thickness (to the nearest 0.01 mm), and weighted (to the nearest 0.01 g) to determine potential growth. The weight of the drained visceral mass and the empty shell are also recorded. These same oysters are used to determine two conditions index: the "Association Française de Normalisation" (AFNOR) condition index (CI) and the Walne an Mann index (Walne and Mann, 1975) respectively calculated as follows:

$$\mathbf{CI_{AFNOR}} = (\mathbf{fleshweight}/\mathbf{totalweight}) \times \mathbf{100}$$
$$\mathbf{CI_{WalneandMann}} = (\mathbf{dryfleshweight}/\mathbf{dryshellweight}) \times \mathbf{100}$$

For histology analysis, oyster cross-sections (5 mm thick) were cut behind the labial palps and fixed in Davidson's solution (1 vol. glycerol, 2 vol. 37% formaldehyde, 3 vol. 95% ethanol and 3 vol. filtered seawater). Tissue samples were then routinely processed, and the 3 µm paraffin-embedded sections were stained according to the trichromatic protocol of Prenant Gabe (Gabe, 1968). For each individual, the sex and stage of gametogenesis were determined. A Mean gonad Index (MGI) reflecting the global cyclic changes in the reproductive tissues of oysters was calculated (Gosling, 2003) and a coefficient was assigned to each stage as follow: stage 0: sexual rest = 0; stage I: beginning of gametogenesis = 1; stage II: active gametogenesis = 2; stage IIIa: beginning of sexual maturity = 3; stage IIIb: beginning of spawning = 3.5; stage IIId: advanced spawning = 1.5.

$$MGI = [(number\ of\ individuals\ at\ stage\ n \times coefficient\ of\ the\ stage\ n) + \cdots + (number\ of\ individuals\ at\ stage\ n+1 \times coefficient\ of\ the\ stage\ n+1)]/(total\ number\ of\ individuals)$$

2.4. Measures of metallic concentrations and bioaccumulation

Seawater samples were collected every week to determine total concentrations of Al and Zn by atomic absorption spectrometry (AAS). The aluminum was analyzed by a Zeeman Atomic Absorption Spectrometer Z-AAS (AA240Z – Varian) equipped with a graphite tube atomizer (FTA 120, Varian) and a programmable sample dispenser (PSD 120, Varian). Analyses were performed with a matrix modifier solution (2 g L^{-1} of Mg $(NO_3)_2$). Each digested sample was analysed five times, and the mean of values was used for evaluation. Zn was analysed with air–acetylene flame atomic absorption spectrometry (F-AAS) using a Varian AA240F apparatus equipped with a deuterium lamp to correct non-specific absorption. Analyses were conducted on each sample in triplicate, and mean values were used for evaluation. The limit of detection for determination of each metallic concentration was defined as mean of blank analyses plus three times standard deviation (SD) of the blank analyses. A SD of 3.1 µg L^{-1} (n = 30) was given for Zn and 6.6 µg L^{-1} (n = 45) for Al.

Ten oysters for each condition and sampling date were dissected to measure metals in tissues. The visceral mass was weighed and then stored at −20 °C. The samples were freeze-drying then crushed with Teflon balls and mineralized with prior microwave acid sample digestion (Multiwave ECO, Anton Paar, Les Ulis, France) (800 µl of concentrated and purified HNO_3 (70%), 200 µl of purified H_2O_2 and 1 ml of ultrapure water for 40 mg DW). All samples were spiked with three internal-standard solutions of Gallium, Rhodium and Iridium for final concentrations of 5, 1 and 1 mg L^{-1}, respectively, diluted to 50 ml with Ultrapure water to obtain solutions containing 2.0% (v/v) of nitric acid, and then filtered at 0.45 mm using a teflon filtration system (Digifiltre, SCP Science, Villebon-sur-Yvette, France). Total concentrations of several elements (Al, Ag, As, Ca, Cd, Cu, Fe, K, Mg, Mn, Ni, Pb and Zn) were measured by High Resolution Inductively Coupled Plasma -Mass Spectrometry (HR ICP-MS Thermo Scientific™ Element 2™, Bremen, Germany and Element XR™). To assess the quality of the biota processing procedure, a standard reference material (SRM NIST-1566b oyster tissue from the National Institute of Standards and Technology, Gaithersburg, MD, USA) was analysed with a series of samples. The relative errors were less than 10% for all metal concentrations recorded in both standard reference materials, excepted for Pb and Al concentrations which ranged between 10% and 40%. Appropriate blanks were also analyzed in series with all the samples. The limit of quantification (LQ) for each element was calculated from 10 times the standard deviation of 25 measures of blanks. The limits of quantification for determination of the Al and Zn concentrations measured in seawaters (10 measures of blanks) were respectively of 6.4 µg L^{-1} and 7.7 µg L^{-1}.

Bioconcentration factors (BCFs) were used to assess the capacity of oysters to accumulate Al and Zn from environment (Adams et al., 2000). BCF was calculated as follow:

BCF = [Total Al or Zn concentration in oysters' flesh (mg kg^{-1}) / Average of the total Al or Zn in seawater (mg L^{-1})]×0.12 (dry weight/ fresh weight in bivalves Papp, 2011).

2.5. Immune parameters of the hemolymph

2.5.1. Hemolymph removing and hemocyte counting

The hemolymph was withdrawed from the posterior adductor muscle using a 25-gauge needle mounted on a 5 mL plastic syringe. The hemolymph from each oyster was transferred into an individual tube on ice. For each sample, a fraction was examined under light microscopy to check the quality of the sample and determine the concentration of cells. A volume of hemolymph was calculated in order to have 100,000 cells per sample.

2.5.2. Phagocytic activity and reactive oxygen species

The phagocyte capacity of hemocytes was determined by adding fluorescent beads (Fluorospheres F8823, Carboxylate, 1 μm diameter yellow-green fluorescent, Invitrogen®) to the medium. These beads are coupled with fluorescein (FITC) which allows, after phagocytosis, their detection by flow cytometry. From a stock solution of 3.6×10^{10} beads mL^{-1}, these were added to the medium at a rate of 100 beads per cell. A stock solution was prepared using 5.6 μl of fluorescent beads added to 194.4 μl of Molluscan Physiological Saline (MPS) (4.10^{-1} M NaCl; 1.10^{-1} M $MgSO_4$; 2.10^{-2} M HEPES; 1.10^{-2} M $CaCl_2$; 1.10^{-2} M KCl). After 40 min incubation at 17 °C, Hoechst 33342 (H3570, Trihydrochloride, Trihydrate, Invitrogen®) was added to 2 μg mL^{-1}. This compound binds to the DNA of living cells and can be detected by fluorescence allowing the elimination of debris from the cytogram. After 20 min incubation time, cells were fixed with 3% formaldehyde. All samples were stored at 4 °C until analysis by flow cytometry (Beckman® Coulter CytoFLEX, Flow Cytometry platform SF4206 ICORE). In total, 10, 000 events were counted for each sample, and the phagocytosis efficiency of cells was evaluated as the percentage of hemocytes that had engulfed at least three beads (Delaporte, 2003).

The determination of reactive oxygen species (ROS) was also performed by flow cytometry on the same individual hemolymph samples. For this, 2,7-dichlorofluorescein diacetate (DCFH-DA, Sigma®, final concentration = 10 μM) which diffuses into the cells during the 1-h incubation time was used. In the cytoplasm, the acetate (-DA) groups are eliminated by esterase and the DCFH is thus trapped in the cells. Intracellular DCFH, which is a non-fluorescent fluorescein analogue, is oxidized to dichlorofluorescein (DCF) by hemocytes to produce ROS. The oxidation of intracellular DCFH is quantitatively related to the oxidative metabolism of hemocytes and is mediated by hydrogen peroxide (H_2O_2). After 1 h of incubation, the samples were fixed with 3% formaldehyde and stored at 4 °C until flow cytometry analysis. The production of DCFH results in green fluorescence, measurable on the FL1 detector of the flow cytometer. The results of ROS production were expressed as the percentage of cells showing fluorescence.

2.5.3. Lysosomal membrane stability

The lysosomal system was evaluated with the neutral red retention assay (NRRA) according to the protocol used by Minguez et al. (2014). The hemolymph was deposited in the wells of a 24-well plate. After 30 min of incubation at 17 °C to allow the hemocytes to adhere to the well bottom, 200 μL of neutral red (NR) solution were added in each well. The solution was prepared with 10 μg of NR (N7005, Sigma Aldrich®) diluted in 347 μl of DMSO and filtered with a 0.22 μm syringe. Then 10 μL of filtrate were diluted with 5 mL of MPS. NR is absorbed into the cells by membrane diffusion where it is trapped in the lysosomal compartment (Lowe and Pipe, 1994). An alteration in its absorption reflects damage to the plasma membrane. Dead cells or cells with damaged membranes cannot accumulate NR and as a result, wells containing dead or damaged cells will be less stained. After 1 h of incubation at 17 °C, wells were rinsed twice with 300 μL of MPS to remove the excess NR not incorporated in the lysosomes. Two hundred microliters of lysis buffer (1:50:49 v/v/v acetic acid, absolute ethanol, Milli-Q water) were added to each well and the plate was darkened for 30 min at room temperature to lysis the cells and release the neutral red from the lysosomes. Absorbance measurement was performed with the microplate reader at 540 nm (Flexstation 3, Molecular Device®, Sunnyvale, CA, USA, Proteogen platform SF4206 ICORE).

2.6. Malondialdehyde content and protein content

Malondialdehyde (MDA) accumulation in the gills and digestive gland was used as a lipid peroxidation marker. Samples were homogenized with a Potter-Elvehjem homogenizer for 1 min at 1400 rpm in PBS (NaCl 500 mM, Na_2HPO_4 10 mM, KH_2PO_4 3.1 mM, pH 7.4) containing butylated hydroxytoluene (antioxidant agent). Samples were then centrifuged (10,000 x g, 10 min, 4 °C) and the supernatants were aliquoted and stored at −80 °C until analysed. The same sample was used for determining both proteins levels and the MDA content. The MDA assay was performed with a test kit (MDA-586 Oxis-Research®) following manufacturer's instructions. The plate was read with the microplate reader at 586 nm. Results were expressed in nmol MDA/mg protein.

2.7. Energy reserves

Glycogen reserves were used as a marker of energy reserves in oysters. After removing the mantle edge and gills, a sample of the remaining visceral mass was taken, frozen in liquid nitrogen and stored at −80 °C. After defrosting on ice, ultra-pure water was added to each tube in a proportion by volume: weight (100 μL for 0.01g). Tissues were homogenized with a Potter-Elvehjem for 1 min at 1400 rpm. The samples were then boiled for 5 min to inactivate the enzymes and then centrifuged (3000 x g, 5 min, 4 °C). The supernatant was recovered and centrifuged again (13 000 x g, 5 min, 4°C). Finally, the supernatants were aliquoted and stored at −80°C until assayed. The glycogen assay was performed with glycogen test kit (MAK016-1KT Sigma-Aldrich®) following manufacturer's instructions. The plate was read with the microplate reader at 570 nm. Results were expressed in mg glycogen/g flesh.

In addition, hemolymph was used to measure the Brix index which is an indicator of the nutritional status, strongly correlated to the concentrations of proteins, triglycerides, cholesterol, calcium and phosphorus in the hemolymph (Berry et al., 2019). Hemolymph of 10 oysters per condition and sampling date was individually analyzed. To this end, 100 μL of hemolymph were centrifuged (3000 x g, 5 min, 4 °C) and supernatant was deposited on the optical reader of a digital refractometer with automatic temperature compensation (Hanna Instruments, HI96801). Results were expressed in °Brix. Ultrapure water was used as a blank between each sample.

2.8. Statistical analyses

Statistical analyses were performed using R software version 3.4.4. The normality of the data was tested with the Shapiro-Wilk test and homoscedasticity with F-test. If the data followed a normal distribution and homoscedasticity was respected, analyses of variance (ANOVAs) followed by Tukey post-hoc tests were used to determine homogeneous groups. When the data did not follow a normal distribution, non-parametric Kruskal-Wallis (K-W) tests followed by Dunn's tests were performed. Differences were considered significant at p-value < 0.05. In addition to these analyses, a correlation matrix (Pearson test) and a principal component analysis were performed on the biomarkers and metals concentrations data. All results are presented as mean ± SEM

Table I
Average metal concentrations in water during the exposure period for the 4 conditions. Metals levels are expressed as ug L^{-1}(mean values ± SEM, n=15).

Concentration (µg L^{-1})	Control	C1	C2	C3
Nominal concentrations of aluminium	0	50	100	300
Aluminium	7.4 ± 1.3	65 ± 6.7	125 ± 11.2	296 ± 29
Zinc	9.7 ± 0.8	15 ± 0.5	22 ± 0.6	35 ± 22.6

(Standard Error of the Mean).

3. Results

For C1, C2 and C3 conditions, effective concentrations of metals measured in exposure tanks during the experiment were about respectively 65, 125 and 296 µg L^{-1} for Al and 15, 22 and 35 µg L^{-1} for Zn (Table I).

3.1. Oyster mortality and biometry

For the 3 months of exposure, 1% on the 120 oysters of the control died and there was no difference between the control and the 3 concentrations (*data not shown*). Regardless of the conditions or time, oysters did not show variations in the different biometric parameters measured during the experiment (ANOVA, $p > 0.05$). In the same way there was no variation for condition index which were on average of 17.61 for the AFNOR condition index and 5.79 for the Walne and Mann index.

3.2. Bioaccumulation

At the beginning of the experiment, the aluminum and zinc levels in the oysters are respectively 19.7 ± 5.7 and 672.5 ± 96.4 mg kg^{-1}. Fig. 2 shows the bioaccumulation kinetics of Al and Zn during the 84 days of exposure for the 4 experimental conditions. After 24 h of exposure, significant differences in aluminum levels in oysters were observed. For condition C3, it reached 109.0 ± 25.2 mg kg^{-1} and subsequently it is necessary to wait 47 days to recover a similar content. Indeed, after 1 week of exposure, a decrease of the aluminum concentration in oysters was observed in all the experimental conditions. Table II lists elements concentrations measured in the total tissues of oysters ($n = 10$) collected for each condition after 84 days. Results for all exposure times are included in supplementary 1. For Al, the contents in oysters were respectively 14.6 ± 6.8, 26.8 ± 3.5, 27.6 ± 3.3 and 103.7 ± 10.3 mg kg^{-1} for the control, C1, C2 and C3. The bioaccumulation kinetics of Zn was different from that of Al. After 29 days, significant differences in the flesh concentrations were observed between the C3 condition and the control, with Zn content of respectively 1532 ± 131.9 mg kg^{-1} and 908 ± 46.3 mg kg^{-1}. The concentration differences between all the conditions gradually increased after 84 days. Then, Zn levels in oysters were respectively 1 052 ± 108, 1 328 ± 132, 1 952 ± 82 and 2 754 ± 419 mg kg^{-1} for the control, C1, C2 and C3. For both metals, the concentration in oyster tissue was significantly higher for condition C3. The other toxicologically important elements analyzed such as copper, lead or cadmium did not show significant variations in oysters and a similar result was obtained for potassium, magnesium and calcium (ANOVAs, $p > 0.05$). Fig. 3 presents kinetics of bioconcentration factors of Al and Zn during the 3 months of exposure for the 4 experimental conditions. For the two metals, highest BCF values were linked to lowest and naturally exposure concentrations. For the three concentrations of exposures (C1, C2 and C3), Al presents close and lowest BCF values, whereas Zn BCF values increased in relation to the decrease of exposure concentrations.

3.3. Gametogenesis stages

Histological analysis (n=13 per condition) did not reveal any significant differences in the progress of gametogenesis between the 4 exposure conditions (Kruskal-Wallis, $p > 0.05$). After 84 days, MGI were, respectively, for the control, C1, C2 and C3 of 3.35 ± 0.15, 3.04 ± 0.24, 3.27 ± 0.22 and 3.35 ± 0.16 (*data not shown*).

3.4. Immune parameters

Fig. 4 shows four immune parameters of oyster hemocytes after 84 days. For all parameters (i.e. hemocyte count, phagocytic activity, ROS production and lysosomal system), we did not record any significant differences between individuals exposed to the four conditions (Kruskal-Wallis, $p > 0.05$). However, some decreasing trends could be observed, particularly for phagocytic efficiency with a 40% decrease for the C3 condition compared to the control. By contrast, there was an increase of 50% in the lysosomal system for condition C2 and 25% for condition C3. The individual variability induced large deviations which probably explains the absence of significance.

3.5. Proteins and MDA levels

Protein concentrations in the digestive gland ranged from 34.55 to 54.73 mg mL^{-1} and in the gills from 15.34 to 27.24 mg mL^{-1}. There were no significant differences between the different exposure conditions throughout the exposure (*data not shown*). After 84 days, MDA levels in the digestive gland of oysters showed variations with a significant decrease only for oysters exposed to C3 (Fig. 5). The values averaged 3.61 ± 0.79, 1.92 ± 0.24, 1.62 ± 0.18, 1.15 ± 0.38 nmol MDA/mg protein for, respectively, the control, C1, C2 and C3. In contrast, MDA

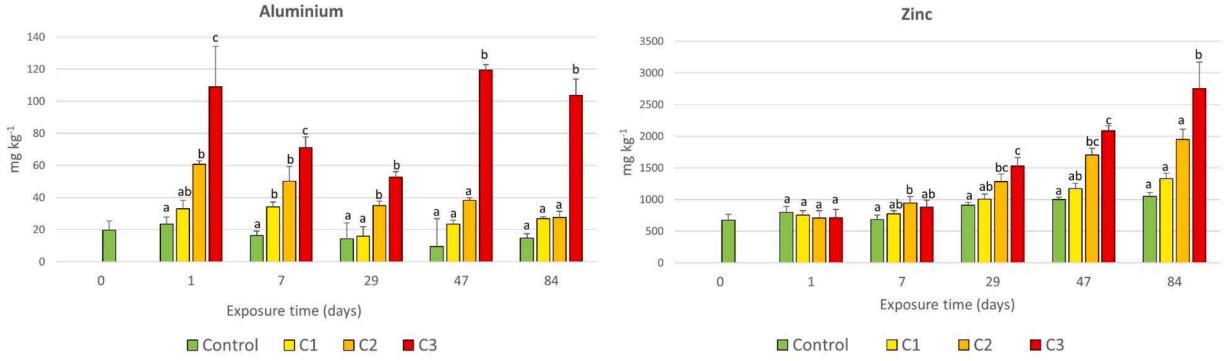

Fig. 2. Bioaccumulation kinetics of aluminium and zinc for the 84 days of exposure under the 4 experimental conditions. Metals levels are expressed as mg kg^{-1} of flesh (mean values ± SEM, n=10). The significance of data is presented for each date compared to the control. A letter common to two conditions indicates that there is no significant difference between them.

Table II
Bioaccumulation kinetics of different elements for 0 day and after 84 days of exposure for the 4 experimental conditions. Elements levels are expressed as mg kg^{-1} of flesh (mean values ± SEM, n=10). These results were obtained by High Resolution ICP-MS.

Concentration (mg kg^{-1})	Aluminium	Zinc	Copper	Manganese	Iron	Arsenic	Silver	Cadmium	Nickel	Lead	Potassium	Calcium	Magnesium
Control (0 day)	19.7 ± 5.7	672.5 ± 96.4	44.2 ± 5.4	33.5 ± 4.7	100.8 ± 4.8	18.15 ± 0.6	2.5 ± 0.4	1.5 ± 0.2	0.4 ± 0.03	0.6 ± 0.02	12112 ± 383.4	4415 ± 809.5	2656 ± 123.7
Control (84 days)	14.6 ± 6.8	1051.7 ± 108.3	53.7 ± 7.7	47.0 ± 9.6	125.5 ± 5.1	11.5 ± 0.9	3.0 ± 0.4	1.8 ± 0.1	0.5 ± 0.1	0.4 ± 0.04	12418 ± 393.8	8636.3 ± 1737.7	4397.9 ± 558.1
C1 (84 days)	26.8 ± 3.5	1327.8 ± 131.9	65.9 ± 8.1	30.2 ± 6.7	107.5 ± 5.7	11.2 ± 0.5	3.2 ± 0.4	1.7 ± 0.1	0.5 ± 0.04	0.4 ± 0.02	13159 ± 436.3	6213.7 ± 1035	4409.6 ± 283.5
C2 (84 days)	27.6 ± 3.3	1952 ± 81.8	79.9 ± 5.5	31.3 ± 9.4	91.9 ± 2.5	13.2 ± 1.6	4 ± 0.3	1.5 ± 0.1	0.3 ± 0.05	0.3 ± 0.02	11960.4 ± 332.2	6245.5 ± 3966.1	3751.1 ± 195
C3 (84 days)	103.7 ± 10.3	2753.6 ± 418.7	63.1 ± 9.8	29.7 ± 4.8	109.5 ± 5.1	13.3 ± 1.3	3.7 ± 0.6	1.6 ± 0.1	0.3 ± 0.12	0.4 ± 0.02	11660.3 ± 314.2	9481.5 ± 2820	3777.4 ± 216

levels in the gills revealed stable even after 84 days; values ranged from 2.38 to 3.13 nmol MDA/mg protein.

3.6. Glycogen and Brix index

The brix index measured in the bivalve hemolymph did not show any variation either as a function of time or exposure conditions (Kruskal-Wallis, $p > 0.05$). Values ranged from 4.20 to 5.05 °brix (*data not shown*). Fig. 6 shows the kinetics of oyster glycogen content during the 3 months of exposure. At the beginning of the experiment, the average glycogen content was of 117.1 ± 5.75 mg/g flesh. Whether after 29 days, 47 days or 84 days of exposure we did not measure any significant differences between the oysters exposed to the 4 conditions. Overall, the glycogen content decreased during the experiment to reach values around the order of 38.4 to 64 mg/g flesh. At 84 days, a trend could be noted because glycogen content was slightly lower for the 2 highest concentrations.

3.7. Principal components analysis and correlation matrix

A correlation matrix (supplementary data 2) performed with the Pearson test between bioaccumulated aluminum and zinc in relation to the biomarkers after 84 days reveals that Al in oysters is negatively correlated with MDA levels in the digestive gland. Such a result can also be illustrated when looking at the principal component analysis (Fig. 7) where Al and Zn concentrations present inverse relationship to both MDA and glycogen levels along F1 (Fig. 7A). All immune parameters go in the same direction along F2. Furthermore, several groups emerged corresponding to the 4 conditions on the F1F2 plane (explained variance: F1: 23.08% and F2: 19.39%) (Fig. 7B).

4. Discussion

Over the last several decades, the evaluation of biological effects of anthropogenic contaminants has become a crucial importance to maintain a good ecological status of coastal environment as recommended by European directives such as the Marine Strategy Framework Directive (MSFD) (2008/56/EC). To our knowledge, the present study is one of the first to focus on the chronic impact of aluminum galvanic anodes on a marine organism. We conducted an experimental integrative approach, designed to complement the previously published results on the effects of galvanic anodes (Caplat et al., 2020, 2012, 2010; Deborde et al., 2015; Mao et al., 2011; Mottin et al., 2012).

The concentrations of contaminants tested during the experiment did not induce significant mortalities of oysters and subletal effects had thus been to be researched. The results of bioaccumulation analyses confirmed that oysters have bioaccumulated the various constituents of the galvanic anode. At the beginning of the experiment, metals concentrations in oysters were close to the natural levels given for coastal seawaters in Normandy (RIN program, *personal data*). BCF measures the ability of an organism to bioconcentrate an element in its tissue considering the concentration of that element in the water. The BCF values measured for Al and Zn in oysters depended on the concentration of these two elements in the chronic exposure medium. At the concentration measured under C3 condition, reverse relationships were observed between metallic BCF values and total concentrations in water. In their review about metals, McGeer et al. (2003) have already reported similar relationships, that they linked to the ability of organisms to regulate uptake of chronically Zn elevated concentrations. In any case, BCFs for Al are low (around 50) indicating that *C. gigas* is not a hyper accumulator of aluminium in contrast to Zn for which BCFs are particularly high (around 10 000). Furthermore, it is observed that the BCFs of Zn decrease when the concentration in water increases. Indeed, the bioaccumulation of metals results from different mechanisms compared to organic substances. Metals in the environments are mostly in hydrophilic and hydrated forms that are not able to cross biological

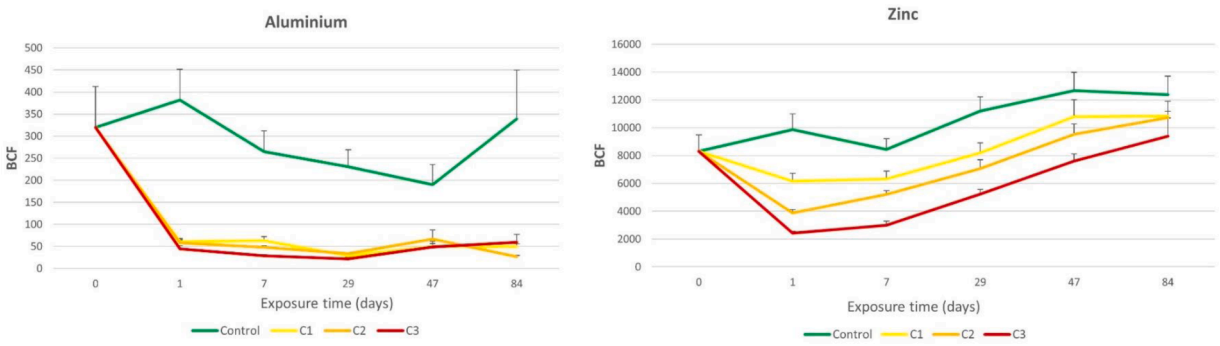

Fig. 3. Bioconcentration factors of aluminium and zinc for the 84 days of exposure under the 4 experimental conditions (mean values ± SEM, n=10).

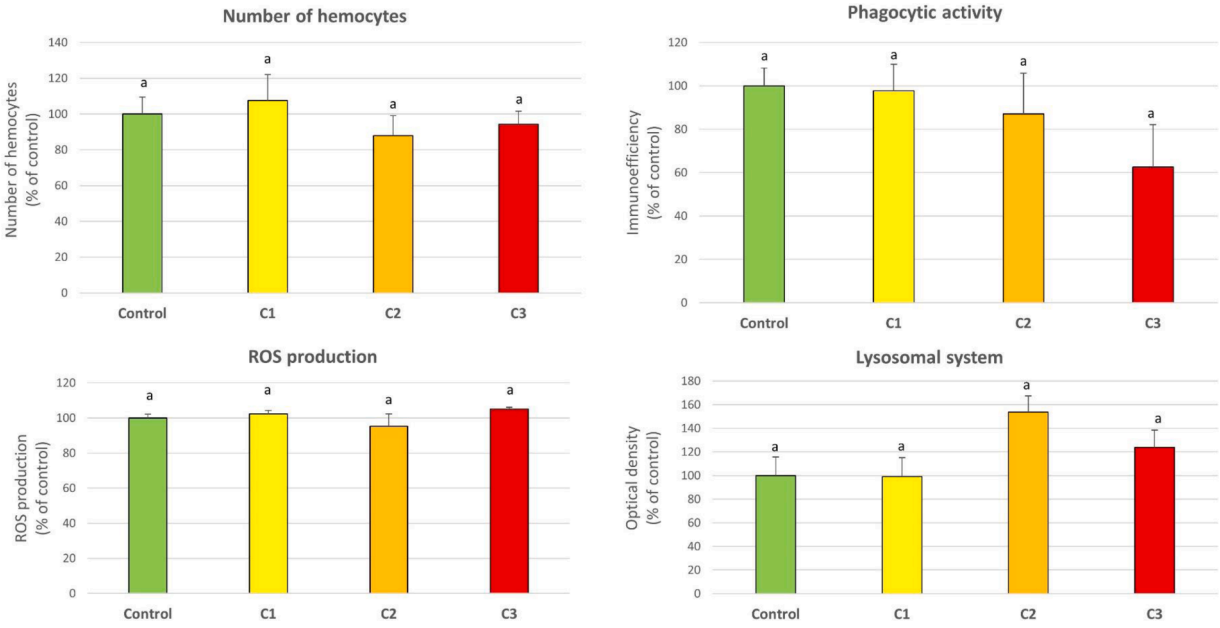

Fig. 4. Immune parameters of oyster hemocytes after 84 days of exposure to metals released from the dissolution of an aluminum galvanic anode at different concentrations. Parameters studied are the number of hemocytes mL^{-1}, phagocytic activity, ROS production and lysosomal system in oyster hemocytes. Results were given compared to the 100% control (mean values ± SEM, n=13). A letter common to two conditions indicates that there is no significant difference between them.

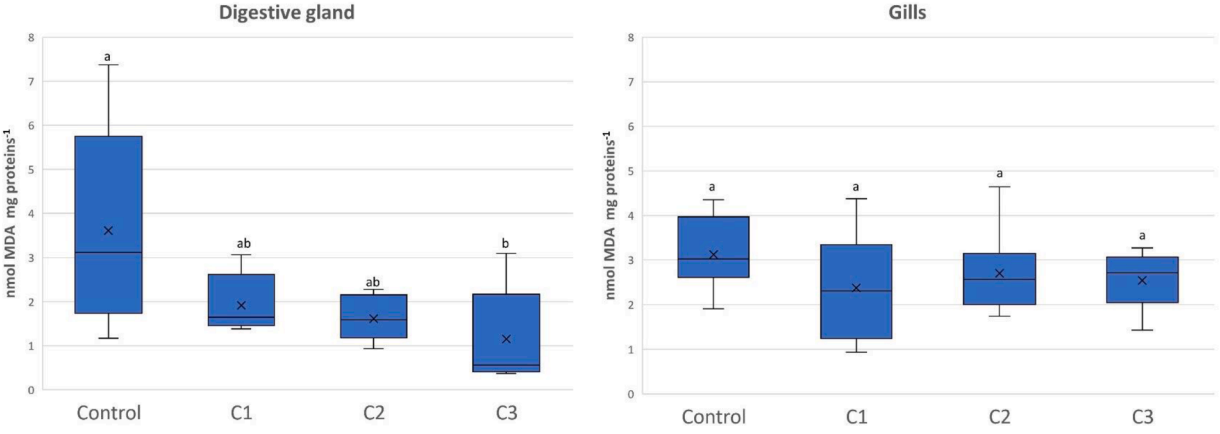

Fig. 5. MDA (malondialdehyde) levels in the digestive gland and gills after 84 days of exposure to metals released from the dissolution of an aluminum galvanic anode at different concentrations. MDA levels are expressed as nmol MDA mg $proteins^{-1}$ (mean values ± SEM, n=8). A letter common to two conditions indicates that there is no significant difference between them

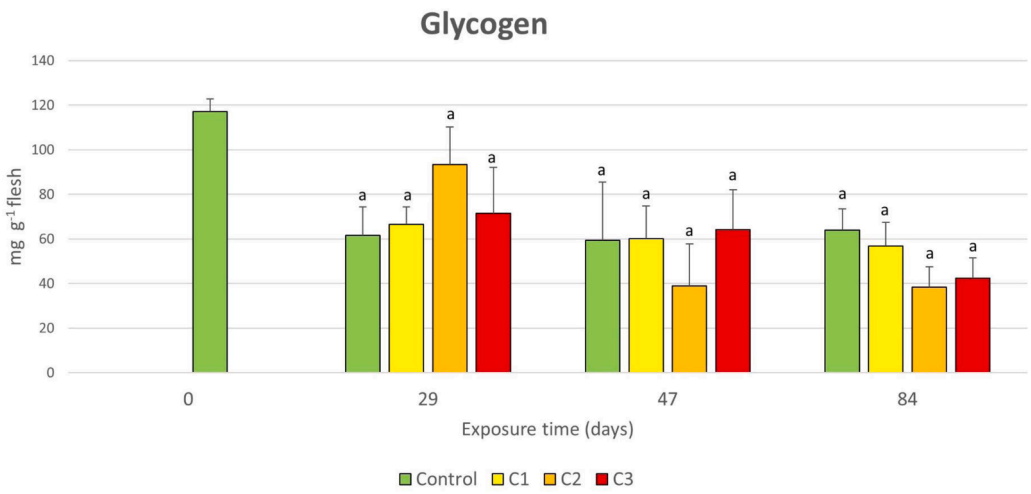

Fig. 6. Glycogen content for 84 days of exposure to metals released from the dissolution of an aluminum galvanic anode for the four conditions. Glycogen levels are expressed as mg g^{-1} of flesh (mean values ± SEM, n=8). A letter common to two conditions indicates that there is no significant difference between them

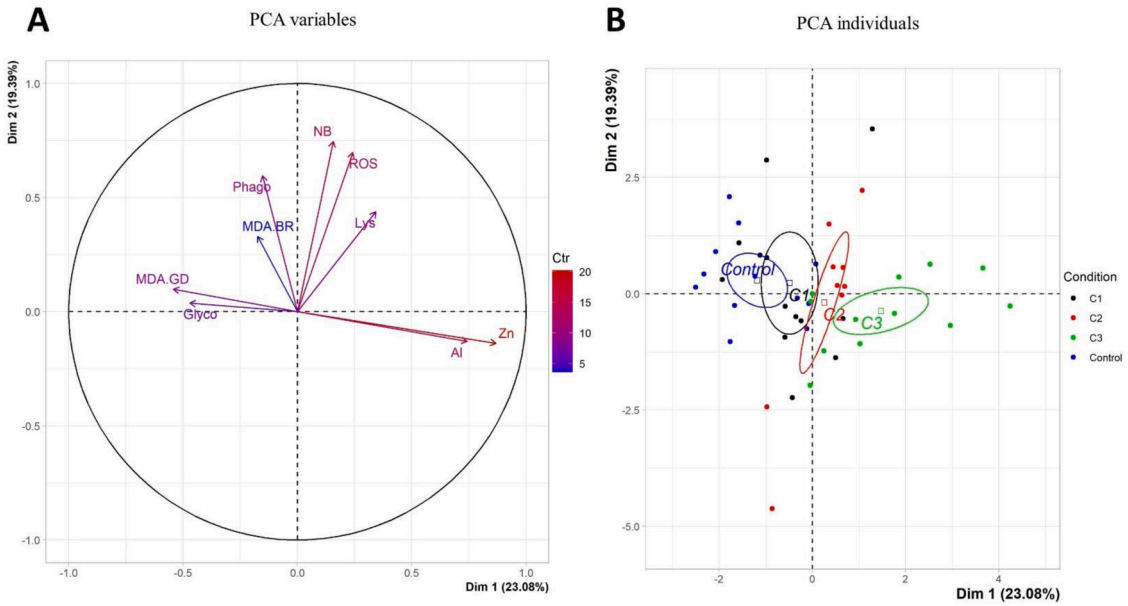

Fig. 7. The principal components analysis (PCA) of the different variables (A) and individuals (B) of the study after 84 days of exposure. Al = aluminium bioaccumulated. Zn = zinc bioaccumulated. MDA GD = MDA levels in the digestive gland. MDA Br = MDA levels in the gills. NB = number of hemocytes. Lys = lysosomal system. Phago = phagocytosis efficiency, ROS = ROS production; Glyco = Glycogene content. BRIX = Brix index.

membranes by simple diffusion. Their uptake thus requires facilitated transport involving protein transporters or transmembrane channels which can reach saturation at high metal concentrations (Pelletier and Campbell, 2008).

The filter feeding of bivalves contributes to the high bioavailability of aquatic contaminants. For an Al concentration of 296 µg L^{-1} (C3 condition) after 24 h of exposure, the Al content in oysters reaches 109 mg kg^{-1}. The detoxification process of Al starts after 7 days of exposure when the concentration drops to 71 mg kg^{-1}, and it is necessary to wait 47 days to measure again a concentration above 100 mg kg^{-1}. Concerning C2 condition, such an increase in Al content is not observed after 47 days of exposure, the oyster physiological processes being sufficiently efficient to detoxify at this Al concentration. One of the few marine studies on Al released from a galvanic anode concerns the bioaccumulation of Al in the mussel *Mytilus edulis* (Mao et al., 2011). Mussels were exposed to 530 µg L^{-1} Al for 8 weeks which is higher than the C3 concentration in our study (i.e. 296 µg L^{-1}). Mao et al. (2011) showed that the Al concentration reached its maximum after 13 days of exposure in the digestive gland (1 700 mg kg^{-1}) and after 8 days of exposure in total tissues (308 mg kg^{-1}). In our study we did not focus on any specific organ but aluminum levels in tissues are about the same order of importance between the two species (71 mg kg^{-1} after 7 days for oyster *versus* 308 mg kg^{-1} after 8 days for mussels).

The zinc profile is totally different because the enrichment in oysters' tissues was gradual over the three months of exposure and no equilibrium level has been reached at the end of the experimentation. At 0 day, the Zn concentration is 672 mg kg^{-1} and it takes 29 days to observe significant differences between conditions. After 84 days, the Zn content in oysters reaches 2753 mg kg^{-1} for C3 condition. This value is comparable to those found in the natural environment for polluted sites on the southern Spanish coast, such as Punta Umbria and Mazagon, where the Zn content in *Crassostrea angulata* oysters were, respectively, 2 240 and 3 016 mg kg^{-1} (Funes et al., 2006). Finally, in the present study, metallic results do not indicate significant enrichment of all other

quantified elements (Cu, Cd, Pb…) in oysters chronically exposed to products of the dissolution of the aluminum-based galvanic anode suggesting that Al and Zn are alone to be of concern.

In natural environments, contaminants adsorb more onto suspended organic matter, but low amount of dissolved or suspended organic matter occur in controlled conditions, which increases the bioavailability of contaminants to organisms. Therefore, the experimental BCF can be higher than that measured *in situ*. For filter-feeding bivalves such as *C. gigas*, a metal can become available by two major ways: dissolved uptake, which is mainly through the gills and probably requires no energy investment; and the ingestion of particulates associated with food particles which becomes available to bivalves. It has been already shown that aluminium dissolved in water can be incorporated into the frustules of diatoms as a detoxification mechanism (Dixit et al., 2001; Gehlen et al., 2002). Aluminium can therefore be ingested by oysters through the trophic pathway. Our results show that the oyster is not a good bioaccumulator of Al, whereas Zn is more bioaccumulated in tissues. In the future, it would be interesting to distinguish the two uptake mechanisms by performing an experiment in which oysters would be placed in non-contaminated water but fed with microalgae previously cultivated in water contaminated with aluminium.

Although Al has no biological function, it can substitute for essential metals in some biochemical processes (Exley and House, 2011). By contrast, zinc is an essential trace element for the functioning of many cellular processes, but in excess it is toxic and generates ROS that damage the organism (Calabrese et al., 1973; Mottin et al., 2012). At the same concentration, Al is known to be less toxic than Zn in marine organismes. To our knowledge, no studies have investigated the biological effects of aluminum-based galvanic anode on bivalves. Bell et al. (2020) investigated the effect of galvanic anodes during acute exposure on an amphipod species, *Corophium volutator*, a diatom species, *Phaeodactylum tricornutum*, and a bacterial species, *Aliivibrio fischeri*. They found no acute toxicity for the bacteria tested and only slight effects on algal growth. Regarding amphipods, significant increases in mortality were observed at the highest concentration of 100 mg L^{-1}. Sublethal effects would have probably occurred at lower concentrations but all tested concentrations were very far from the environmental maximums. It is interesting to note that in the study by Caplat et al. (2010), it is shown that the dissolution of Al and Zn from a galvanic anode was less toxic for the urchin *Paracentrotus lividus* than sulphate salts. The dissolution of the anode could result in hydroxide precipitation due to a local increase in pH or associations of metallorganic complexes that could reduce the levels of free ionic Al (III) and Zn (II) species and therefore their availability. As a consequence, this could result in a lower toxicity of the degradation products of galvanic anodes compared to Al and Zn in the form of sulphate salts for which there is a higher proportion of free metal forms due to the sulphate anion association. Using aluminum salts is consequently not the right solution for ecotoxicological studies of galvanic anodes. Considering Al in all its forms in literature, various LC_{50} (lethal concentration) values have been reported: 3 000 μg L^{-1} in the coral *Acropora tenuis* (Negri et al., 2011); 10 000 μg L^{-1} in the copepod *Nitocra spinipes* (Bengtsson, 1978), while no effect is observed in annelids *Ctenodrilus serratus* and *Neanthes arenaceodentata* at 2 000 μg. L^{-1} (Petrich and Reish, 1979), and at 28 000 μg L^{-1} in the sea urchin *Heliocidaris turberculata* (Golding et al., 2015). Nevertheless, at these elevated total concentrations of Al, it can be assumed that the element was principally under a non-bioavailable particulate form ($Al(OH)_3$) since the Al solubility limit is 500 μg L^{-1} (Angel et al., 2016; Golding et al., 2015).

The quantification of ROS and the number of circulating hemocytes remained stable throughout the experiment. However, after 84 days, phagocytic efficiency and the NRRA tended to, respectively, decrease and increase. Regarding NRRA, a change in DO can be interpreted as a change in the stability of lysosomal membranes or a change in the number or size of lysosomes. The decrease of almost 40% in phagocytic efficiency associated with a modulation of the lysosomal system may be indicative of a weakened immune system. A study on the effect zinc-based galvanic anode in *C. gigas* have reported a stimulation of the immune system during a 10 weeks chronic exposure (0.53 ± 0.04 mg L^{-1}) while immune parameters were inhibited during the one week acute exposure (10.2 ± 1.2 mg L^{-1}) (Mottin et al., 2012). In the present study, the absence of significant effects on the immune system can be explained by the low doses of contaminant in the water and probably the adaptation of the molluscs to chronically exposure concentrations until elevated value. Indeed, Mottin et al. (2012) investigated concentrations in Zn approximatively 30 times higher than in our C3 condition.

In bivalves, energy reserves and the reproductive cycle are described as being closely linked (Heude-Berthelin, 2000). Glycogen represents an important part of energy reserves; it is particularly used as an energy source for gametogenesis. The measurement of glycogen levels in oysters gives us information about the metabolic state of individuals. The overall decrease in glycogen levels after 29 days can be explained by active gametogenesis, with a majority of oysters being in stages 2 (active gametogenesis) and 3a (beginning of sexual maturity). There was a slight trend of decrease for conditions C2 and C3 after 84 days but this result was not related to the MGI. It is possible that oysters spend more energy to detoxify. Actually, the observation of histological sections showed no differences in the progress of gametogenesis between the 4 conditions. Concerning the BRIX index measured on hemolymph, it indicated no significant variation according to time or condition. To our knowledge, our study is the first one to use this type of index on bivalves during exposure to contaminants.

Reactive oxygen species (ROS) have the capacity to damage tissues and cellular components of organisms, which is known as oxidative stress. It is well established that marine organisms and particularly bivalves show a large panel of antioxidant defenses to protect themselves from exposure to contaminants with oxidative potential (Pipe and Coles, 1995). The role of these antioxidant systems and their sensitivity may be of great importance in ecotoxicological studies. MDA accumulation is one of the manifestations of oxidative stress, an indicator of lipid peroxidation corresponding to the oxidation of polyunsaturated fatty acids by ROS or enzymes. In our study, individuals exposed to C3 concentration showed significantly lower levels of MDA in the digestive gland after 84 days, while no variation in MDA levels in the gills wad observed. The MDA values recorded are in agreement with those found in other studies in which *C. gigas* was exposed to pesticides (Mottier et al., 2015; Séguin et al., 2016). Various laboratory studies showed an increase in MDA levels in several bivalve species following metal exposures (Khebbeb et al., 2010; Moore et al., 2020). However, it has also been shown that a 21-day exposure to cadmium, silver, and mercury induced a decrease in MDA levels in the gills of *C. gigas* (Géret, 2002). A clear decrease of MDA in *P. perna* gills was recorded during the cycle of contamination by *Staphylococcus aureus* (Bendjoudi et al., 2013). It has also been shown a significant positive correlation between zinc and copper levels in *C. angulata* oysters and biomarkers involved in defense against oxidative stress such as catalase (CAT), superoxide dismutase, glutathione peroxidase, glutathione-S-transferases as well as metallothioneins (Funes et al., 2006). In chronic exposures to contaminants released from the estuaries of Huelva and Guadalquivir, low levels of MDA were associated with high activity of metallothioneins in the clams *Chamaelea gallina* (Rodríguez-Ortega et al., 2002). Therefore, the decrease in MDA levels in the digestive gland may be explained by antioxidant defense provided by sensitive enzymes of which activity increases in target organs depending on contaminant concentration (Damiens et al., 2006). CATs, a family of key enzymes in the regulation of oxidative stress, as well as metallothioneins, proteins particularly important in metal sequestration and detoxification, are examples of defense mechanisms against oxidative stress that were able to overtake lipid peroxidation. We did not have measure these enzymatic activities but in further studies, it would be interesting to evaluate these biomarkers in the digestive gland of oysters exposed to the C3 concentration for which MDA levels are the lowest. In addition, the absence of

variation in ROS production measured in hemocytes by flow cytometry suggests that the digestive gland is impacted before the circulating system.

The principal component analysis confirms this antagonist relationship between MDA levels in the digestive gland with aluminum and zinc concentrations in water. The emergence of 4 distinct clusters on the PCA along dimension 1 corresponds to the 4 concentrations tested in our experiment. These results demonstrate a sensitivity of oysters even if only the highest concentration induced pronounced biological effects.

Conclusion

On the basis of the concentrations tested and experimental conditions, the assessment of the chronic toxicity of the degradation products of an aluminum-based galvanic anode on the Pacific oyster *C. gigas*, does not seem to indicate a direct environmental risk given the absence of significant mortality. Nevertheless, results demonstrate a sensitivity of oysters even if only the highest concentration induced pronounced biological effects. It is important to relate these results to the context of climate change, with the ocean acidification that has been shown to amplify metal bioaccumulation and may exacerbate their toxicity in *C. gigas* (Cao et al., 2018). As a non-essential element, Al shows low bioconcentration factors but galvanic anodes may facilitate the entry of Al into the food chain and its biomagnification in higher trophic level organisms considering that invertebrates are food sources of many species. It is known that Al in freshwater can be transferred from a primary to a secondary consumer at a pH of 7.1 (Walton et al., 2010). Zinc is also important to monitor because it represents 5% of an aluminum-based galvanic anode. Furthermore, in view of the bioaccumulation results, the Zn level equilibrium has not been achieved in oysters even after 84 days and it would be interesting to extant the exposure period. The sustainability of marine organisms sensitive to contaminants is essential in terms of ecosystem services as well as to limit the health risks associated with the consumption of organisms grown near areas rich in galvanic anodes. As the biological effects of aluminum-based galvanic anode are poorly studied in the literature, further investigations on larval stages and other taxonomic groups remain necessary.

Declaration of Competing Interest

The authors declare that they have no known competing financials interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106223.

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