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S. Guilherme, R. Crespo, D. Azevedo, A. Marques, M.A. Santos, R. Serradeiro, M. Pacheco

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DNA and chromosomal damage in Senegalese sole (*Solea senegalensis*) as side effects of ozone-based water treatment - Contribution to optimization of fish-farming practices

Guilherme, S.[1]*, Crespo, R.[1], Azevedo, D.[1], Marques, A. [1], Santos, M.A.[1], Serradeiro, R.[2], Pacheco, M.[1]

[1] Department of Biology and CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

[2] Riasearch, Rua do Farol, Nº 131, Torrão do Lameiro 3880-394 Ovar

* Corresponding author: Sofia Guilherme (sofia.g.guilherme@ua.pt)

Abstract

The progressive growth of aquaculture implicates a dependence on large water amounts, which are submitted to disinfection processes, namely ozonation. Considering the importance of genomic integrity, it is critical to improve the knowledge on ozone-related genotoxic hazard to organisms reared in recirculating aquaculture systems (RAS) applying ozonation. Therefore, genetic damage induced by ozone exposure in the Senegalese sole (*Solea senegalensis*) was assessed, combining the comet and the erythrocytic nuclear abnormalities (ENA) assays, reflecting different damage levels, i.e. DNA and chromosomal damage, respectively. Fish were subjected to a daily 6-h ozone (0.15 mg L^{-1}) exposure, repeated for 3 consecutive days, simulating a short-term event of overozonation. To assess the temporal impact of the previous event, the progression of damage was evaluated 7 days later, following transference to ozone-free water or to 0.07 mg L^{-1} ozone, a routinely adopted level in RAS. Both endpoints pointed to the ozone genotoxic potential, displaying DNA oxidation as a possible mechanism of damage. Overall, the present findings pointed out the genotoxic hazard of ozone to fish, highlighting the importance of these types of

studies and contributing to improve aquaculture practices, namely in RAS systems. These early genotoxic signals may be a prelude to negative repercussions on fish health, which may affect the aquaculture productivity. The present findings recommend precautions in relation to accidental or intentional overozonation in fish-farming, even when short-term events are considered. The strategies to mitigate the impact of ozonation in *S. senegalensis* may include a dietary extra supplementation of antioxidants (regularly, or punctually in cases of overozonation).

Keywords: DNA damage; ozone; fish, RAS

Introduction

Global fish production has grown steadily worldwide in the last five decades (FAO, 2014) and the subsequent increasing dependence on large water amounts made the ozone-based methods a widespread strategy for disinfection and water quality improvement, particularly in recirculation aquaculture systems (RAS) (Coman et al., 2005; Liltved et al., 2006; Good et al., 2011). Nevertheless, despite the apparent advantages in using these methods, the biological basis for the establishment of safety margins is still a critical issue due to the ozone-related toxicity. Ozone is recognized as an extremely reactive molecule (Tango and Gagnon, 2003; Sharrer and Summerfelt, 2007; Reiser et al., 2011), namely when injected into seawater. Under those conditions, it reacts with halogen ions (Liltved et al., 2006; Schröder, 2010; Schroeder et al., 2010), namely bromide ion (Summerfelt, 2003; Tango and Gagnon, 2003; Perrins et al., 2006; Sharrer and Summerfelt, 2007; Schroeder et al., 2010), and/or organic matter (Tanaka and Matsumura, 2002; Schroeder et al., 2010), generating ozonation by-products (OBP) (Tango and Gagnon, 2003; Liltved et al., 2006; Schroeder et al., 2010, 2011; Reiser et al., 2011). Since ozone reactions are fast and produce more stable compounds (Liltved et al., 2006; Schröder, 2010; Reiser et al., 2010; Reiser et al., 2011), OBP tend to accumulate in RAS (Schröder, 2010).

This knowledge raised concerns on the potential toxicity of ozone and/or OBP to reared organisms, namely fish. Previous studies supported the OBP toxicity to fish (Perrins et al., 2006; Reiser et al., 2011, 2010; Tango and Gagnon, 2003). In addition, Jones and co-workers (2006) attested to the damage that ozone treated seawater can cause to marine organisms, despite defending that ozone, by itself, is unlikely to be responsible for the toxicity in RAS systems. Nevertheless, ozone is considered toxic to fish since it can cause oxidative stress in blood cells (Ritola et al., 2000) and the histopathological evaluation revealed increased levels of gill epithelial hyperplasia and hypertrophy, as well as hepatic lipidosis (Good et al., 2011), depending on the species, life stages, concentration and exposure time (Coman et al., 2005). Moreover, Hébert

and co-workers (2008) found that ozone, used as disinfection strategy, can modify the immune system in fish at the level of T lymphocyte proliferation.

It is also known that oxygen-supersaturation or ozonated water can produce reactive oxygen species (ROS), such as anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical, ($OH^{\bullet}$) or ozonide radical ($O_3^{\bullet-}$), in an organism tissues (Liu et al., 2007a; Ritola et al., 2002, 2000). A few studies investigated the ozone and/or OBP induced oxidative stress in fish (Reiser et al., 2011). Large amounts of ROS can inactivate antioxidant defense enzymes, which might lead to oxidative damage in lipids, proteins and DNA (Bellagamba et al., 2011; Liu et al., 2007a; Liu et al., 2007b; Niu et al., 2013; Ritola et al., 2002, 2000). Thus, assuming the critical importance of DNA, its eventual loss of integrity may result in deleterious effects (Kienzler et al., 2013), with potential negative repercussions on fish health and, subsequently, on aquaculture productivity. It was already demonstrated that the ability of OBP to induce genetic damage, expressed as chromosome aberrations found in lymphocytes of chinese hamsters and chromatid deletions induced in pulmonary macrophages in rats (Victorin, 1992). However, in studies concerning ozonation-related genotoxicity in fish, a single study was performed (Silva et al., 2011), illustrating an induction of erythrocytic nuclear abnormalities (ENA). Though this study highlighted a chromosomal damaging effect, which extended beyond the exposure period, it didn't clarify the underlying toxicity mechanisms, namely the involvement of oxidative damage. This understanding can be crucial to provide clues to the definition of risk mitigation measures.

Keeping in view the aquaculture potential of the Senegalese sole (*Solea senegalensis*), the present work intended to evaluate the genotoxic impact of ozonation to this species, commonly reared in RAS, elucidating the DNA damaging mechanisms involved. For this purpose, a combination of standard/enzyme-linked comet and ENA assays was applied to blood cells, to detect DNA and chromosomal damage, respectively. The ENA assay is based on the detection of micronuclei and other nuclear anomalies (Pacheco and Santos, 1997), signalling clastogenicity (chromosome

breakage) or aneugenicity (chromosome loss and mitotic spindle apparatus dysfunction) events (Fenech, 2000; Stoiber et al., 2004), corresponding to irreparable lesions. On the other hand, the comet assay detects DNA strand breaks and alkali labile sites (Andrade et al., 2004; Lee and Steinert, 2003), reflecting damage at the molecular level, amenable to repair. The comet assay procedure improved with an extra-step involving digestion with lesion-specific repair endonucleases enabling the detection of oxidative DNA damaging pathways.

Simulating a short-term event of overozonation likely to occur in RAS, intentionally (to cope with sudden peaks of nitrogenous compounds) or accidentally (due to inexperience or technical problems), fish were daily exposed (6 h/day) to ozone 0.15 mg L^{-1}, repeatedly for 3 consecutive days. Moreover, in order to assess the progression of damage after the end of overozonation, fish were also evaluated 7 days later, following conveyance to ozone-free water and to 0.07 mg L^{-1} ozone, representing a routinely adopted level in intensive rearing of Senegalese sole in RAS.

Materials and methods

Fish and holding conditions

Specimens of Senegalese sole (*S. senegalensis*), weighing 57.52 ± 12.74 g and measuring (total length) 16.90 ± 1.21 cm, supplied by Aquacria Piscícolas, S.A. (Torreira, Portugal), were acclimated to the experimental tanks/conditions for 2 weeks. Fish were kept in 1.95 m long, 0.75 m wide and 0.20 m height tanks, with a water volume of 146 L, under recirculation. Three recirculating sub-systems were prepared (assigned to the experimental lines C, O_w and O_o; see figure 1 and description below), incorporating the respective replicate tanks and a biological filter composed by corrugated plastic sheets as biofilter media. Water parameters were kept as follow: flow rate 12.0 L min^{-1}, 21 ‰ salinity, 19.0 ± 0.6 ^{0}C temperature, 12.3 ± 2.8 mg L^{-1} dissolved oxygen, 0.024 ± 0.016 mg L^{-1} nitrite and 0.008 ± 0.005 mg L^{-1} ammonia. Salinity, temperature and dissolved oxygen were determined with an YSI 556 MPS probe.

Water was obtained from a groundwater aquifer (40 m deep well, at 500 m from the coastline). Fish were held under a 17:7 light:dark photoperiod, at an initial density of 11.8 kg m^{-2} (300 animals per tank). This fish density was defined to maintain an adequate and realistic level (considering the recommended rearing practices) up to the end of the experiment, taking into account the successive fish subtraction along the experiment.

Fish were fed by hand with a commercial dry food (in intervals of two hours), according to defined feeding tables. In the sampling days, fish were not fed at least in the 12 hours preceding handling, being just fed in late afternoon (after the sampling).

Experimental design and sampling

Simulating a short-term event of overozonation likely to occur in RAS, intentionally (to cope with sudden peaks of nitrogenous compounds) or accidentally (due to inexperience or technical problems), fish were daily exposed (6 h/day) to ozone 0.15 mg L^{-1} (O groups), repeatedly for 3 consecutive days (Fig.1). Moreover, in order to assess the progression of damage after the end of overozonation, fish were also evaluated 7 days later, following conveyance to ozone-free water (O_w group) and to 0.07 mg L^{-1} ozone (O_o group), representing a routinely adopted level in intensive rearing of Senegalese sole in RAS.

The ozone gas used in the experiment was produced by a commercial ozone generator (Ozonia, Switzerland) and mixed with seawater, using an injector connected to the water circulation system. Ozone concentration in the tanks was monitored within intervals of 1 hour and the ozone production of the generator continuously adjusted to provide the intended level. Ozone concentrations were measured spectrophotometrically as bromine (Br_2), using the colorimetric N,N-diethyl-p-phenylenediamine (DPD) method, recommended for the quantification of OBP in seawater.

The experiment was carried out using triplicate groups for each condition (ozone treatments or control). Samplings were carried out at first, second and third days of exposure, as well as at the seventh day of the post-exposure period (10th day of the experiment; see Fig. 1). In each sampling time point, 3 fish were collected per replicate tank (total of 9 fish per condition, per time point), anesthetized with tricaine methanesulfonate (MS-222) during approximately 15 min, and then measured and weighed. Thereafter, fish were dissected, and the blood was collected with heparinised Pasteur pipettes from the cardinal vein. Two microliters of blood were immediately diluted in 1 mL of a freezing solution, constituted by phosphate-buffered saline (PBS) and dimethyl sulfoxide (DMSO) 9:1 (v/v), slowly frozen and kept at -80 ^{0}C according to the slow freeze/fast thaw method (Collins, 2004), until further comet assay procedures. Additionally, blood smears were immediately prepared for the ENA assay. Following sampling, fish were sacrificed by cervical transection.

Evaluation of genetic damage

Comet assay

The conventional alkaline version of the comet assay was performed according to the Collins (2004) methodology, as adapted by Guilherme et al. (2012), with the proper adjustments to the extra step, concerning the nucleoid digestion with endonucleases. A system of eight gels per slide was adopted, based on a model created by Shaposhnikov et al. (2010). Briefly, 20 µL of cell suspension (previously prepared in PBS) were mixed with 70 µL of 1% low melting point agarose (in PBS). Six drops of 6 µL were placed onto the glass microscope slide, precoated with 1% normal melting point agarose, as two rows of 4 (each individual is represented by 2 replicate gels x 4 individuals = 8 gels), each drop/gel containing approximately 1500 cells. The gels were left for ± 5 min at 4 ºC in order to solidify the agarose, and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) at 4 ºC, for 1 h. After lysis of agarose-embedded cells, slides were washed 3 times with enzyme

buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, 0.2 mg mL^{-1} bovine serum albumin, pH 8) at 4 ºC.

Three sets of slides were prepared: two were incubated with endonucleases FPG (1) and EndoIII (2), that convert oxidized purines and pyrimidines into DNA single strand breaks, respectively (Azqueta et al., 2009). The third set (3) was incubated only with enzyme buffer. Hence, 30 µL of each enzyme diluted in enzyme buffer (and only buffer in the third set) was applied to each mini-gel, with coverslip, and the slides were incubated at 37 °C, during 30 min, in a humidified chamber. Then, slides were immediately placed in the electrophoresis tank, immersed in electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH > 13) for ± 20 min (alkaline treatment). Electrophoresis was performed during 15 min at a fixed voltage of 25 V, which results in 0.7 V cm^{-1} and a current of 300 mA (achieved by adjusting the buffer volume in the electrophoresis tank).

The slides were stained with ethidium bromide (20 µg mL^{-1}) and 50 nucleoids were scored per gel, using an Olympus BX41 fluorescence microscope (400x of magnification). Nucleoids were classified by visual scoring into 5 comet classes, according to the tail length and intensity from 0 (no tail) to 4 (almost all DNA in tail) (Collins, 2004). The final score, expressed as genetic damage indicator (GDI), was obtained by multiplying the mean percentage of nucleoids in each class by the corresponding factor, according to the formula:

$$\mathrm{GDI} = \sum \%\ \text{nucleoids class}\ i\ \times i$$

where *i* is the number of each defined class (ranging within 0-4).

GDI values were expressed as “arbitrary units” in a range of 0–400 per 100 scored nucleoids (as average value for the two mini-gels observed per fish).

When the comet assay was performed with additional FPG or EndoIII steps, GDI values were calculated in the same way but the parameter designated GDI_{FPG} and

$GDI_{EndoIII}$, respectively. Additional DNA breaks corresponding to net enzyme-sensitive sites alone (NSS_{FPG} and $NSS_{EndoIII}$ parameters) were also expressed.

Moreover, the frequency of nucleoids observed in each comet class considering GDI_{FPG} and $GDI_{EndoIII}$ was also expressed, as recommended by Azqueta et al. (2009).

ENA assay

This assay was performed in mature peripheral erythrocytes, as described by Pacheco and Santos (1997). One blood smear per animal was fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min. Slides were coded and scored blind. From each smear, 1000 erythrocytes were scored, under 1000x magnification (microscope Olympus BX50), to evaluate the relative frequency of the following nuclear lesions: kidney shaped nuclei (K), lobed nuclei (L), binucleate or segmented nuclei (S), vacuolated nuclei (V) and micronuclei (MN) (Carrasco et al., 1990; Pacheco and Santos, 1996). Blebbed and lobed nuclei were considered in a single category – lobed nuclei – and not differentially scored due to some ambiguity in their distinction, as suggested by Guilherme et al. (2008). Though the frequency (‰) of each nuclear abnormality category was individually reported, the results of ENA assay were expressed as the sum of frequencies for all the categories considered (K + L + S + V + MN).

Statistical analysis

Statistica 8.0 software (StatSoft, Inc., OK, USA) was used for statistical analysis. Data were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). Since the statistical demands for the application of parametric analysis were not met, the non-parametric Kruskal-Wallis test was performed, to compare groups within the same exposure/post-exposure time. For statistical purposes, and since no significant differences were found between replicates (considering 3 organisms per replicate), the total number of organisms per condition was considered

representative, resulting in a statistical n=9. As well, since no significant differences were observed between the two groups subjected to overozonation (considering 1, 2 and 3 days of exposure, for all the studied parameters), they were merged into a single group (O). The differences between means were considered significant when $p < 0.05$ (Zar, 1996).

Results

Non-specific DNA damage

Analysing GDI values (Fig. 2A) during the exposure period, O groups recurrently display significant increases of non-specific DNA damage, in relation to the respective controls (C). In line, the post-exposure period revealed that both previously treated groups (O_W and O_O) kept significantly higher GDI levels, in comparison with the control (Fig. 2A).

Oxidative DNA damage

The detection of oxidized bases was achieved by the comet assay with an extra step where nucleoids were incubated with the DNA lesion-specific repair enzymes FPG and EndoIII, and is depicted in figures 2B-E.

FPG associated DNA breaks

Regarding to the exposure period, GDI_{FPG} parameter (Fig. 2B) showed significantly higher levels in O groups (1, 2 and 3 days), in comparison with the respective controls. In what concerns to the post-exposure period, both pre-treated groups (O_w and O_o) presented an increased DNA damage (Fig. 2B). On the other hand, the NSS_{FPG} parameter (Fig. 2C) only distinguished the O_o group (in the post-exposure period), depicting a significant increase when compared to control.

The damage classes considering the GDI_{FPG} parameter were also individually reported (Table 1). During both exposure and post-exposure periods, all the points in time displayed significant decreases for class 1 and increases for class 2 in O groups comparing to their respective controls. When class 3 is considered, a pattern similar to class 2 was found, with the exception of O_w group that didn´t differ from the control (O_O group also displayed significantly higher values than O_W). Considering sub-total values, significant increases were observed in O groups at all sampling moments. In what concerns the identification of the prevalent class, class 1 was pointed out for control groups, while O groups displayed an unclear pattern. Hence, for 1-day exposure, classes 1 and 2 presented the highest frequencies. The 2nd exposure time pointed to class 2 as prevalent, while on the third day class 1 remained dominant.

Endo III associated DNA breaks

Similarly to GDI and GDI_{FPG}, during the exposure period, $GDI_{EndoIII}$ (Fig. 2D) in O groups also revealed an overall damage significantly higher than the respective controls. Regarding the post-exposure samples, no significant differences were found. Nevertheless, the $NSS_{EndoIII}$ parameter (Fig. 2E) detected the existence of oxidative DNA damage after the first day of exposure. Surprisingly, during the post-exposure period, the $NSS_{EndoIII}$ parameter indicated significantly lower values corresponding to treated groups, in relation to control.

Considering the discrimination of DNA damage by classes, the parameter $GDI_{EndoIII}$ (Table 2) showed the same pattern of significances displayed by GDI_{FPG} (Table 1), when the exposure period was considered. During this period, class 1 appeared as the most frequent in all control groups. In what concerns to O groups, all the exposure times showed classes 1 and 2 as predominant. Sub-total values displayed significant increases in comparison with control for all the exposure times. The post-exposure

period did not distinguish any group, since all groups displayed class 1 as the most prevalent.

Chromosomal damage

The exposure period showed significant increases of ENA frequency in O groups (Fig. 3) for all sampling time points, when compared with the respective controls. Looking to the post-exposure period, it was only possible to observe a significant increment in the O_O group, also in relation to control.

The results in terms of frequency of each nuclear lesion category (Table 3), and in what concerns the exposure period, lobed (L) and segmented (S) categories, as well as the sub-total (K + L + S + V), showed significantly higher values in all sampling time points for O groups, relatively to the respective control. Additionally, vacuolated (V) nuclei frequency, associated to O groups, showed to be significantly higher after the second and the third day of exposure, comparing with their respective controls. Overall, the kidney shaped nuclei appeared to be the most common abnormality. The post-exposure period (Table 3) displayed significant increments of V category and the sub-total (K + L + S + V) when O_O and control groups were compared. Additionally, the frequency of vacuolated nuclei associated with O_O group presented higher values, when compared to both C and O_W. On the other hand, during this experimental period, ozone groups displayed L category as the most frequent in the O_W group while O_O presented V as preferential.

Discussion

Despite the body of knowledge concerning ozone and/or OBPs toxicity to fish (Costa et al., 2014; Gonçalves and Gagnon, 2011; Jones et al., 2006; Reiser et al., 2010; Ritola et al., 2000; Yan et al., 2014), their genotoxicity remains scarcely explored. Thus, and considering the vast use of ozone-based treatments in RAS, the *leitmotiv* of

this study was the increment of knowledge concerning ozone genotoxic potential to aquaculture species, namely *S. senegalensis*.

The ozone levels adopted in RAS, and particularly in sole farming, are routinely under 0.10 mg L^{-1} (expressed as total residual oxidants). Nevertheless, ozone levels may raise up to 0.15 mg L^{-1}, still considered realistic within a time scale of hours to days, as previously stated. In order to better understand an eventual recovery from the damage inflicted by overozonation, a post-exposure period was considered, with the decision of its duration being based on the literature (Pacheco and Santos 2002; Santos and Pacheco 1996) where it was reported that 7 days after a short-term exposure was enough to allow fish recovery from both cytogenetic and hematological effects, caused by physical and/or chemical stressors.

Thus, the genotoxic potential of a sub-lethal concentration of ozone (0.15 mg L^{-1}) was evaluated using comet and ENA assays, in order to reflect genetic damage at different levels, in a complementary point of view (Guilherme et al., 2014).

The occurrence of genetic damage results from the balance between the genotoxicity promotion and anti-genotoxic mechanisms. The DNA integrity may be affected by a direct attack of the genotoxicant (e.g. formation of DNA adducts) and/or its metabolites (Bonfanti et al., 1992), as well as by an indirect result of the ROS over-generation that may damage the DNA and/or affect the function of DNA repair enzymes (Shimura-Miura et al., 1999).

The influence of ozone on DNA and RNA molecules concerning the disinfection mechanism has already been highlighted (Flyunt et al. 2002; Cataldo 2006; Silva et al. 2011b). According to Theruvathu et al. (2001), ozone induces an oxidizing action on bacteria and viruses nucleic acids, promoting their inactivation. Moreover, a few studies demonstrated the pernicious effects in fish related to the ozonation process, such as histological and physiological alterations (Reiser et al., 2010), as well as oxidative stress (Reiser et al., 2011). In regards to the genotoxic effects of ozonated water in fish

raised in RAS, only a study with turbot (*Scophthalmus maximus*) demonstrated the genotoxic potential of ozone at a cytogenetic level (Silva et al., 2011).

DNA damage

The ability of ozone and its by-products to induce DNA damage, as mentioned above, was demonstrated. This adverse effect was only assessed through the use of comet assay in two mussels species (Gagné et al., 2007; Stalter et al., 2010). In the same direction, the present results regarding to the non-specific DNA damage, represented by GDI values, showed that an exposure of hours to ozone was enough to cause damage in blood cells of *S. senegalensis*. The same results were also observed when the daily exposure was prolonged for three consecutive days. However, the decrease of ozone levels (O_O group) or even its total abolishment (O_W group), in the post-exposure period, was not enough to return the damage levels to control baselines, pointing out an incapacity to recover.

In the same way, the innovative approach concerning the assessment of oxidative DNA damage (through the use of DNA lesion-specific repair enzymes, namely FPG - GDI_{FPG}) showed that all exposure times lead to the occurrence of DNA damage, and the post-exposure conditions were not able to invert that tendency. Furthermore, the reduction of ozone level (from 0.15 mg L^{-1} to 0.07 mg L^{-1}) in the post-exposure period (O_O group), in contrast to its complete exclusion (O_w group), pointed out the occurrence of DNA oxidation, namely purines, as indicated by FPG enzyme (NSS_{FPG}). This was evident that the post-exposure period can be even more critical for manifestation of this kind of damage.

The adoption of the other DNA lesion-specific repair enzyme (EndoIII), which indicates oxidized pyrimidines, showed that, during the exposure period (3 days), fish continuously presented DNA damage ($GDI_{EndoIII}$), while the absence of damage was visible in all groups after 7 days (post-exposure period), either in ozone-free water (O_W) or in exposure to 0.07 mg L^{-1} ozone (O_O). Additionally, and despite being in line with

the $GDI_{EndoIII}$, $NSS_{EndoIII}$ gave different information when compared to the homologous FPG parameter. The EndoIII enzyme demonstrated an early oxidative damage (oxidized pyrimidines) after the first 6-h exposure (day 1). Interestingly, the post-exposure period presented a successful recovery, since fish were able to repair the oxidative damage previously detected, reaching levels of oxidative DNA damage below the control levels. This scenario may result from the activation of cell defense mechanisms concerning DNA repair and/or action of the antioxidant system (Marques et al., 2014b), which resulted in a particular protection of the pyrimidines. This recovery of damage relies on the tendency of the anti-genotoxic processes to gain preponderance in relation to the genotoxic pressures due to the decrease/cessation of the exposure to ozone. This effect was already observed in human peripheral blood leukocytes exposed to ozone, that repaired the observed damage after a post-treatment incubation in a PBS solution (Díaz-Llera et al., 2002)

Overall, the integration of these results points to a recognized risk to the integrity of DNA molecules caused by overozonation. Concerning the ozonation working level, routinely adopted in intensive Senegalese sole RAS, it was not possible to confirm its healthy nature, since almost all the parameters pointed to an absence of recovery from genotoxic actions under those conditions. Moreover, the participation of oxidative stress on the damage induced by ozone can be considered as an underlying damaging mechanism.

Chromosomal damage

As mentioned above, the chromosomal damage has already been stated to occur as a result of overozonation in another flatfish species – turbot (*Scophthalmus maximus*) (Silva et al., 2011). Considering this, and without surprise, the current ENA data showed that this kind of damage was evidenced soon, at the first day exposure, remaining during the entire exposure period. However, despite chromosomal damage being less transient and irreparable than that detected by comet assay, looking to the

post-exposure period, it was possible to observe that, after 7 days in ozone-free water (O_w), this damage disappeared. The observed decrease may be due to a removal of erythrocytes containing abnormal nuclei and/or a dilution effect resulting from erythropoiesis (releasing new normal cells into circulation) (Marques et al., 2014a). On the other hand, fish transfer from ozone levels of 0.15 (O) to 0.07 mg L^{-1} (O_o) was not enough to promote the reparation of chromosomal damage.

It is known that the comparative analysis of comet and MN (or ENA) assays in terms of their sensitivity is a controversial matter. Thus, data resulting from both assays were considered in parallel, as reflecting different expressions of genetic damage. In line with this idea, it was possible to observe a slightly different pattern related to the temporal evolution of the damage. In general, parameters related to the comet assay didn't distinguish different conditions in the post-exposure period, while for the ENA assay O_W and O_O groups responded differently. Considering this, it can be stated that these different genotoxic endpoints jointly applied provide complementary information, leading to a more integrated evaluation of the genotoxic effect caused by ozone.

Overall, the current work highlights the importance of studies concerning the genotoxic potential of ozone which may lead to negative repercussions on fish health and, consequently, on aquaculture productivity. Bearing all this in mind, the ozonation overdose in aquaculture should be avoided, even when short-terms events are considered. In addition, the risk to wild aquatic populations associated to discharges of ozone primary-treated effluents should not be neglected, since the demonstrated genotoxic potential may eventually lead to malformations and neoplastic lesions.

Contribution to optimization of fish-farming practices

The decrease of health conditions, added to the energy allocated to mechanisms related, for example, to DNA damage repair, may interfere with the main proposition concerning aquaculture profitability: to improve the fish growth while

maintaining their welfare. In this way, the current results point out that genotoxicity can then bring negative effects at the organism level, having consequently undesirable repercussions on fish health towards the sustainability of aquaculture productivity. Besides the induction of DNA damage after an exposure (of 3 days) to ozonated water, this study also demonstrated a long-lasting damage, which remained during the post-exposure period (10 days in ozone-free water). Considering this evidence, it is recommended that, under eventual similar real scenarios (even short-term events), a recovery period in ozone-free water be provided to the affected fish.

Moreover, considering that overozonation might induce oxidative DNA damage, it is emphasized the need for antioxidant protection. In this view, Gao et al. (2014) showed that lipid peroxidation was reduced by increasing the dietary levels of vitamins C or E. Thus, it is possible to infer that an eventual increment of antioxidants (namely vitamins) in the fish diet would promote a consequent decrease of the unwanted effects caused by the ozone (and overozonation), thereby minimizing the above-mentioned consequences.

The increasing scale of production in intensive aquaculture systems demands accurate and efficient tools to screen and guarantee the health status of fish during rearing. In this perspective, the present findings contribute to the establishment of the safety margins for the use of ozone in RAS, as well as providing measures to mitigate eventual impacts, thus improving aquaculture practices.

Conclusions

The present study confirmed the genotoxic potential of ozonation to fish, namely to *S. senegalensis*. The genetic damage was jointly demonstrated by comet and ENA assays, which demonstrated DNA and chromosomal damage, respectively. Shedding light on the mechanisms involved in the damaging action, it was revealed that ozone induced DNA oxidation in fish cells, in particular when pyrimidic basis were considered.

Therefore, these findings contribute to knowledge concerning the ozone toxicity, helping to establish safety margins for the use of ozone in aquaculture practices, namely in RAS. The strategies to mitigate the impact of ozonation in *S. senegalensis* may include a reinforced antioxidant supplementation (regularly, or punctually in cases of overozonation). The recovery from overozonation events is favored by the complete absence of ozone. Upgrading these practices will promote aquaculture profitability, by affecting fish growth and welfare.

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References

Andrade, V., Silva, J., Silva, F., Heuser, V., Dias, J., Lu, M., 2004. Fish as Bioindicators to Assess the Effects of Pollution in Two Southern Brazilian Rivers Using the Comet Assay and Micronucleus Test. Environ. Mol. Mutagen. 468, 459–468. doi:10.1002/em.20070

Azqueta, A., Shaposhnikov, S., Collins, A.R., 2009. DNA oxidation: investigating its key role in environmental mutagenesis with the comet assay. Mutat. Res. 674, 101–108. doi:10.1016/j.mrgentox.2008.10.013

Bellagamba, F., Caprino, F., Busetto, M.L., Francis, D.S., Vasconi, M., Turchini, G.M., Moretti, V.M., 2011. Assessment of oxidatively generated DNA damage in rainbow trout (Oncorhynchus mykiss) fed with different lipid sources. Aquaculture 317, 124–132. doi:10.1016/j.aquaculture.2011.04.012

Bonfanti, M., Taverna, P., Chiappetta, L., Villa, P., D'Incalci, M., Bagnati, R., Fanelli, R., 1992. DNA damage induced by alachlor after in vitro activation by rat hepatocytes. Toxicology 72, 207–219. doi:10.1016/0300-483x(92)90113-s

Carrasco, K., Tilbury, K., Myers, M., 1990. Assessment of the piscine micronuclei test as an in situ biological indicator of chemical contaminant effects. Can. J. Fish. Aquat. Sci. 47, 2123–2136.

Cataldo, F., 2006. DNA degradation with ozone. Int. J. Biol. Macromol. 38, 248–54. doi:10.1016/j.ijbiomac.2006.02.029

Collins, A.R., 2004. The Comet Assay for DNA Damage and Repair. Mol. Biotechnol. 26, 249–261. doi:10.1385/MB:26:3:249

Coman, G.J., Sellars, M.J., Morehead, D.T., 2005. Toxicity of ozone generated from different combinations of ozone concentration (C) and exposure time (T): a comparison of the relative effect of C and T on hatch rates of Penaeus (Marsupenaeus) japonicus embryos. Aquaculture 244, 141–145. doi:10.1016/j.aquaculture.2004.11.041

Costa, J.B., Rodgher, S., Daniel, L.A., Espíndola, E.L.G., 2014. Toxicity on aquatic organisms exposed to secondary effluent disinfected with chlorine, peracetic acid, ozone and UV radiation. Ecotoxicology 23, 1803–1813. doi:10.1007/s10646-014-1346-z

Díaz-Llera, S., González-Hernández, Y., Prieto-González, E.., Azoy, A., 2002. Genotoxic effect

of ozone in human peripheral blood leukocytes. Mutat. Res. Toxicol. Environ. Mutagen. 517, 13–20. doi:10.1016/S1383-5718(02)00022-0

FAO, 2014. The State of World Fisheries and Aquaculture 2014 [WWW Document]. URL http://www.fao.org/3/a-i3720e/index.html (accessed 6.8.15).

Fenech, M., 2000. The in vitro micronucleus technique. Mutat. Res. 455, 81–95.

Flyunt, R., Theruvathu, J.A., Leitzke, A., von Sonntag, C., 2002. The reactions of thymine and thymidine with ozone. J. Chem. Soc. Perkin Trans. 2 1572–1582. doi:10.1039/b204067k

Gagné, F., André, C., Cejka, P., Gagnon, C., Blaise, C., 2007. Toxicological effects of primary-treated urban wastewaters, before and after ozone treatment, on freshwater mussels (Elliptio complanata). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 145, 542–552. doi:10.1016/j.cbpc.2007.01.019

Gao, J., Koshio, S., Ishikawa, M., Yokoyama, S., Mamauag, R.E.P., 2014. Interactive effects of vitamin C and E supplementation on growth performance, fatty acid composition and reduction of oxidative stress in juvenile Japanese flounder Paralichthys olivaceus fed dietary oxidized fish oil. Aquaculture 422–423, 84–90. doi:10.1016/J.AQUACULTURE.2013.11.031

Gonçalves, A.A., Gagnon, G.A., 2011. Ozone Application in Recirculating Aquaculture System: An Overview. Ozone Sci. Eng. 33, 345–367. doi:10.1080/01919512.2011.604595

Good, C., Davidson, J., Welsh, C., Snekvik, K., Summerfelt, S., 2011. The effects of ozonation on performance, health and welfare of rainbow trout Oncorhynchus mykiss in low-exchange water recirculation aquaculture systems. Aquac. Eng. 44, 97–102. doi:10.1016/j.aquaeng.2011.04.003

Guilherme, S., Gaivão, I., Santos, M.A.A., Pacheco, M., 2012. DNA damage in fish (Anguilla anguilla) exposed to a glyphosate-based herbicide – Elucidation of organ-specificity and the role of oxidative stress. Mutat. Res.-Gen. Tox. En. 743, 1–9. doi:10.1016/j.mrgentox.2011.10.017

Guilherme, S., Santos, M.A., Gaivão, I., Pacheco, M., 2014. DNA and chromosomal damage induced in fish (Anguilla anguilla L.) by aminomethylphosphonic acid (AMPA)-the major environmental breakdown product of glyphosate. Environ. Sci. Pollut. Res. Int. doi:10.1007/s11356-014-2803-1

Guilherme, S., Válega, M., Pereira, M.E., Santos, M.A., Pacheco, M., 2008. Erythrocytic nuclear abnormalities in wild and caged fish (*Liza aurata*) along an environmental mercury contamination gradient. Ecotox. Environ. Safe. 70, 411–421. doi:10.1016/j.ecoenv.2007.08.016

Hébert, N., Gagné, F., Cejka, P., Bouchard, B., Hausler, R., Cyr, D.G., Blaise, C., Fournier, M., 2008. Effects of ozone, ultraviolet and peracetic acid disinfection of a primary-treated municipal effluent on the Immune system of rainbow trout (Oncorhynchus mykiss). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 148, 122–127. doi:10.1016/J.CBPC.2008.04.007

Jones, A.C., Gensemer, R.W., Stubblefield, W., Van Genderen, E., Dethloff, G.M., Cooper, W.J., 2006. Toxicity of ozonated seawater to marine organisms. Environ. Toxicol. Chem. 25, 2683–91.

Kienzler, A., Bony, S., Devaux, A., 2013. DNA repair activity in fish and interest in ecotoxicology: a review. Aquat. Toxicol. 134–135, 47–56. doi:10.1016/j.aquatox.2013.03.005

Lee, R., Steinert, S., 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutat. Res. 544, 43–64.

Liltved, H., Vogelsang, C., Modahl, I., Dannevig, B.H., 2006. High resistance of fish pathogenic viruses to UV irradiation and ozonated seawater. Aquac. Eng. 34, 72–82. doi:10.1016/j.aquaeng.2005.05.002

Liu, H., Wu, Y., Chen, H., 2007a. Production of ozone and reactive oxygen species after welding. Arch. Environ. Contam. Toxicol. 53, 513–8. doi:10.1007/s00244-007-0030-1

Liu, Y., Wang, W., Wang, A., Wang, J., Sun, R., 2007b. Effects of dietary vitamin E supplementation on antioxidant enzyme activities in Litopenaeus vannamei (Boone, 1931) exposed to acute salinity changes. Aquaculture 265, 351–358. doi:10.1016/j.aquaculture.2007.02.010

Marques, A., Custódio, M., Guilherme, S., Gaivão, I., Santos, M.A., Pacheco, M., 2014a. Assessment of chromosomal damage induced by a deltamethrin-based insecticide in fish (Anguilla anguilla L.) - a follow-up study upon exposure and post-exposure periods. Pestic. Biochem. Physiol. 113, 40–6. doi:10.1016/j.pestbp.2014.06.003

Marques, A., Guilherme, S., Gaivão, I., Santos, M.A., Pacheco, M., 2014b. Progression of DNA

damage induced by a glyphosate-based herbicide in fish (Anguilla anguilla) upon exposure and post-exposure periods--insights into the mechanisms of genotoxicity and DNA repair. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 166, 126–33. doi:10.1016/j.cbpc.2014.07.009

Niu, J., Lin, H., Jiang, S., Chen, X., Wu, K., Liu, Y., Wang, S., Tian, L., 2013. Comparison of effect of chitin, chitosan, chitosan oligosaccharide and N-acetyl-d-glucosamine on growth performance, antioxidant defenses and oxidative stress status of Penaeus monodon. Aquaculture 372–375, 1–8. doi:10.1016/j.aquaculture.2012.10.021

Pacheco, M., Santos, M.A., 2002. Biotransformation, genotoxic, and histopathological effects of environmental contaminants in European eel (Anguilla anguilla L.). Ecotoxicol. Environ. Saf. 53, 331–47.

Pacheco, M., Santos, M.A.A., 1997. Induction of EROD activity and genotoxic effects by polycyclic aromatic hydrocarbons and resin acids on juvenile eel Anguilla anguilla L. Ecotoxicol. Environ. Saf. 38, 252–259. doi:10.1006/eesa.1997.1585

Pacheco, M., Santos, M.A.A., 1996. Induction of micronuclei and nuclear abnormalities in the erythrocytes of Anguilla anguilla L. exposed either to cyclophosphamide or to bleached kraft pulp mill ef uent. Fresen. Environ. Bull. 5, 746–751.

Perrins, J.C., Cooper, W.J., van Leeuwen, J., Herwig, R.P., 2006. Ozonation of seawater from different locations: formation and decay of total residual oxidant--implications for ballast water treatment. Mar. Pollut. Bull. 52, 1023–33. doi:10.1016/j.marpolbul.2006.01.007

Reiser, S., Schroeder, J.P., Wuertz, S., Kloas, W., Hanel, R., 2010. Histological and physiological alterations in juvenile turbot (Psetta maxima, L.) exposed to sublethal concentrations of ozone-produced oxidants in ozonated seawater. Aquaculture 307, 157–164. doi:10.1016/j.aquaculture.2010.07.007

Reiser, S., Wuertz, S., Schroeder, J.P., Kloas, W., Hanel, R., 2011. Risks of seawater ozonation in recirculation aquaculture--effects of oxidative stress on animal welfare of juvenile turbot (Psetta maxima, L.). Aquat. Toxicol. 105, 508–17. doi:10.1016/j.aquatox.2011.08.004

Ritola, O., Livingstone, D.., Peters, L.., Lindström-Seppä, P., 2002. Antioxidant processes are affected in juvenile rainbow trout (Oncorhynchus mykiss) exposed to ozone and oxygen-supersaturated water. Aquaculture 210, 1–19. doi:10.1016/S0044-8486(01)00823-7

Ritola, O., Lyytikainen, T., Molsa, H., Lindstrom-seppa, P., 2000. Glutathione-dependent defence system and monooxygenase enzyme activities in Arctic charr SalÕelinus alpinus ž L . / exposed to ozone 219–233.

Santos, M.A., Pacheco, M., 1996. Anguilla anguilla L. stress biomarkers recovery in clean water and secondary-treated pulp mill effluent. Ecotoxicol. Environ. Saf. 35, 96–100. doi:10.1006/eesa.1996.0086

Schröder, J., 2010. Potential and Limitations of Ozone in Marine Recirculating Aquaculture Systems: Guidelines and Thresholds for a Safe Application.

Schroeder, J.P., Croot, P.L., Von Dewitz, B., Waller, U., Hanel, R., 2011. Potential and limitations of ozone for the removal of ammonia, nitrite, and yellow substances in marine recirculating aquaculture systems. Aquac. Eng. 45, 35–41. doi:10.1016/j.aquaeng.2011.06.001

Schroeder, J.P., Gärtner, a., Waller, U., Hanel, R., 2010. The toxicity of ozone-produced oxidants to the Pacific white shrimp Litopenaeus vannamei. Aquaculture 305, 6–11. doi:10.1016/j.aquaculture.2010.03.030

Shaposhnikov, S., Azqueta, A., Henriksson, S., Meier, S., Gaivão, I., Huskisson, N.H., Smart, A., Brunborg, G., Nilsson, M., Collins, A.R., 2010. Twelve-gel slide format optimised for comet assay and fluorescent in situ hybridisation. Toxicol. Lett. 195, 31–34. doi:DOI: 10.1016/j.toxlet.2010.02.017

Sharrer, M.J., Summerfelt, S.T., 2007. Ozonation followed by ultraviolet irradiation provides effective bacteria inactivation in a freshwater recirculating system. Aquac. Eng. 37, 180–191. doi:10.1016/j.aquaeng.2007.05.001

Shimura-Miura, H., Hattori, N., Kang, D., Miyako, K., Nakabeppu, Y., Mizuno, Y., 1999. Increased 8-oxo-dGTPase in the mitochondria of substantia nigral neurons in Parkinson's disease. Ann. Neurol. 46, 920–924.

Silva, J., Laranjeira, A., Serradeiro, R., Santos, M.A., Pacheco, M., 2011. Ozonated seawater induces genotoxicity and hematological alterations in turbot (Scophthalmus maximus) — Implications for management of recirculation aquaculture systems. Aquaculture 318, 180–184. doi:10.1016/j.aquaculture.2011.05.009

Stalter, D., Magdeburg, A., Oehlmann, J., 2010. Comparative toxicity assessment of ozone and

activated carbon treated sewage effluents using an in vivo test battery. Water Res. 44, 2610–2620. doi:10.1016/j.watres.2010.01.023

Stoiber, T., Bonacker, D., Bohm, K., Bolt, H., Thier, R., Degen, G., Unger, E., 2004. Disturbed microtubule function and induction of micronuclei by chelate complexes of mercury (II). Mutat.Res. 563, 97–106.

Summerfelt, S.T., 2003. Ozonation and UV irradiation—an introduction and examples of current applications. Aquac. Eng. 28, 21–36. doi:10.1016/S0144-8609(02)00069-9

Summerfelt, S.T., Hochheimer, J.N., 1997. Review of Ozone Processes and Applications as an Oxidizing Agent in Aquaculture. Progress. Fish-Culturist 59, 94–105. doi:10.1577/1548-8640(1997)059<0094:ROOPAA>2.3.CO;2

Tanaka, J., Matsumura, M., 2002. Kinetic studies of removal of ammonia from seawater by ozonation. J. Chem. Technol. Biotechnol. 77, 649–656. doi:10.1002/jctb.624

Tango, M.S., Gagnon, G. a, 2003. Impact of ozonation on water quality in marine recirculation systems. Aquac. Eng. 29, 125–137. doi:10.1016/S0144-8609(03)00061-X

Theruvathu, J.A., Flyunt, R., Aravindakumar, C.T., von Sonntag, C., 2001. Rate constants of ozone reactions with DNA, its constituents and related compounds. J. Chem. Soc. Perkin Trans. 2 269–274. doi:10.1039/b009388m

Victorin, K., 1992. Review of the genotoxicity of ozone. Mutat. Res. 277, 221–38.

Yan, Z., Zhang, Y., Yuan, H., Tian, Z., Yang, M., 2014. Fish larval deformity caused by aldehydes and unknown byproducts in ozonated effluents from municipal wastewater treatment systems. Water Res. 66, 423–429. doi:10.1016/j.watres.2014.08.019

Zar, J., 1996. Biostatistical analysis. Prentice Hall International Inc., New Jersey.

Fig. 1. Schematic representation of the experimental design comprising the 6-h daily exposure of *S. senegalensis* to 0.15 mg L^{-1} ozone (O), repeated for 3 consecutive days, and the post-exposure period (7 days) in ozone-free water (O_W), or in 0.07 mg L^{-1} ozone (O_O). In parallel, a control group (C), kept permanently in ozone-free water, was also considered.

Fig. 2. Mean values of DNA damage, measured by comet assay in blood cells of *S. senegalensis* subjected to daily 6-h ozone (O - 0.15 mg L^{-1}) exposure, for 3 consecutive days, plus 7-day post-exposure either in ozone-free water (O_W) or in 0.07 mg L^{-1} ozone (O_O); (A) genetic damage indicator (GDI) measured by the standard (alkaline) comet assay; (B) overall damage (GDI_{FPG}) and partial scores, namely genetic damage indicator (GDI; grey) and additional DNA breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; black); (C) NSS_{FPG} alone; (D) overall damage ($GDI_{EndoIII}$) and partial scores, namely genetic damage indicator (GDI; light grey) and additional DNA breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey); (E) $NSS_{EndoIII}$ alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (a) in relation to control (C), within the same exposure/post-exposure time.

Fig. 3. Mean frequency (‰) of erythrocytic nuclear abnormalities (ENA) in peripheral erythrocytes of *S. senegalensis* subjected to daily 6-h ozone (O - 0.15 mg L^{-1}) exposure, for 3 consecutive days, plus 7-day post-exposure either in ozone-free water (OW) or in 0.07 mg L^{-1} ozone (OO). Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (a) in relation to control (C), within the same exposure/post-exposure time (a).

Table 1: Mean frequencies (%) of each DNA damage class and sub-total of damaged nucleoids (± standard error), measured by the comet assay including the incubation with the FPG enzyme in blood cells of *S. senegalensis* subjected to daily 6-h ozone (O - 0.15 mg L^{-1}) exposure, for 3 consecutive days, plus 7-day post-exposure either in ozone-free water (O_W) or in 0.07 mg L^{-1} ozone (O_O). Statistically significant differences ($p < 0.05$) are: (a) in relation to control (C) and (b) in relation to O_W groups.

Experimental Conditions			GDI_{FPG} DNA Damage Classes					
			0	1	2	3	4	Sub-total (2+3+4)
Exposure	1 day	C	0.00±0.00	62.89±2.41	36.78±2.40	0.33±0.33	0.00±0.00	37.11±2.14
		O	0.00±0.00	44.34±2.16 **a**	51.38±1.88 **a**	4.03±0.91 **a**	0.25±0.14	55.66±2.10 **a**
	2 days	C	0.00±0.00	58.95±1.97	40.94±2.20	0.11±1.45	0.00±0.22	41.05±2.83
		O	0.00±0.00	26.00±4.09 **a**	68.00±3.11 **a**	5.80±1.29 **a**	0.20±0.11	74.00±4.09 **a**
	3 days	C	0.00±0.00	86.56±1.62	13.44±1.62	0.00±0.00	0.00±0.00	13.44±1.62
		O	0.00±0.00	62.09±2.30 **a**	31.06±2.25 **a**	6.48±1.06 **a**	0.36±0.20	37.91±2.31 **a**
Post-exposure	7 days	C	0.00±0.00	80.94±1.09 **a**	19.06±1.09 **a**	0.00±0.00	0.00±0.00	19.06±1.09 **a**
		O_W	0.00±0.00	63.11±1.68 **a**	35.89±1.47 **a**	1.00±0.41 **ab**	0.00±0.00	36.89±1.68 **a**
		O_O	0.00±0.00	54.58±1.47	39.88±1.37	5.17±1.01	0.38±0.18	45.42±1.47

Table 2: Mean frequencies (%) of each DNA damage class and sub-total of damaged nucleoids (± standard error), measured by the comet assay including the incubation with the EndoIII enzyme in blood cells of *S. senegalensis* subjected to daily 6-h ozone (O - 0.15 mg L^{-1}) exposure, for 3 consecutive days, plus 7-day post-exposure either in ozone-free water (O_W) or in 0.07 mg L^{-1} ozone (O_O). Statistically significant differences ($p < 0.05$) are: (a) in relation to control (C).

	Experimental Conditions		$GDI_{EndoIII}$ DNA Damage Classes					
			0	1	2	3	4	Sub-total (2+3+4)
Exposure	1 day	C	0.11±0.12	71.64±1.52	28.03±1.48	0.22±0.16	0.00±0.00	28.25±1.49
		O	0.00±0.00	52.42±1.09 [a]	41.93±1.16 [a]	5.42±0.88 [a]	0.24±0.18	47.58±1.09 [a]
	2 days	C	0.00±0.00	59.96±3.13	40.04±3.13	0.00±0.00	0.00±0.00	40.04±3.13
		O	0.00±0.00	40.07±4.11 [a]	53.19±2.75 [a]	6.17±1.95 [a]	0.56±0.33	59.93±4.11 [a]
	3 days	C	0.00±0.00	71.78±1.35	28.22±1.35	0.00±0.00	0.00±0.00	28.22±1.35
		O	0.00±0.00	53.14±2.77 [a]	42.84±1.68 [a]	3.94±1.29 [a]	0.08±0.08	46.86±2.77 [a]
Post-Exposure	7 days	C	0.00±0.00	69.56±1.19	30.22±1.20	0.22±0.15	0.00±0.00	30.44±1.19
		O_w	0.00±0.00	70.42±1.19	29.44±1.18	0.14±0.14	0.00±0.00	29.58±1.19
		O_O	0.00±0.00	69.67±1.34	30.01±1.28	0.32±0.21	0.00±0.00	30.33±1.34

Table 3: Mean frequency (‰) of each nuclear abnormality category (± standard error) in peripheral erythrocytes of *S. senegalensis* subjected to daily 6-h ozone (O - 0.15 mg L^{-1}) exposure, for 3 consecutive days, plus 7-day post-exposure either in ozone-free water (O_W) or in 0.07 mg L^{-1} ozone (O_O). Statistically significant differences ($p < 0.05$) are: (a) in relation to control (C) and (b) in relation to O_W groups.

Experimental Conditions			Nuclear Abnormalities Categories					
			Kidney shaped (K)	Lobed (L)	Segmented (S)	Vacuolated (V)	Sub-total (K+L+S+V)	Micronuclei (MN)
Exposure	1 day	C	3.22±0.83	0.00±0.00	0.11±0.11	0.00±0.00	3.33±0.78	0.11±0.11
		O	4.29±0.44	1.47±0.27 a	2.00±0.36 a	2.00±0.89	9.11±0.98 a	0.00±0.00
	2 days	C	4.67±0.53	0.00±0.00	0.11±0.11	0.00±0.00	4.78±0.62	0.00±0.00
		O	3.88±0.54	4.24±0.51 a	3.12±0.52 a	7.06±1.07 a	17.28±1.75 a	0.06±0.06
	3 days	C	2.78±0.28	0.44±0.18	0.78±0.22	0.33±0.17	4.33±0.44	0.00±0.00
		O	3.56±0.61	3.22±0.55 a	2.17±0.43 a	4.06±1.11 a	13.00±1.25 a	0.06±0.06
Post-exposure	7 days	C	1.44±0.24	2.33±0.65	0.44±0.29	2.78±0.62	7.00±0.73	0.00±0.00
		O_W	0.78±0.28	5.11±0.84	1.33±0.53	2.89±0.81	10.11±1.38	0.00±0.00
		O_O	1.67±0.24	3.89±0.93	1.11±0.42	8.89±0.90 ab	15.56±0.96 a	0.00±0.00

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Highlights

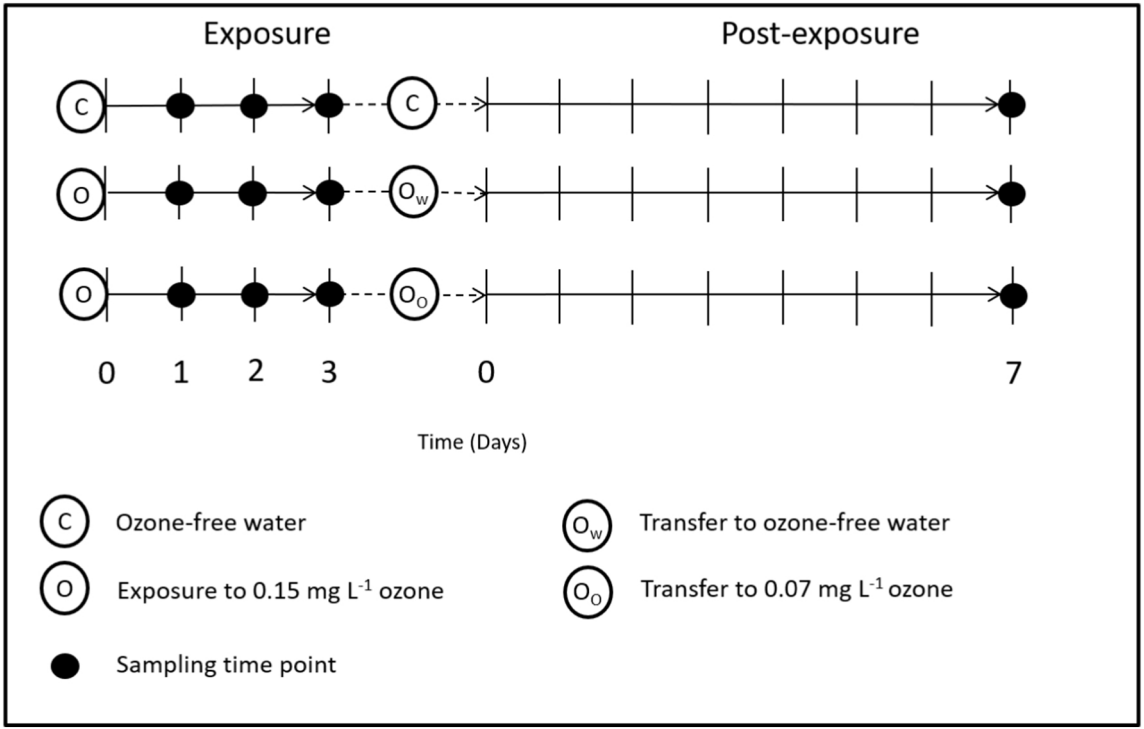
- Knowledge gap concerning the ozone-related genotoxic hazard to organisms reared in recirculating aquaculture systems (RAS) applying ozonation;

- Early genotoxic signals may preconize negative repercussions on fish health, which may affect the aquaculture productivity;

- Genetic damage induced by ozone exposure in the Senegalese sole (Solea senegalensis), assessed through the comet and the erythrocytic nuclear abnormalities (ENA) assays;

- Both endpoints point the ozone genotoxic potential, displaying DNA oxidation as a possible mechanism of damage;

- Present findings may contribute to improve aquaculture practices, namely in RAS systems.


Exposure
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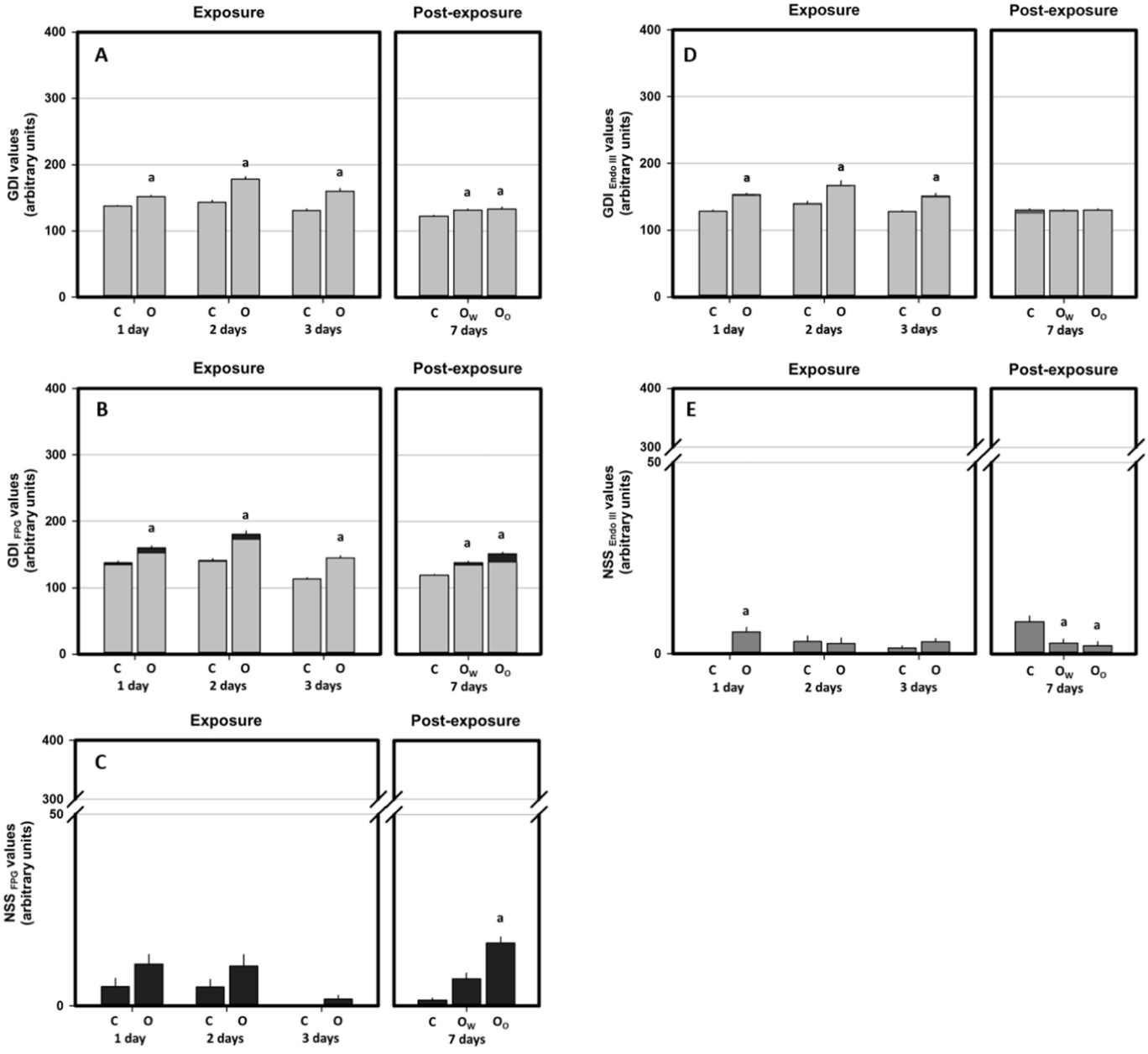
Transfer to ozone-free water
O_O
Transfer to 0.07 mg L^{-1} ozone

Figure 1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GDI values (arbitrary units)
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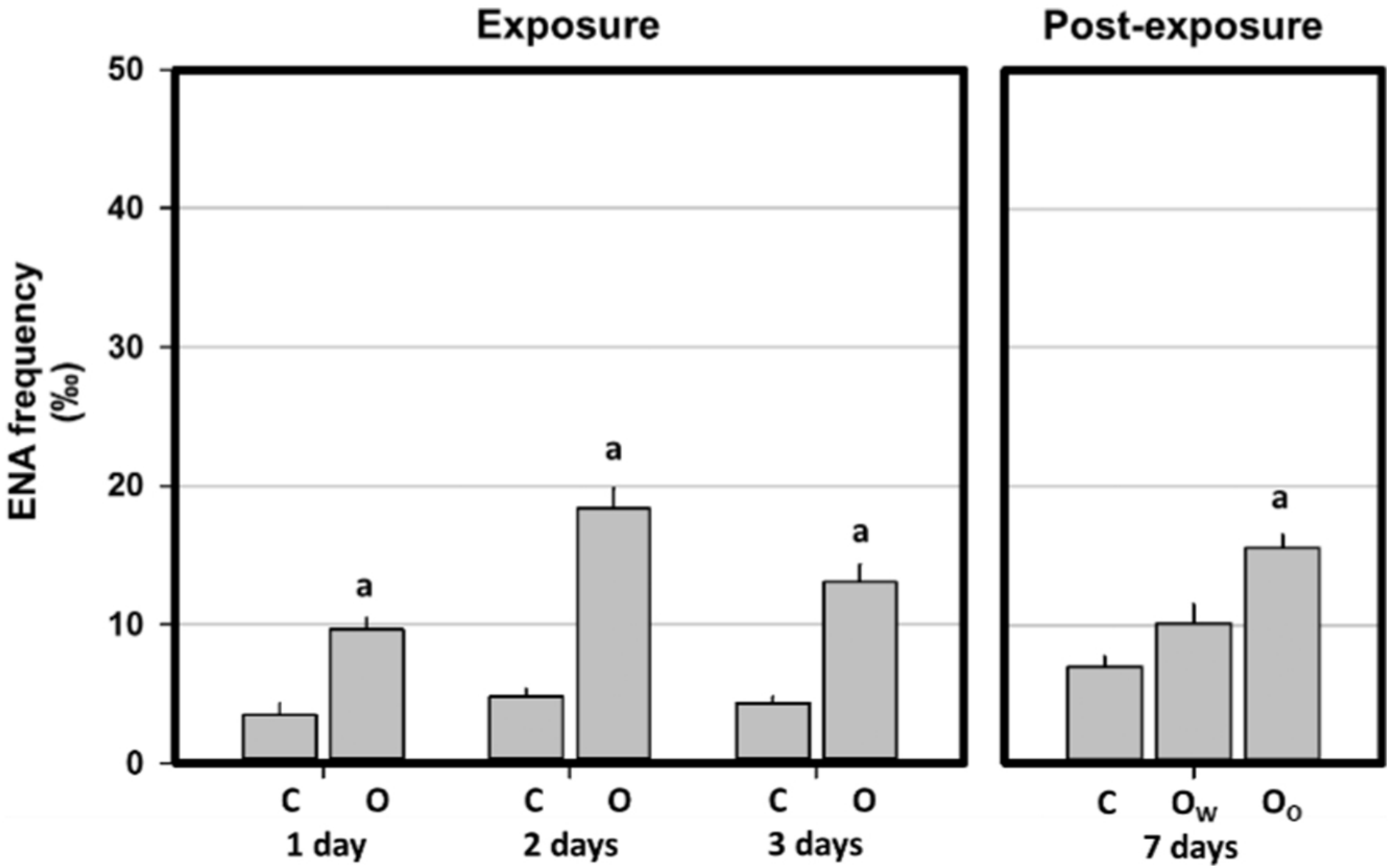
Figure 2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ENA frequency (‰)
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1 day
2 days
3 days
7 days

Figure 3