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Sperm quality assessment in *Ficopomatus enigmaticus* (Fauvel, 1923): Effects of selected organic and inorganic chemicals across salinity levels

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ABSTRACT

Contamination by organic and inorganic compounds remains one of the most complex problems in both brackish and marine environments, causing potential implications for the reproductive success and survival of several broadcast spawners. *Ficopomatus enigmaticus* is a tubeworm polychaete that has previously been used as a model organism for ecotoxicological analysis, due to its sensitivity and ecological relevance. In the present study, the effects of five trace elements (zinc, copper, cadmium, arsenic and lead), one surfactant (sodium dodecyl sulfate, SDS) and one polycyclic aromatic hydrocarbon (benzo(a)pyrene, B(a)P) on the sperm quality of *F. enigmaticus* were investigated. Sperm suspensions were exposed *in vitro* to different concentrations of each selected contaminant under four salinity conditions (10, 20, 30, 35). Possible adverse effects on sperm function were assessed by measuring oxidative stress, membrane integrity, viability and DNA damage. Sperm quality impairments induced by organic contaminants were more evident than those induced by inorganic compounds. SDS exerted the largest effect on sperm. In addition, *F. enigmaticus* sperm showed high tolerance to salinity variation, supporting the wide use of this species as a promising model organism for ecotoxicological assays. Easy and rapid methods on polychaete spermatozoids were shown to be effective as integrated sperm quality parameters or as an alternative analysis for early assessment of marine and brackish water pollution.

1. Introduction

In recent decades, the continuous introduction of several anthropogenic contaminants into terrestrial and marine environments has caused alarm within the scientific community due to their potential ecotoxicological impact on biota (Gallo and Tosti, 2019). In particular, aquatic contamination by trace elements, polycyclic aromatic hydrocarbons (PAHs) and surfactants is known to be of critical environmental concern (Châtel et al., 2017). Trace elements can be released into water bodies both by natural and anthropogenic sources (Hudspith et al., 2017). Moderate to very high concentrations of these elements have been recorded (Al-Masri et al., 2002; Hudspith et al., 2017). In particular, the presence of zinc (Zn), copper (Cu), cadmium (Cd), arsenic (As) and lead (Pb) in aquatic systems has been a topic of concern due to their abundance, persistence and toxicity in different organisms (Carolin et al., 2017). Studies have shown that bioavailable forms of trace elements can accumulate within tissues, inducing biochemical responses and toxic effects on reproduction, growth, development and survival in several aquatic species, such as oysters, mussels, sea-urchins, crabs and fish (Avigliano et al., 2015; Hudspith et al., 2017; Oliva et al., 2019a). Benzo(a)pyrene (B(a)P) is one of the most ubiquitously distributed PAHs in aquatic matrices (Châtel et al., 2017). In vertebrates, B(a)P is a well-known pro-carcinogen that can be activated to its carcinogenic form by highly reactive intermediates that are able to covalently bind to DNA (Shiizaki et al., 2017). The genotoxic effects of B(a)P on aquatic invertebrates have been extensively studied, and include increased oxidation of proteins and DNA, as well as a positive correlation between DNA strand breakages and abnormalities in oyster embryos (Wessel et al., 2007). Regarding surfactants, the wide variety of their applications and the associated concern over their environmental influence is well known. In particular, sodium dodecyl sulfate (SDS) is a commercialized compound found in household and industrial cleaners, personal

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care products, cosmetics, industrial manufacturing and oil-spill clean-up processes (Rosety et al., 2001). SDS toxicity has been demonstrated in bivalves, crustaceans, echinoderms and fish (Freitas et al., 2020; Mariani et al., 2006). The key toxic effects of SDS are believed to be alterations in membrane potential, cellular ionic balance disruption and increased oxidative stress, resulting in the generation of other downstream physiological and biochemical stresses (Messina et al., 2014).

The polychaete *Ficopomatus enigmaticus* (Fauvel, 1923) is a filter-feeding serpulid tubeworm, found in temperate waters in the Northern and Southern hemispheres (Ten Hove and Kupriyanova, 2009). *F. enigmaticus* is particularly tolerant to salinity variation and is physiologically well adapted to brackish water conditions, though its optimum salinity environment ranges between 10 and 35 (Dittmann et al., 2009). However, its reproduction and larval settlement can be affected by temperature and salinity, which results in the absence of reproduction in certain, albeit few, periods of the year. *F. enigmaticus* is a free-spawning species with a high fecundity rate, producing planktotrophic larvae. We recently described the reliability of using this species as a candidate model organism for ecotoxicological purposes, by assessing contaminant effects on the first developmental stages (Oliva et al., 2019b). Although most studies have focused on juvenile and adult stages of polychaetes (Lewis and Galloway, 2008; Oliva et al., 2019b), few studies have been devoted to the effects of pollutants on polychaetes spermatozoids (Gopalakrishnan et al., 2008; Lewis and Galloway, 2008) and none have been carried out on *F. enigmaticus*. Sperm quality is defined as the ability of spermatozoa to successfully fertilize an oocyte (Bobe and Labbé, 2010), so investigating the spermiotoxic impact that environmental pollutants have on marine species represents an important issue, because adverse effects could lead to the transmission of damage to offspring and may have potential implications for reproductive fitness, survival and population preservation.

The aim of the present study was to determine the putative effects of some of the most abundant pollutants in aquatic environments (Zn, Cu, Cd, As, Pb, B(a)P and SDS) on the reproductive efficiency of *F. enigmaticus* under different salinity conditions (10, 20, 30 and 35). To carry out this investigation, we used some parameters crucial for sperm functionality, such as lipid peroxidation, reactive oxygen species production, sperm viability and DNA integrity, evaluating the suitability of these methods on *F. enigmaticus* spermatozoids.

2. Materials and methods

2.1. Chemicals

Zinc, copper, cadmium, arsenic and lead were purchased from Romil Pure Chemistry™ as 1.00 g/L standard solutions for atomic absorption spectrometry (AAS). 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), 4,4-difluoro-5-(4- phenyl-1,3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY$^{581/591}$) and dihydro ethidium (DHE) were purchased from Invitrogen™ as analytical grade reagents. The Pico488 was purchased from Lumiprobe GmbH. Sodium dodecyl sulfate (SDS), benzo (a) pyrene (B(a)P), Calf thymus DNA standard, ethanol and pyrogallol were purchased from Sigma-Aldrich.

2.2. Organisms collection and sperm-release induction

Adults of *F. enigmaticus* were collected from the S. Rossore-Migliarino Regional Park (Pisa, Italy). The specimens were transported to the laboratory under wet conditions. Organisms were then washed with natural seawater (NSW) filtered at 0.45 μm and maintained in separate aquaria (1 per salinity condition), filled with fresh NSW under continuous aeration, at 22 ± 1 °C and pH 8.1 ± 0.1 for an acclimation period of 1 week. During this period, the salinity (S) was initially adjusted to the sampling site value (9) and then raised daily at a maximum of 5/day, until the selected test salinities were reached. Organisms were fed three times per week with an algal mix (*Rhodomonas salina* and *Isochrysis galbana*) at a concentration of 3×10^5 cells/mL, as outlined in Oliva et al. (2019b).

The spawning of *F. enigmaticus* was induced mechanically by breaking the calcareous tube and the sperm-release was induced within 10 min. Following the protocol described by Oliva et al. (2019b), each well of a 24-well plate was filled with one *F. enigmaticus* individual in 0.5 mL of NSW at the selected salinity (10, 20, 30, 35). After emission, sperm suspensions from 10 organisms were pooled in sterile test tubes for each tested salinity condition. The sperm concentration for each pool was then determined using a Burker counting chamber and an Olympus CH-2 optical microscope.

2.3. Experimental design

In order to test the effect of salinity on polychaete biomarker levels, four salinities (10, 20, 30 and 35) were assessed so that the influence of salinity as a stressor could be investigated both by itself and in combination with contaminant effects. The different salinity conditions were chosen based on: I) seasonal variation of the sampling site (Langeneck et al., 2015) and II) the optimum range for *F. enigmaticus* (Dittmann et al., 2009). To understand the combined effect of each salinity and contaminant condition, for each salinity level, four concentrations of each toxicant were chosen: 0.05–0.2-0.5–1 mg/L for the trace elements, 0.5-1-2-4 mg/L for SDS and 0.5-1-2-4 μg/L for B(a)P. The different concentrations of compounds were selected based on: I) environmentally relevant concentrations (Konieczka et al., 2018; Sarker et al., 2018) and II) levels where toxic effects were demonstrated in previous literature for other marine and brackish species (Gopalakrishnan et al., 2008; Oliva et al., 2019a; Freitas et al., 2020). Contaminant stock solutions (100-fold) were prepared in ultrapure water for each concentration so that small volumes could be added to the sperm suspensions. For every salinity/pollutant condition, 3 replicates of the sperm suspension (200 μL each) were incubated with 2 μL of toxicant for 30 min at room temperature (RT).

After exposure, a battery of sperm quality biomarker tests was used to assess the spermiotoxic effects of the selected chemicals. These included measurements of sperm viability (MTT), lipid peroxidation (LPO), intracellular reactive oxygen species (ROS) and DNA damage. The staining was carried out by adding the relevant probe for each biomarker (C11-BODIPY $^{581/591}$ for LPO, DHE for ROS production, MTT for sperm viability and Pico488 for DNA damage) to the sperm suspensions, which were then incubated in darkness for 30 min at 20 °C. Plates were analysed with a microplate reader Synergy HT (Bio-Tek®Inc.). Before performing spectrofluorimetric analysis of LPO and ROS, the correct and specific localization of each fluorochrome was evaluated and confirmed using fluorescence microscopy. Briefly, aliquots of sperm suspension (200 μL) were incubated with the fluorescent probes C11-BODIPY$^{581/591}$ and DHE, as outlined in Boni et al. (2016) and immediately observed. Microscopic evaluation was performed with a fluorescence microscope Nikon Eclipse 80i (Nikon Instruments, Calenzano, Italy) at a 60X objective.

2.3.1. Lipid peroxidation (LPO)

LPO was determined by incubating sperm suspensions (1.5×10^7 spermatozoids/200 μL) with the fluorescent membrane probe C11-BODIPY $^{581/591}$, according to Gallo et al. (2018). The fluorescence intensity was measured by setting the excitation at 490 nm and reading the emission at 520 and 590 nm. Results were expressed as a percentage depicting fluorescence intensity ratio (FIR) and calculated using the following equation:

$$\frac{F_e(520)}{F_e(520) + F_e(590)}\ x\ 100$$

where $F_e(520)$ = fluorescence emission value at 520 nm; $F_e(590)$ =

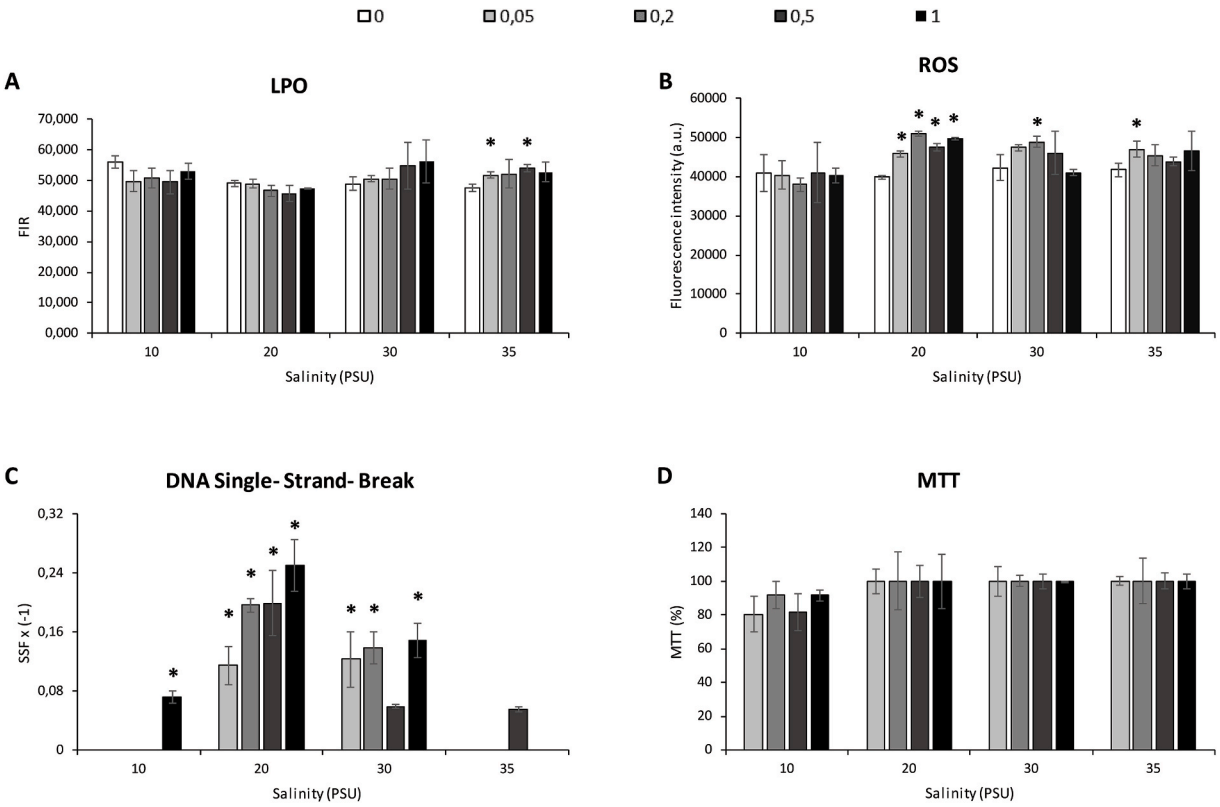

Fig. 1. Results of *F. enigmaticus* spermatozoids exposed to different concentrations (0.05, 0.2, 0.5 and 1 mg/L) of copper under four salinity conditions (10, 20, 30, 35). For each salinity, significant differences between control and Cu concentrations were represented with an asterisk (*) ($p < 0.05$). Mean values (±Standard deviation) for: **A:** Lipid peroxidation (LPO), expressed as fluorescence intensity ratio (FIR); **B:** Reactive oxygen species (ROS), expressed as fluorescence intensity (a.u.); **C:** Negative DNA Single-Strand-Break, expressed as strand scission factors (SSF) x (−1) and the control corresponded to the x-axis; **D:** Sperm viability (MTT), expressed as percentage (%) of MTT and the controls representing 100% of viability.

fluorescence emission value at 590 nm.

2.3.2. Reactive oxygen species (ROS) production

The production of ROS was detected with the superoxide-specific fluorescent probe DHE outlined in Gallo et al. (2018). Samples of sperm suspensions (1.5×10^7 spermatozoids/200 µL) were incubated with 5 mM DHE for 30 min at 20 °C, in darkness. A 30 µM pyrogallol solution was used as a positive control, incubated with the other samples, under the same conditions. The concentration of superoxide anions was evaluated by setting the excitation at 350 nm and the emission at 590 nm. Results were expressed as arbitrary units of fluorescence intensity (a.u.).

2.3.3. Sperm viability (MTT)

Sperm viability was evaluated using the MTT assay (Van Meerloo et al., 2011) with slight modifications. Briefly, after exposure to the selected compound, as described above, samples were centrifuged at 5000 rpm, at 4 °C for 2 min. The supernatant was discarded and the pellet was resuspended in 200 µL of NSW to obtain a sperm concentration of 7×10^7 cells. From this, 150 µL of the suspension from each sample was placed into a 96-well plate and 15 µL of MTT solution (5 mg/mL) was added to each well. The plate was then incubated for 4 h at 20 °C. Before analysis, 150 µL of absolute ethanol was added to each well, and the plate was incubated for a further 10 min, allowing the formazan crystals to thoroughly dissolve.

The absorbance was measured at two different wavelengths: 540 nm (for samples and controls) and 720 nm (for blanks). The living cell percentage was determined using the following calculation:

$$\frac{\overline{OD}\ TW\ -\ B}{\overline{OD}\ CW\ -\ B}\ x\ 100$$

where OD TW = optical density of treated well; B = blank; OD CW = optical density of control well.

2.3.4. DNA damage (Fast Micromethod ®)

DNA damage estimation was determined using the Fast Micromethod®, according to Schröder et al. (2006) with slight modifications. This method is based on the ability of the dye fluorochrome Pico488 to interact preferentially with high integrity dsDNA rather than ssDNA and proteins in the presence of a high alkaline medium. Because this method required 30 ng of DNA, a calibration curve was built using Calf thymus DNA standard (1–1000 ng/mL) in order to calculate the adequate sperm concentration for the assay, which was estimated to be 3.5×10^8 cells/mL. After exposure to the selected toxicants at under the different salinities, sperm samples were centrifuged at 5000 rpm at 4 °C for 2 min. The supernatant was discarded and the pellet was resuspended in 200 µL of TE buffer. 25 µL of the samples were transferred into microplates, adding 25 µL of lysing solution supplemented with Pico488 (20 µL/mL of lysing solution), and incubated in darkness at RT for 1 h. DNA denaturation started immediately after the addition of 250 µL of NaOH-EDTA solution (pH 12.4) and the fluorescence intensity reduction of the Pico488-dsDNA complex was monitored for 10 min directly by the microplate reader, set at 485 nm in excitation and 520 nm for emission. Results were expressed as strand scission factors (SSF) and calculated by:

$$-\ log_{10}\left(\frac{\text{Fluorescence units of samples}}{\text{Fluorescence units of control}}\right)$$

2.4. Statistical analysis

All data are reported as the mean ± standard deviation (SD).

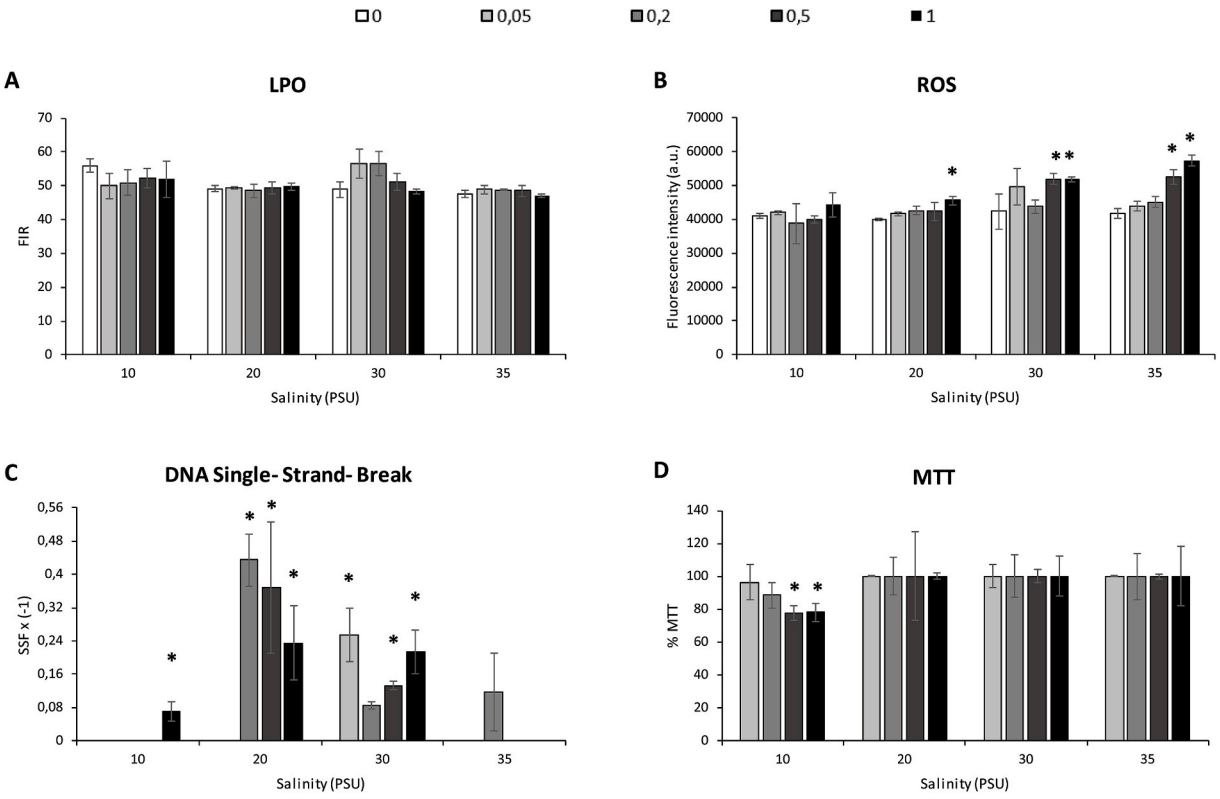

Fig. 2. Results of *F. enigmaticus* spermatozoids exposed to different concentrations (0.05, 0.2, 0.5 and 1 mg/L) of lead under four salinity conditions (10, 20, 30, 35). For each salinity, significant differences between control and Pb concentrations were represented with an asterisk (*) ($p < 0.05$). Mean values (±Standard deviation) for: **A:** Lipid peroxidation (LPO), expressed as fluorescence intensity ratio (FIR); **B:** Reactive oxygen species (ROS), expressed as fluorescence intensity (a.u.); **C:** Negative DNA Single-Strand-Break, expressed as strand scission factors (SSF) x (−1) and the control corresponded to the x-axis; **D:** Sperm viability (MTT), expressed as percentage (%) of MTT and the controls representing 100% of viability.

Statistical analyses were performed using GraphPad Prism 6.00 software (version for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) and results were analysed using: I) one-way analysis of variance (ANOVA) followed by the Dunnett multiple comparison test to evaluate significant differences between uncontaminated sperm at different salinities, and differences between concentrations of each contaminant relative to controls; II) two-way ANOVA with Bonferroni testing to compare all exposures at different salinity levels and contaminant concentrations.The significance levels were set at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

3. Results

3.1. Salinity conditions and interaction with chemicals

Table S1reports the control mean values of LPO levels and ROS production for all tested salinities (10, 20, 30, 35) without any contaminant present. No significant differences in membrane damage or oxidative stress were seen in the uncontaminated sperm suspensions treated at different salinity levels.

3.2. Chemicals

3.2.1. Copper

Cu-exposed sperm suspensions showed no significant differences in LPO levels compared to controls across all tested concentrations and salinity conditions, with the exception of salinity 35 which exhibited an increase of 9 and 13% in fluorescence intensity ratio (FIR) at 0.05 and 0.5 mg/L (Fig. 1A). For the Cu concentrations, significant differences between salinities were observed at 0.05 mg/L between 20 vs 30 and 20 vs 35 salinities, and at 1 mg/L, between 20 vs 30 (Table S3). Higher ROS production was found in samples exposed to concentrations of 0.05 mg/L to 1 mg/L under salinity 20, with the highest percentage value (27%) being seen in the 0.2 mg/L concentration compared to controls. At salinities 30 and 35, a significant increase of ROS concentration was observed when specimens were exposed to 0.2 (16%) and 0.05 mg/L (13%) of Cu, respectively (Fig. 1B). Significant differences among salinities at each concentration were observed for the 0.05 mg/L (salinity 10 vs 30), 0.2 mg/L (salinity 10 vs 20,30,35) and 1 mg/L (salinity 10 vs 20 and salinity 20 vs 30) (Table S3) concentrations. Only the highest Cu concentration significantly induced DNA integrity loss in salinity 10, while a significantly concentration-dependent response was observed for salinity 20, with major DNA breakage occurring at the highest Cu concentration. In salinity 30, significant DNA damage was detected in the 0.05, 0.2 and 1 mg/L concentrations of Cu compared to controls (Fig. 1C) and this effect was significantly different in comparison to salinity 20 (from 0.2 to 1 mg/L) and 30 (0.05 and 0.2 mg/L). At all concentrations, significant differences were observed between salinities 10 and 20, 10 and 30 and 20 and 35 (Table S3). No significant differences in sperm viability (MTT) were observed in exposed sperm across the different salinity levels compared to controls (Fig. 1D), or between salinities at each Cu concentration (Table S3).

3.2.2. Lead

No significant differences in LPO were detected in any of the exposed sperm suspensions or salinity conditions in comparison to controls (Fig. 2A). When comparing different salinities with each Pb concentration, significant differences were observed between salinity 10 vs 30, 20 vs 30 and 30 vs 35 at both 0.05 and 0.2 mg/L, respectively (Table S3). Significantly higher ROS production was measured in salinities 20 (1 mg/L), 30 and 35 (0.5 and 1 mg/L) compared to controls (Fig. 2B), showing an increase of 14%, 22%, 23%, 26% and 37%, respectively.

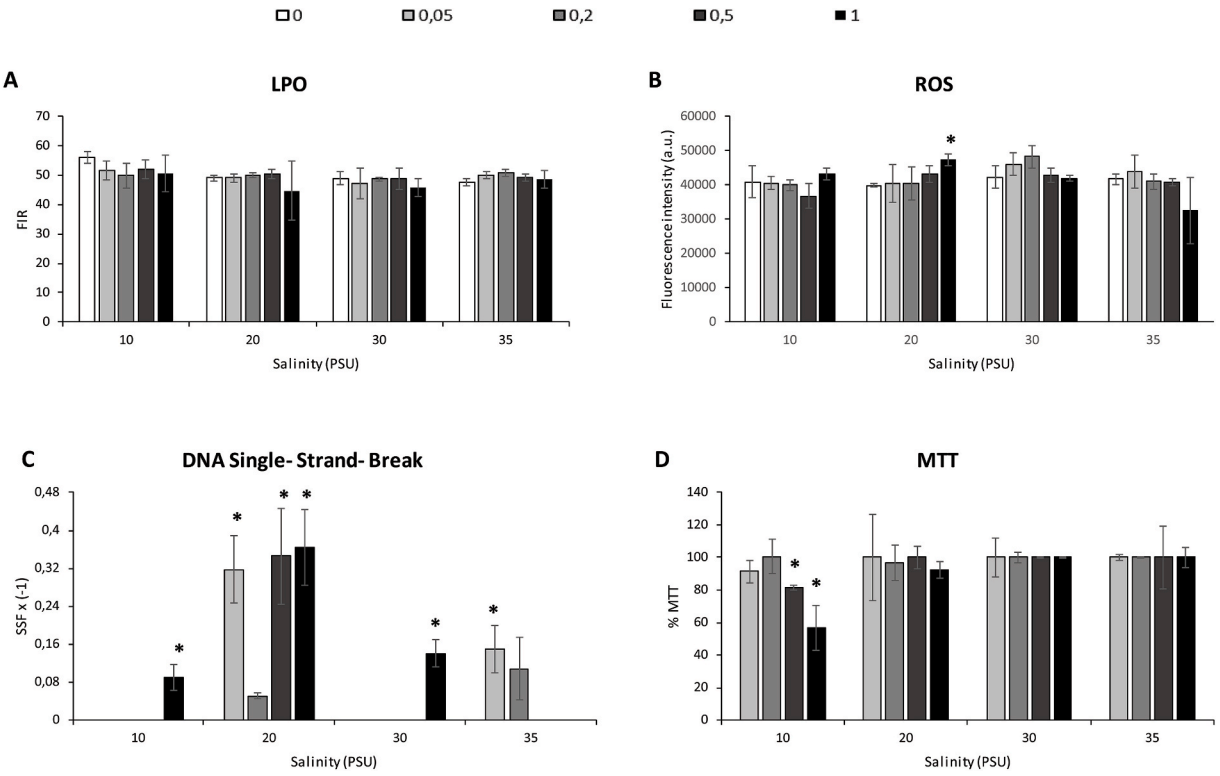

Fig. 3. Results of *F. enigmaticus* spermatozoids exposed to different concentrations (0.05, 0.2, 0.5 and 1 mg/L) of arsenic under four salinity conditions (10, 20, 30, 35). For each salinity, significant differences between control and As concentrations were represented with an asterisk (*) ($p < 0.05$). Mean values (±Standard deviation) for: **A:** Lipid peroxidation (LPO), expressed as fluorescence intensity ratio (FIR); **B:** Reactive oxygen species (ROS), expressed as fluorescence intensity (a.u.); **C:** Negative DNA Single-Strand-Break, expressed as strand scission factors (SSF) x (−1) and the control corresponded to the x-axis; **D:** Sperm viability (MTT), expressed as percentage (%) of MTT and the controls representing 100% of viability.

When comparing the salinities at the two highest Pb concentrations, significant differences were detected between 10 and 20 vs 30 and 35, whereas for the two lower concentrations, significant differences were seen between salinity 10 vs 30 and 20 vs 30 for the 0.05 mg/L concentration, and between salinity 10 vs 35 for 0.2 mg/L (Table S3). Sperm suspensions exposed to 1 mg/L of Pb demonstrated a significant increase in DNA damage at salinities 10, 20 and 30. At these two highest salinities a loss of DNA integrity was also observed at low concentrations as well (0.2 and 0.5 mg/L for the 20 condition; 0.05 and 0.5 mg/L for the 30 condition) (Fig. 2C). Significant differences between salinities were detected between 10 vs 20 and 20 vs 35 when the samples were exposed to the range of Pb concentrations (0.2–1 mg/L). Moreover, significant differences were found for the following concentrations: 0.05 mg/L (salinity 10 vs 30; 20 vs 30; 30 vs 35), 0.2 mg/L (salinity 20 vs 30), 0.5 mg/L (salinity 10 vs 30; 20 vs 30; 30 vs 35), 1 mg/L (salinity 10 vs 30; 30 vs 35) (Table S3). Significant differences in MTT were observed only at the two highest concentrations under the lowest salinity (Fig. 2D), while no significant differences between salinities were detected in sperm exposed to each concentration (Table S3).

3.2.3. Arsenic

There were no significant differences in LPO rates in As-exposed sperm suspensions at all salinity levels in comparison to controls (Fig. 3A) or between salinities at each As concentration (Table S3). No significant increases in ROS concentration were detected under any of the As concentrations or salinities, with the exception of 1 mg/L at salinity 20, which showed a 19% increase compared to controls (Fig. 3B). Considering differences among salinities at each As concentration, significant differences were detected at the 0.2 mg/L (salinity 10 vs 30) and 1 mg/L (salinity 10 vs 35, 20 vs 35 and salinity 30 vs 35) concentrations (Table S3). Significant single strand DNA breakages were detected for the following salinity levels and As concentrations; 10: 1 mg/L; 20: 0.05, 0.5 and 1 mg/L; 30: 1 mg/L and 35: 0.05 mg/L (Fig. 3C). Significant differences among salinities were identified between 10 vs 30 as well as 10 vs 30 and 35 conditions when exposed to 0.05 and 1 mg/L of As, respectively. Moreover, significant differences were also detected at 0.2 mg/L (salinity 10 vs 35 and salinity 30 vs 35) (Table S3). A concentration-dependent decrease in MTT was found in the sperm suspensions for the salinity level 10 with a decrease of 37% in the highest As concentration (Fig. 3D). Similar results were obtained when comparing salinity conditions, with significant differences seen only for salinity 10 compared to other levels when the samples were subjected to 1 mg/L of As (Table S3).

3.2.4. Zinc

An 8% increase in the rate of LPO was observed in sperm exposed to 0.05 mg/L of Zn at salinity 20 as well as to 0.05, 0.2 and 0.5 mg/L of Zn at salinity 35 (12%, 15% and 11% respectively), in comparison to controls (Fig. 4A). If comparing salinity conditions for each Zn concentration, significant differences were observed between salinity 30 vs 35 at 0.2 mg/L and between salinity 10 vs 30 as well as 30 vs 35 for the 0.5 mg/L concentration (Table S3). Significant increases in ROS production were seen at the two lowest Zn concentrations with a 12% increase for the salinity levels of 20 and 30 compared to controls (Fig. 4B). Regarding the comparison between salinities, significant differences were detected only for the 0.2 mg/L (salinity 10 vs 30) and 1 mg/L concentrations (salinity 10 vs 35, 20 vs 35 and salinity 30 vs 35) (Table S3). DNA was significantly damaged in spermatozoids in concentrations of 0.05, 0.5 and 1 mg/L at salinity 10. Similar results were also detected at salinity 20 (0.05, 0.2 and 1 mg/L), while significant DNA damage was observed only at the highest element concentration at salinity 30. Considering the salinity 35, 0.2 and 1 mg/L were the only Zn concentrations that showed significant loss of DNA integrity in comparison to controls (Fig. 4C). Moreover, at the two lowest Zn concentrations, significant differences

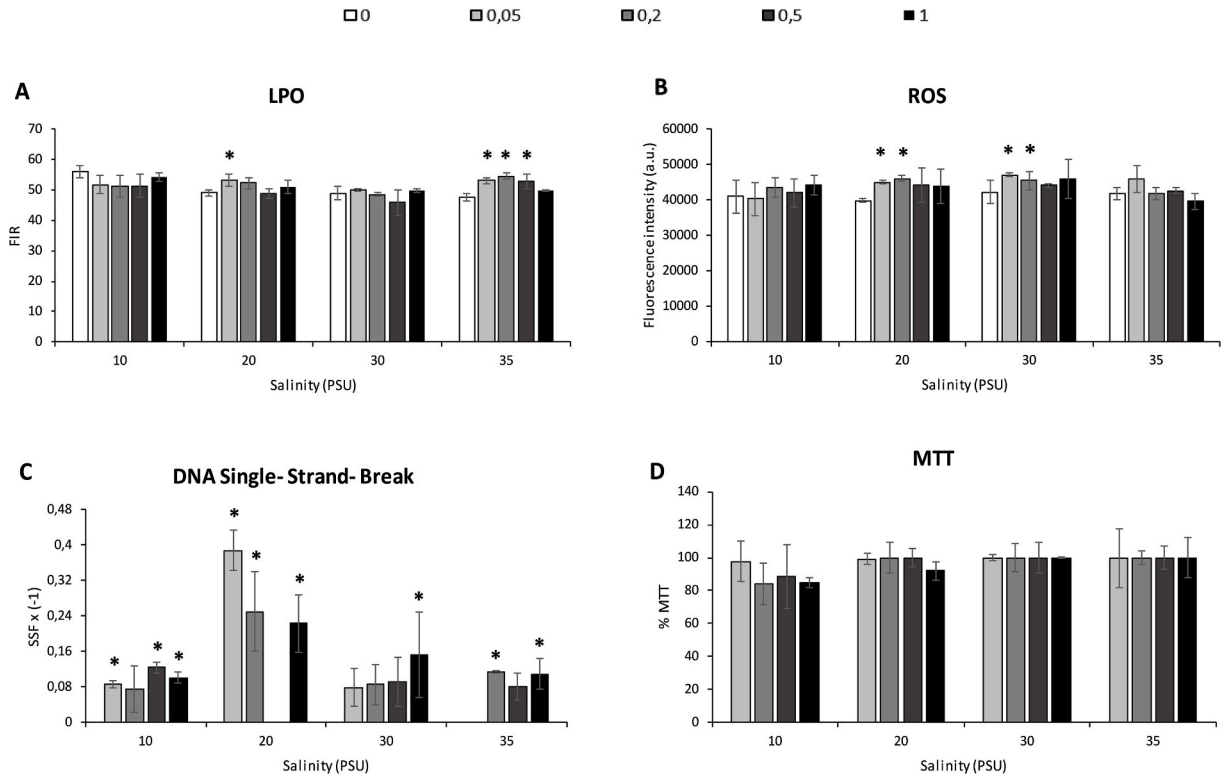

Fig. 4. Results of *F. enigmaticus* spermatozoids exposed to different concentrations (0.05, 0.2, 0.5 and 1 mg/L) of zinc under four salinity conditions (10, 20, 30, 35). For each salinity, significant differences between control and Zn concentrations were represented with an asterisk (*) ($p < 0.05$). Mean values (±Standard deviation) for: **A:** Lipid peroxidation (LPO), expressed as fluorescence intensity ratio (FIR); **B:** Reactive oxygen species (ROS), expressed as fluorescence intensity (a.u.); **C:** Negative DNA Single-Strand-Break, expressed as strand scission factors (SSF) x (−1) and the control corresponded to the x-axis; **D:** Sperm viability (MTT), expressed as percentage (%) of MTT and the controls representing 100% of viability.

were detected between salinity 10 and 30 and salinity 20 to 30 and 35. At the other concentrations, significant differences were evident between salinities 10 vs 20 at 0.5 mg/L and between salinity 10 vs 20 and 20 vs 35 at 1 mg/L (Table S3). No significant differences in sperm viability were detected, regardless of Zn concentration or salinity level (Fig. 4D; Table S3).

3.2.5. Cadmium

No significant differences in the percentage of LPO levels were detected between contaminated and non-contaminated sperm suspensions (Fig. 5A) as well as between different salinity conditions across all the Cd concentrations (Table S3). When the specimens were exposed to concentrations of 0.2 and 1 mg/L Cd under a salinity of 20, higher percentage of ROS production was found in comparison to the control, with a maximum increase value of 14% at the highest concentration (Fig. 5B). There were no significant differences between salinities for each Cd concentration (Table S3). The two highest Cd concentrations induced a significant loss of DNA integrity in cells at salinity 10, while for salinity 20 significant DNA damage was detected for the 0.05 and 0.5 mg/L concentrations. At salinity 30, significant DNA damage was observed for all concentrations, with the exception of 0.5 mg/L, which was the only concentration that showed a significant effect at salinity 35 (Fig. 5C). When comparing salinity levels, significant differences were found across all the Cd concentrations: 0.05 mg/L (salinity 10 vs 20 and 30; 20 vs 30; 30 vs 35), 0.2 mg/L (salinity 10 vs 30; 30 vs 35), 0.5 mg/L (salinity 10 vs 20, 30 and 35; salinity 30 vs 35), 1 mg/L (salinity 10 vs 20 and 35; 30 vs 35) (Table S3). No significant differences in cell viability were observed in sperm exposed to all Cd concentrations compared to controls (Fig. 5D) or between the different salinity conditions at each Cd concentration (Table S3).

3.2.6. Sodium dodecyl sulfate

At salinity 10, significantly higher LPO levels were detected only for the SDS concentrations of 0.5 and 4 mg/L, with increases of 10% and 21% compared to controls. Significant concentration-dependent responses (concentrations from 0.5 to 4 mg/L) were observed for all the other salinities, with the highest LPO levels (≈38%) at the higher exposure concentrations (Fig. 6A). Significant differences between salinities at different SDS concentrations were only detected for the 2 mg/L (salinity 10 vs 30 and 20 vs 35) and 4 mg/L (salinity 10 vs 30) concentrations (Table S3). A salinity-dependent increase in ROS production percentage was observed for all of the SDS concentrations under salinities 20, 30 and 35, ranging from ≈22% to ≈70% at the highest salinity level (Fig. 6B). Similar results were also found between salinities, showing significant differences for all of the SDS concentrations (Table S3). Sperm suspensions that were exposed to SDS showed a significant decrease in DNA integrity across all SDS concentrations and salinity levels (Fig. 6C), while significant differences between salinity levels were only evident for the 0.5 mg/L (salinity 20 vs 30 and 35) and 4 mg/L (salinity 10 vs 35) concentrations (Table S3). The percentage of MTT significantly decreased only at the highest SDS concentration for all of the tested salinities (Fig. 6D), and decreases ranged between 16% and 34% compared to controls. Moreover, significant differences between salinities were found at the 2 mg/L concentration between salinity 10 and all other salinity levels (Table S3).

3.2.7. Benzo(a)pyrene

Significant increases in LPO were detected in sperm suspensions exposed to 0.5 and 4 μg/L B(a)P at salinity 10 (6% and 11%, respectively), all concentrations at salinity 20 (15%) and 1 and 2 μg/L at salinity 30 (15% and 9%, respectively) compared to controls (Fig. 7A). Significant differences between salinities were observed for all the B(a)P

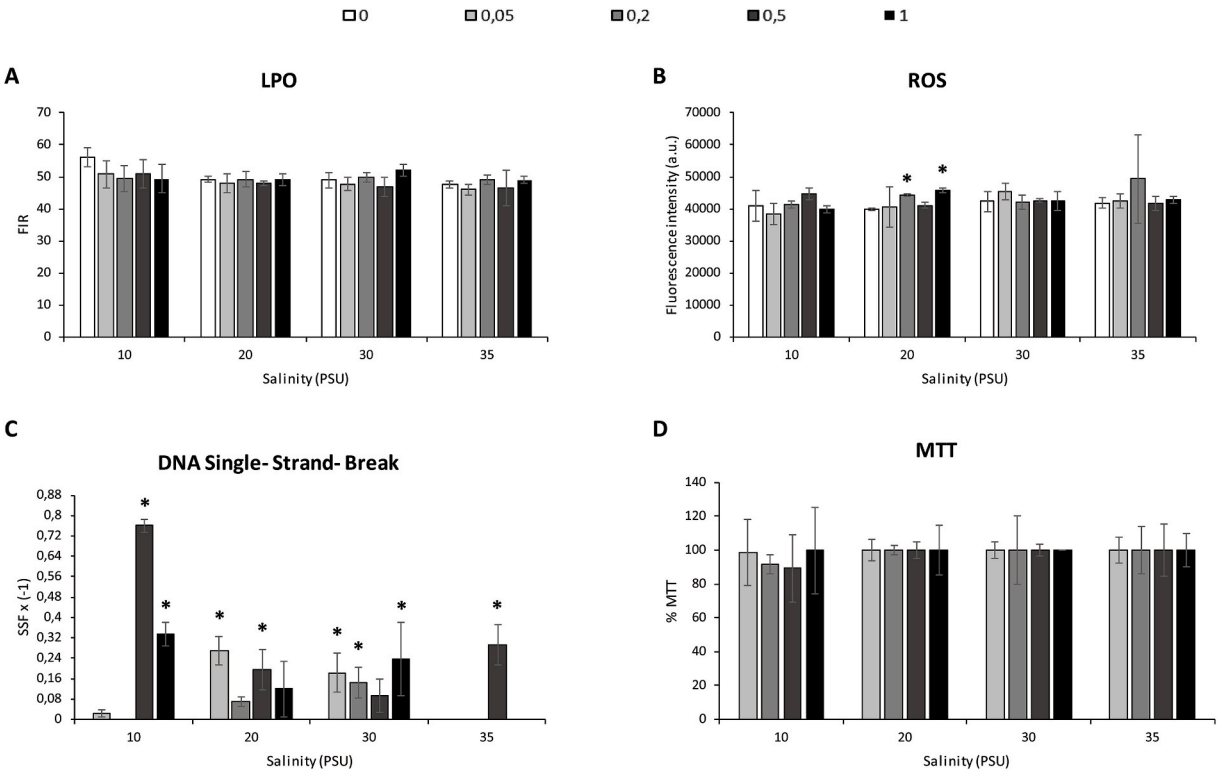

Fig. 5. Results of *F. enigmaticus* spermatozoids exposed to different concentrations (0.05, 0.2, 0.5 and 1 mg/L) of cadmium under four salinity conditions (10, 20, 30, 35). For each salinity, significant differences between control and Cd concentrations were represented with an asterisk (*) ($p < 0.05$). Mean values (±Standard deviation) for: **A:** Lipid peroxidation (LPO), expressed as fluorescence intensity ratio (FIR); **B:** Reactive oxygen species (ROS), expressed as fluorescence intensity (a.u.); **C:** Negative DNA Single-Strand-Break, expressed as strand scission factors (SSF) x (−1) and the control corresponded to the x-axis; **D:** Sperm viability (MTT), expressed as percentage (%) of MTT and the controls representing 100% of viability.

concentrations at 10 vs 35 and 20 vs 35 salinities. Moreover, significant differences were also seen in the 0.5 μg/L (salinity 10 vs 30), 1 μg/L (salinity 30 vs 35), 2 μg/L (salinity 10 vs 30) and 4 μg/L (salinity 10 vs 20 and 30) concentrations (Table S3). Salinity-dependent increases in ROS production were detected for salinity 20, 30 and 35, with increases ranging from ≈15% to ≈52% in comparison to controls (Fig. 7B). Significant differences were found between all of the salinities at the 1 and 2 μg/L concentrations, with the exception of 20 vs 30. At 0.5 and 4 μg/L B(a)P, significant differences were observed between salinities 10 and 20 when compared with both 30 and 35 (Table S3). Significant DNA single strand break was detected in the 0.5 and 4 μg/L B(a)P concentrations for salinities 20 and 30, while at level 35 significant DNA damage was seen for all concentrations, except for the 4 mg/L concentration (Fig. 7C). When the specimens were exposed to the 1 and 4 μg/L concentrations, significant differences were detected between all of the salinity levels, apart from levels 10 vs 35 and 20 vs 30. Significant differences were also detected at the 0.5 μg/L (salinity 10 vs all others, salinity 30 vs 35) and 2 μg/L (salinity 10 vs 35, 20 vs 35 and 30 vs 35) concentrations (Table S3). Significant decreases in sperm viability were observed only at the highest B(a)P concentrations across all salinity conditions (25%, 36%, 29% and 28%, respectively) compared to controls (Fig. 7D), while no differences were detected between salinities and each B(a)P concentration (Table S3).

3.3. Fluorescence microscopy analysis

Fluorescence microscopy images (Figs. S1A and B) confirmed the correct positioning of each fluorochrome that we used to evaluate certain sperm quality parameters. C11-BODIPY$^{581/591}$ fluorophore had two different spectral forms: a non-oxidized one at 595 nm and an oxidized one at 520 nm. These were acquired simultaneously using a double emission wavelength, and the spermatozoids that were exposed to oxidative stress showed an intense green fluorescence (IG) in the presence of high membrane lipid peroxidation, particularly over the acrosomal cap and midpiece. A faint red fluorescence (FR) was instead seen for non-peroxidized membranes (Fig. S1A). Due to the redox status of cells, DHE was oxidized intracellularly into 2-hydroxyethidium (2-OH-E$^+$) and ethidium cation (E$^+$). As shown in Fig. S1B, by setting the excitation wavelength at 350 nm, this probe emitted a blue fluorescence, which can be attributed to the small amount of E$^+$ that bound to DNA and localized within the nuclei, whereas the red fluorescence signifies the oxidized form of 2-OH-E$^+$, so was used to detect superoxide anions produced in mitochondria.

4. Discussion

In the present study, the effects of trace elements (Zn, Cu, Cd, As and Pb) and organic compounds (B(a)P and SDS) on *F. enigmaticus* male gametes at different salinity levels were investigated. In addition, the suitability of spectrofluorimetric or photometric analyses for assessing biomarkers related to sperm function were evaluated.

Sperm quality analysis represents a rapid, accurate and sensitive methodology, useful for predicting how pollutants affect the fertilizing ability and reproductive success of organisms (Gallo and Tosti, 2019). Fertilization success depends on the production of high quality gametes, with undamaged DNA and appropriate sperm motility responses to oocytes (Lewis and Ford, 2012). In this study, spermatozoids were initially examined at different salinity levels to evaluate if this abiotic factor alone could affect short-term sperm quality. *F. enigmaticus* is a free spawning species and its gametes are released into the environment so are constantly exposed to biotic and abiotic factors that could alter their quality. Several studies have previously reported that *F. enigmaticus* is a euryhaline species, thus able to tolerate a wide range of salinity fluctuations even though the optimum range in terms of fertilization success

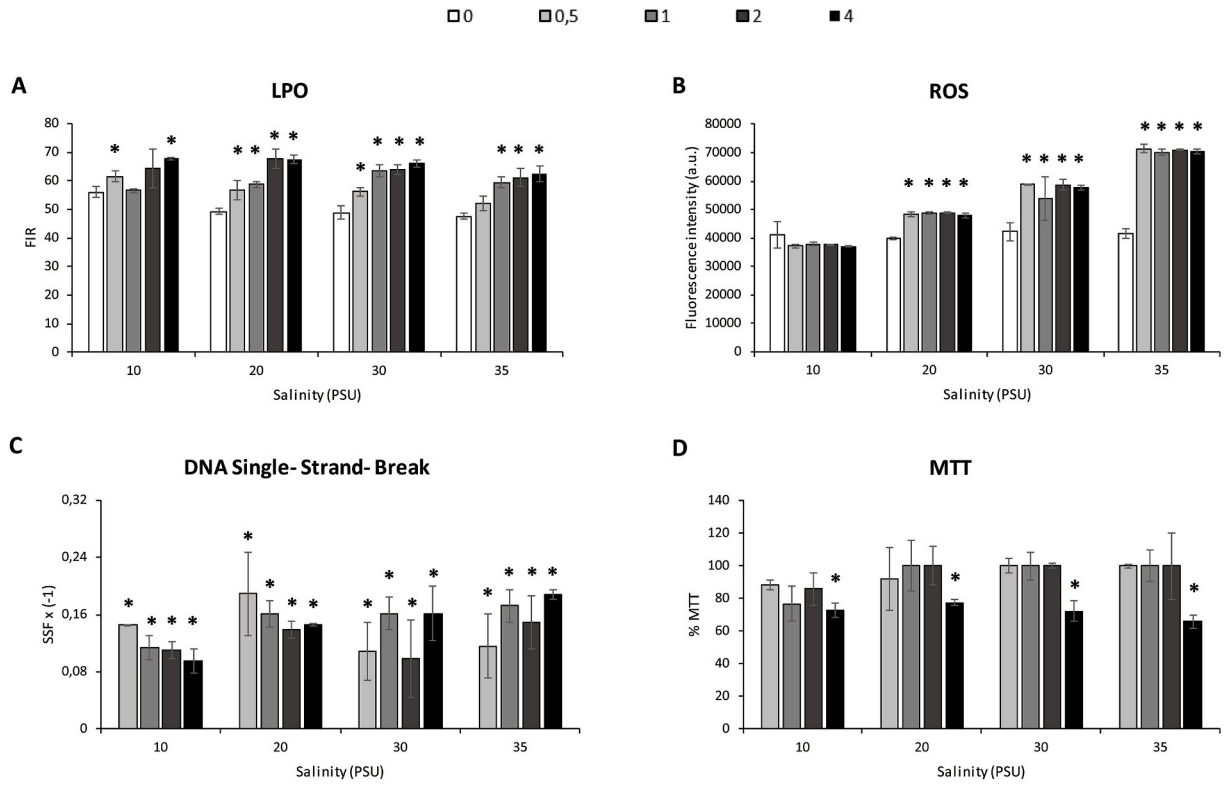

Fig. 6. Results of *F. enigmaticus* spermatozoids exposed to different concentrations (0.5, 1, 2, 4 mg/L) of sodium dodecyl sulfate (SDS) under four salinity conditions (10, 20, 30, 35). For each salinity, significant differences between control and SDS concentrations were represented with an asterisk (*) ($p < 0.05$). Mean values (±Standard deviation) for: **A:** Lipid peroxidation (LPO), expressed as fluorescence intensity ratio (FIR); **B:** Reactive oxygen species (ROS), expressed as fluorescence intensity (a.u.); **C:** Negative DNA Single-Strand-Break, expressed as strand scission factors (SSF) x (−1) and the control corresponded to the x-axis; **D:** Sperm viability (MTT), expressed as percentage (%) of MTT and the controls representing 100% of viability.

and larval development is between 10 and 35 (Dittmann et al., 2009; Oliva et al., 2019b; Peria and Pernet, 2019). In line with these studies (Dittmann et al., 2009; Oliva et al., 2019b; Peria and Pernet, 2019), similar results were obtained in the current study across salinity levels, confirming the high tolerance of this species to salinity variation. Instead, when the specimens were exposed to contaminants at different salinity conditions, they showed variations in biomarker responses. Previous work has already demonstrated that selected pollutants, acting alone, exert effects on reproduction, growth, development and survival by directly and indirectly affecting cellular physiology, metabolism, apoptosis and other cellular activities (Gopalakrishnan et al., 2008; Rani et al., 2014). Other evidence suggests that pollutant exposure induces genetic instability through direct interaction with DNA or by altering its repair mechanisms (Valko et al., 2005; Aina et al., 2006; Rani et al., 2014). The present data, resulting from the combined action of both measured stressors (i.e. salinity level and contaminant exposure), could be due to different factors, such as the relation between salinity and contaminant availability, changes in contaminant toxicity according to salinity level, and/or interactive effects that could be additive or synergistic (Owojori et al., 2009). In particular, when male gametes were exposed to trace elements, differences in sperm impairments were observed at the higher and lower salinity conditions. According to Forsberg et al. (2006), this could be due to the influence of salinity on physio-chemical speciation of inorganic compounds. Consequently, it would have an effect on their availability and toxicity for cells, since no differences were observed in response to salinity alone. Other studies have already confirmed this hypothesis, demonstrating that trace element bioavailability mostly depends upon the water conductivity and salinity. In turn, these physical parameters may cause variations in metal toxicity, competing with metal ions in binding to biological molecules (Baysoy et al., 2012). Bryant et al. (1985) highlighted an antagonistic interaction between Zn concentration and salinity, showing more effects at high salinities and low Zn concentrations. In comparison, spermatozoids that were exposed to the organic compounds SDS and B(a)P at different salinity levels showed differential effects on cellular physiology, homeostasis, and DNA integrity. These results could be due to the direct interference of this abiotic factor on contaminant toxic action and/or combined effects between both stressors. To support this hypothesis, Belhaj et al. (2020) demonstrated that salinity had the potential to influence surfactant stability, especially for anionic ones like SDS, due to the negative charge of the head group in aqueous solutions. Moreover, Whitehouse (1984), assessed salinities ranging from 0 to 36.7 and highlighted that at the same temperature, PAHs solubility is sensitive to changes in salinity.

Since there is limited knowledge on the consequences of the selected contaminants on male fertility in aquatic invertebrates, the present work assessed possible sperm impairments relating to oxidative stress, sperm viability and DNA damage.

Oxidative status is related to the balance between the production and scavenging of ROS to maintain cell homeostasis. Oxyradical damage to lipids, proteins and DNA as a consequence of ROS production and/or variation in antioxidant defence levels (Regoli and Winston, 1999) are widely recognized as key causes of reduced sperm function (Balercia et al., 2003). As reported previously by several authors and confirmed in the present study, both inorganic and organic compounds, alone or in combination with salinity levels, can induce an increase in ROS production in *in vitro* exposed sperm suspensions. Trace elements are able to generate reactive oxygen and nitrogen species through Harber–Weiss and Fenton reactions (Valko et al., 2005; Kim et al., 2014) while organic pollutants, like anionic detergents and PAHs, act as pro-oxidants inducing oxidative stress, altering cell osmotic balance (Messina et al., 2014; Nunes et al., 2008). Moreover, higher levels of salinity enhance

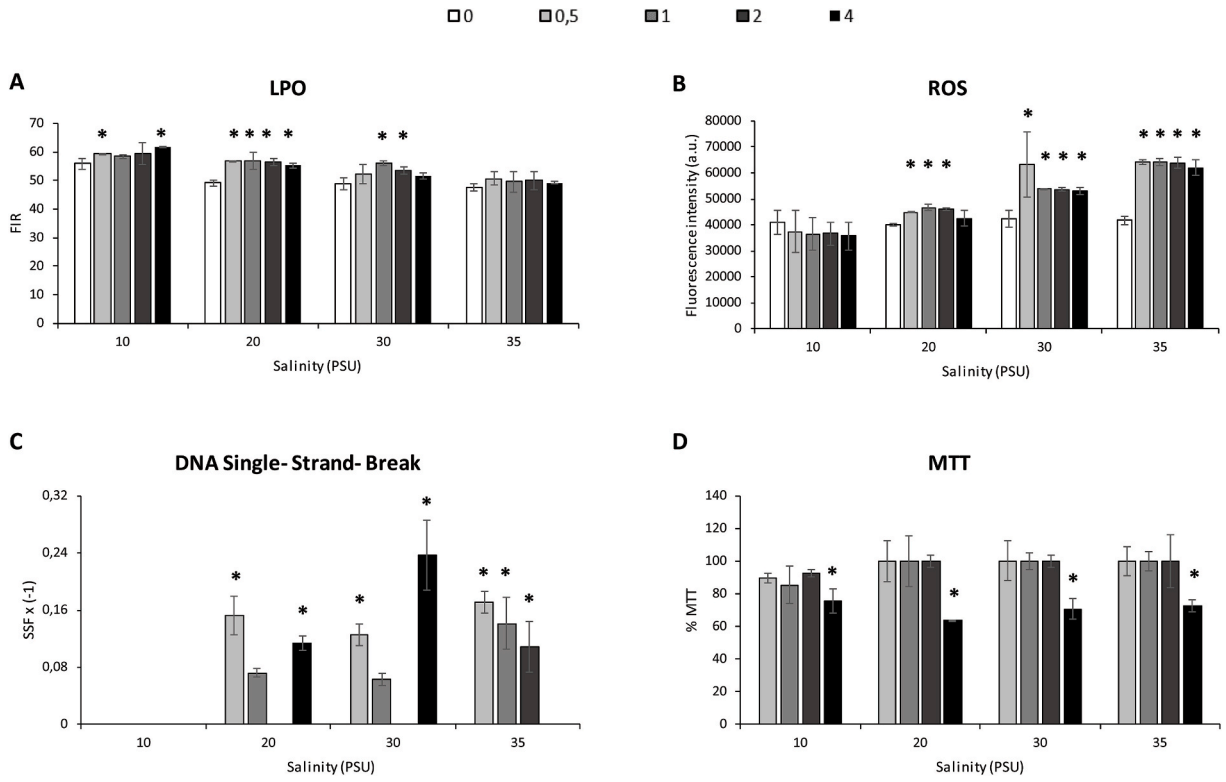

Fig. 7. Results of *F. enigmaticus* spermatozoids exposed to different concentrations (0.5, 1, 2, 4 μg/L) of benzo (a) pyrene (B(a)P) under four salinity conditions (10, 20, 30, 35). For each salinity, significant differences between control and B(a)P concentrations were represented with an asterisk (*) ($p < 0.05$). Mean values (±Standard deviation) for: **A:** Lipid peroxidation (LPO), expressed as fluorescence intensity ratio (FIR); **B:** Reactive oxygen species (ROS), expressed as fluorescence intensity (a.u.); **C:** Negative DNA Single-Strand-Break, expressed as strand scission factors (SSF) x (−1) and the control corresponded to the x-axis; **D:** Sperm viability (MTT), expressed as percentage (%) of MTT and the controls representing 100% of viability.

the concentration-dependent effects of organic compounds. This could be due to the alteration of physio-chemical properties of the tested contaminants. Salinity can modify contaminant solubility (Whitehouse, 1984) or reduce the repulsive forces between molecules (Belhaj et al., 2020). As a direct consequence of ROS concentration increase, the primary mechanism of oxidative cellular damage is lipid peroxidation of membranes (LPO). Sperm membranes contain a high concentration of polyunsaturated fatty acids (PUFA) making them highly susceptible to peroxidative damage (Aitken et al., 2014). In this study, ROS overproduction induced an increase of LPO when spermatozoids were exposed to Cu, Zn, SDS and B(a)P, at different salinity levels. Moreover, significant interaction between salinity level and chemical concentration were evident for Zn and SDS ($p < 0.01$ and $p < 0.001$, respectively). These pollutants could induce an inactivation or insufficient protection provided by antioxidant defence systems, likely due to the accumulation of superoxide anions which, in turn, are able to increase LPO. Despite limited evidence on the toxic effects of the studied contaminants on the male reproductive system, previous studies in other marine invertebrates can support this hypothesis (Chandran et al., 2005; Lewis and Galloway, 2008; Freitas et al., 2020). In particular, Freitas et al. (2020) detected a decrease of SOD and CAT activity in mussels exposed to concentrations of SDS similar to those used in the present study, highlighting an inhibitory capacity against these enzymes. In contrast, when the specimens were exposed to Pb, As and Cd at different salinities, the increase of ROS was not accompanied by changes in LPO levels, which indicates that the enhanced generation of ROS can be overwhelmed by the intrinsic antioxidant defences of cells (Rani et al., 2014).

In addition, mitochondrial status represents another important sperm quality parameter, strictly related to viability and motility. In this study, succinate dehydrogenase activity (MTT assay) was used to estimate sperm viability in the polychaete *F. enigmaticus*. Considering that ROS interaction with PUFAs can alter the structure, as well as the properties of sperm membranes, this will interfere with sperm motility (Tvrdá et al., 2011). Results confirmed this hypothesis, showing a decrease of MTT when spermatozoids were exposed to Pb, As, SDS and B(a)P at different salinity levels. With regards to the trace elements, the data only found a salinity-dependant decrease in sperm viability at the highest contaminant concentrations in the lowest salinity level. Moreover, with the exception of As, the effects exerted by inorganic and organic pollutants were independent of salinity level. These compounds potentially affect sperm viability by disrupting metabolic capacity (Freitas et al., 2020), cellular ATP production and the mitochondrial respiration chain (Bergquist et al., 2009), by inducing morphological changes and surface tensions (Prat and Giraud, 1964) or by reducing protein synthesis (Geraci et al., 2004).

In addition to measuring metabolism, cell damage and oxidative status related parameters, we also evaluated DNA integrity. Many authors have previously described and applied various DNA damage detection techniques in ecotoxicological studies. In the present work, Fast Micromethod® resulted in the most sensitive assessed biomarker, confirming it to be the best choice in terms of rapidity, efficiency and applicability on a wide spectrum of samples (Schröder et al., 2006). Our results show that all of the studied contaminants are able to generate DNA single-strand breakages. Previous literature has demonstrated that organic and inorganic compounds can induce loss of DNA integrity in freshly-spawned sperm (Lewis and Ford, 2012). Spermatozoids are considered to be particularly susceptible to contaminant-induced mutations due to their lack of DNA repair mechanisms as well as their inability to respond to DNA damage with programmed cell death (Lewis and Galloway, 2009). Nevertheless, little attention was given to potential genotoxic damage in the sperm of marine invertebrates (Lewis and

Ford, 2012). Therefore, the toxicity of trace elements involved in genotoxic damage may be due to the oxyradical attack on DNA together with the inhibition of relevant repair mechanisms (Chiarelli and Roccheri, 2014; Valko et al., 2005). In the present study, Zn, Cu and Cd induced DNA damage under all salinity conditions, indicating a genotoxic effect related to the interaction between salinity variation and chemical concentration ($p < 0.0001$). As a consequence, an increase in DNA breakage could induce a reduction in fertilization success, as demonstrated previously by several authors in different marine species (Fitzpatrick et al., 2008; Gopalakrishnan et al., 2008; Reichelt-Brushett and Harrison, 2005). The integrity of sperm DNA was adversely affected by exposure to surfactants and PAHs (Lewis and Galloway, 2008). According to previous studies (Liu et al., 2009; Rosety et al., 2001), SDS induces negative effects on DNA integrity in sperm across all salinity conditions and also shows a significant interaction between these two stressors ($p < 0.05$). As reported by Rosety et al. (2001), a probable consequence of impaired sperm function is the disruption of reproductive processes, as they measured a fertilization rate of only 7% in samples under a SDS concentration of 6 mg/L. It is well known that genotoxic and teratogenic effects induced by B(a)P are due to covalent interaction with the nucleophilic centres of DNA, causing nucleic acid strand breakage (Aina et al., 2006). Data obtained in this study supports these findings, and underlines the influence of salinity across all B(a)P concentrations ($p < 0.0001$). The accumulation of unrepaired DNA lesions could be related to changes in fertilization kinetics and embryotoxicity (Lacaze et al., 2011). In accordance with what was observed in this study, a negative relationship was highlighted between B(a)P-induced DNA damage in sperm and the percentage of well-developed embryos (Lewis and Galloway, 2008).

5. Conclusion

In this study, *in vitro* exposure of male gametes to selected pollutants showed that greater sperm quality impairments were induced by organic contaminants compared to inorganic ones. Moreover, the high tolerance shown by *F. enigmaticus* spermatozoids to salinity variation can be considered an added value supporting: i) the investigation of lower biological organization levels without the interference of this abiotic factor; ii) the suitability of this species as a promising model organism in contaminated matrices assessment. The present study provides evidence for the effectiveness of used fluorometric and photometric methods to investigate the spermiotoxicity of pollutants, allowing for an improved ecotoxicological toolbox that includes integrative or alternative endpoints as well as traditional ones. Further studies are needed to better understand if sperm function impairments are also linked to other developmental stages, potentially caused by transmission of damage to offspring or adverse effects on population structure.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111219.

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