

Polluted water exacerbates *Barbus callensis* oocyte oxidative status

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Abstract. The deleterious effects of environmental pollutants on cellular components and tissues damage in fish have been studied extensively. However, there is no data about the oxidative status of fish oocytes once released into water. This study aimed to investigate the effects of polluted (Soummam River) and unpolluted (Agrioun River) fresh water on the oxidative biomarkers of *Barbus callensis* (= *Lucibarbus callensis*) (Val.) oocytes. The experimental design consisted of collecting fish oocytes from polluted and unpolluted rivers and then activating these oocytes separately in water collected from each site. Four groups were considered: oocytes from the Agrioun River activated in Agrioun fresh water (A-oocytes/A-fresh water); oocytes from the Agrioun River activated in Soummam fresh water (A-oocytes/S-fresh water); oocytes from the Soummam River activated in Agrioun fresh water (S-oocytes/A-fresh water); and oocytes from the Soummam River activated in Soummam fresh water (S-oocytes/S-fresh water). Oxidative stress biomarkers were evaluated by measuring total antioxidant status (TAS), catalase (CAT) activity, and cell-free hemoglobin (Hb) concentrations. The results showed that the oxidative status of fish oocytes was significantly affected by the quality of fresh water. Unpolluted fresh water improved the antioxidant

activity of the fish oocytes. The results of this study suggest that once oocytes are released into polluted water, antioxidant protection is affected with subsequent cellular oxidative damage and potential reproduction impairment.

Keywords: Oxidative stress, oocytes, biomarkers, *Barbus callensis*, pollution.

Introduction

The pollution of aquatic ecosystems is undoubtedly the main environmental threat to aquatic organisms. Pollution can impair the reproductive success of adult organisms by decreasing the quality and/or quantity of gametes, which can lead to the impairment of fertilization success, embryonic development, larval viability, and, consequently, species fitness and survival (Arizza et al. 2009). Many other factors can also affect gamete quality including age, management, feeding, exposure to xenobiotics, and the physicochemical quality of water (Brooks et al. 1997, Valdebenito et al. 2015).

Very often, the chemical analysis of water is an inadequate tool for properly assessing the adverse effects of complex mixtures of contaminants (He et al. 2011). However, it has been established that reactive oxygen species (ROS) produced under environmentally stressful conditions are potent indicators of

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water quality. These species induce oxidative damage to membrane lipids, DNA, and proteins, and modulate antioxidant enzymes (Valavanidis et al. 2006, Ruder et al. 2008, Wells et al. 2009). In fact, the production of ROS by living organisms under environmental stress is very common and represents an important part of the stress response in aquatic organisms. Therefore, quantitative measurement of biomarkers, which indicate toxicological-induced changes in biological systems, could serve as potent indicators of ecosystem health (Maria et al. 2009). In addition, these biomarkers could also reflect the effects of deleterious factors affecting water quality (Al-Gubory 2014, Xie et al. 2015).

Abnormalities concerning the biology of reproduction in parallel with dramatic population declines at polluted sites have prompted major research efforts to understand stress factor-induced changes that could serve as early warning indicators (Thomas 1990). In recent decades, stocks of barbel, *Barbus barbus* L. (Teleostei: Cyprinidae) have dramatically decreased throughout the world because of the deterioration and/or loss of microhabitats (Vilizzi et al. 2006, 2013). Moreover, *Barbus barbus* is an attractive sport fish which remains under strong angling pressure. Consequently, overfishing has significantly contributed to the reduction of fish numbers (Poncin and Philippart 2002, Kottelat and Freyhof 2007).

The Soummam River is the major water body of the region of Bejaia (Algeria), which is characterized by the presence of various pollutants discharged from industrial, agricultural facilities, and human sources. A previous study addressed the impact of continuous exposition to contaminants on intersexuality in *Barbus callensis* (Val.) inhabiting this river (Djoudad-Kadji et al. 2012). In contrast, the Agrioun River is widely known for its wilderness, and it is advertised as one of the least polluted rivers in the region of Bejaia. There are numerous studies on polluted water for large number of toxic compounds showing increased oxidative stress levels in fish (Bainy et al. 1999, Livingstone 2001, Wilhelm Filho et

al. 2001); however, to the best of our knowledge, there is no experimental study on the influence of water quality on oocyte oxidative status once they are released into the water. Thus, the aim of the present work is to perform a comparative study on the impact of polluted (Soummam River) and unpolluted (Agrioun River) fresh water on oocyte oxidative biomarkers during the spawning period.

Materials and Methods

Description of study site

The Soummam River is a heavily polluted river in Bejaia (northeast Algeria). Most industrial factories and agricultural areas are located along this river, consequently, various pollutants reach it. Previously, Mouni et al. (2009) reported the presence of numerous micropollutants in Soummam River. In the present study, fish samples were collected from two different sites, Soummam River (S1: 36° 34' 42.5'' N/5° 4' 37.3'' E) and Agrioun River (S2: 36° 38' 31.3'' N, 5° 20' 21.2'' E), the latter is considered to be less affected by pollution and was chosen as a reference site (Fig. 1).

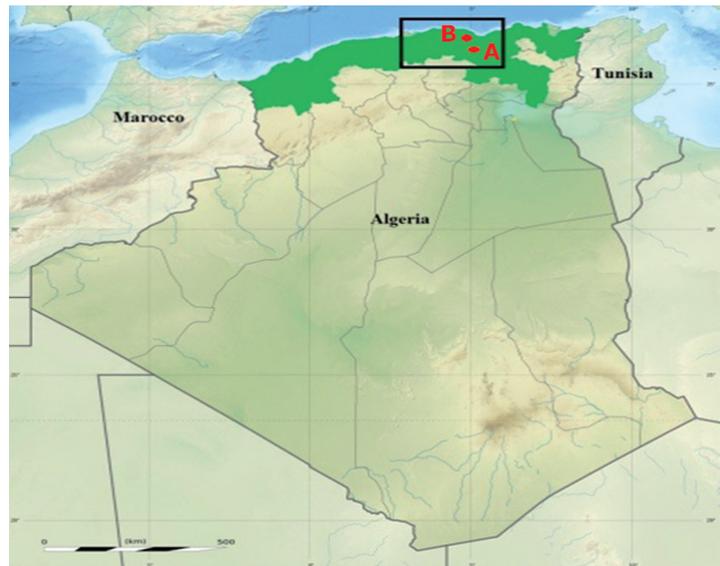


Figure 1. Geographic locations of the study areas (A: Agrioun River, B: Soummam River).

Collection of river fresh water

Water samples were collected from each river in sterilized glass bottles with polyethylene caps and a capacity of one liter (1 L). All containers were rinsed three times with fresh water then filled. They were immersed by hand to approximately 15-30cm below the surface. Water samples were transported to the laboratory in a cooler (4°C).

Collection of fish samples

A total of 12 mature females were captured from the Agrioun River (n = 6) and the Soummam River (n = 6), Bejaia (Algeria) in June 2015. Sampling was performed during the spawning period. The live fish collected were immediately transported to the laboratory. Body weight and total length were individually measured and recorded. The fish were then sacrificed, and their ovaries were removed. Ovary weights were recorded for the calculation of the gonadosomatic index.

Oocyte collection and preparation

Oocytes were carefully collected from ovaries, washed with cold saline (0.9% NaCl), and divided into two sets. The first set (oocytes of fish caught from the Agrioun River) was divided into two groups of 3 g of oocytes and each one was activated separately with Agrioun fresh water or Soummam fresh water for 5 minutes. Likewise, the second set (oocytes of fish caught from the Soummam River) was prepared and treated similarly. Thus, four groups of activated oocytes were obtained: Agrioun oocytes activated with Agrioun fresh water (A-oocytes/A-fresh water); Agrioun oocytes activated with Soummam fresh water (A-oocytes/S-fresh water); Soummam oocytes activated with Agrioun fresh water (S-oocytes/A-fresh water); and Soummam oocytes activated with Soummam fresh water (S-oocytes/S-fresh water). This procedure was performed in triplicate. After incubating for 5 minutes, the fresh water was drained from the oocytes using blotting paper, and then they

were homogenized with a cold phosphate buffer (50 mM, pH7.4) at a ratio of 1/10 (w/v) at 4°C using a motor-driven Teflon Potter homogenizer. The supernatant was prepared by centrifuging the oocyte homogenate (10% w/v in phosphate buffer) at $2500 \times g$ for 10 min at 4°C.

Biochemical analysis

Total antioxidant status

The total antioxidant status (TAS) was measured with the radical cation decolorization assay (Re et al. 1999). This assay is based on the inhibition by antioxidants of the absorbance of the free radical cation from ABTS (2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt. In brief, ABTS was dissolved in deionized water to make a 7 mmol L^{-1} concentration solution. $\text{ABTS}^{\circ+}$ was produced by mixing ABTS stock solution with 2.45 mmol L^{-1} potassium persulfate (final concentration), and the mixture was allowed to stand in the dark at room temperature for 12-16 h before use. In our study, the $\text{ABTS}^{\circ+}$ solution was diluted with PBS (pH 7.4) to an absorbance of $0.7 (\pm 0.02)$ at 734 nm. After the addition of 2 mL of diluted $\text{ABTS}^{\circ+}$ to 20 μL of sample in PBS, the absorbance reading was taken exactly 6 min after initial mixing. PBS blanks were run in each assay. The free radical scavenging capacity of the biological sample was expressed as the percentage inhibition of $\text{ABTS}^{\circ+}$.

Catalase activity

Catalase activity was determined using the method by Aebi (1984). This assay involves the change in absorbance at 240 nm from the catalase decomposition of hydrogen peroxide. Catalase activity was calculated using the molar extinction coefficient ($\epsilon=43.6 \text{ l/mol cm}$) and the results are presented in $\mu\text{mol/min/mL}$. Briefly, 20 μL of supernatant was mixed with 1255 μL of phosphate buffer (50 mM, pH 7.0), and the reaction was started by adding 725 μL

of H₂O₂ (54 mM) at 25°C for 1 min. The blanks contained 20 µL of supernatant and 1980 µL of phosphate buffer (50 mM, pH 7.0).

Evaluation of the antioxidant activity using cell-free hemoglobin assay

The antioxidant activity of the intracytoplasmic constituents of the oocytes was estimated using a recently proposed method based on cell-free hemoglobin (Hb) analysis (Bellik and Iguer-Ouada 2015). In this method, isolated Hb is studied under oxidative conditions (H₂O₂) and in the presence of antioxidants (intracytoplasmic constituents of the oocytes). The antioxidative activity is determined directly from the concentration of Hb by measuring its optical density at a specific wavelength of 412 nm. The decrease of absorbance at 412 nm represents the degree of hemoglobin breakdown. Briefly, Hb suspension was incubated with the intracytoplasmic contents of the oocytes (1/1) (v/v) for 30 minutes at 37 °C. After this, the mixture was diluted in a 50-fold volume of distilled water then treated with H₂O₂ (50 mM) for 30 min. The hemoglobin breakdown was recorded after 24 h of incubation at room temperature.

Results and discussion

Biometric indices

All of the female fish sampled were sexually mature. As shown in Tables 1 and 2, the length and weight of the fish from the Soummam River were significantly lower than that of the fish from the Agrioun River. The mean weight and length of the fish collected from the Agrioun River were 378.05 ± 64.02 g and 31.6 ± 1.81 cm, respectively. While the average weight and length of the fish collected from the Soummam River were 261.7 ± 11.85 g and 27.95 ± 0.71 cm, respectively. The mean GSI (%) was 5.68 ± 0.76 with a range of 4.56-6.91 for the fish collected from the Agrioun River and 5.84 ± 0.41 with a range

of 5.18-6.3 for the fish collected from Soummam River (Tables 1 and 2).

Table 1

Values of total length (cm), total weight (g), and gonadosomatic index (%) of *B. callensis* collected from the Agrioun River

Samples	Total length (cm)	Total weight (g)	GSI (%)
1	30	396.9	5.53
2	29.5	302	5.75
3	33	441	4.56
4	34	458,5	5.91
5	32.5	345.7	6.91
6	30.6	324.2	5.47

Table 2

Values of total length (cm), total weight (g), and gonadosomatic index (%) of *B. callensis* collected from the Soummam River

Samples	Total length (cm)	Total weight (g)	GSI (%)
1	27	270	5.77
2	27.5	264.4	6.3
3	29	260	5.72
4	28.5	277	5.18
5	27.7	256	5.8
6	28	242.8	6.27

ABTS radical scavenging capacity

The ABTS decolorization assay is one of the most widely used analytical techniques for evaluating total antioxidant status. It is based on measurements of the disappearance of the free radical (ABTS^{•+}) directly related to the antioxidant capacity of the analyzed sample (Re et al. 1999). Figure 2 illustrates the results of the antiradical activity of the analyzed oocytes expressed as the percentage inhibition of ABTS.

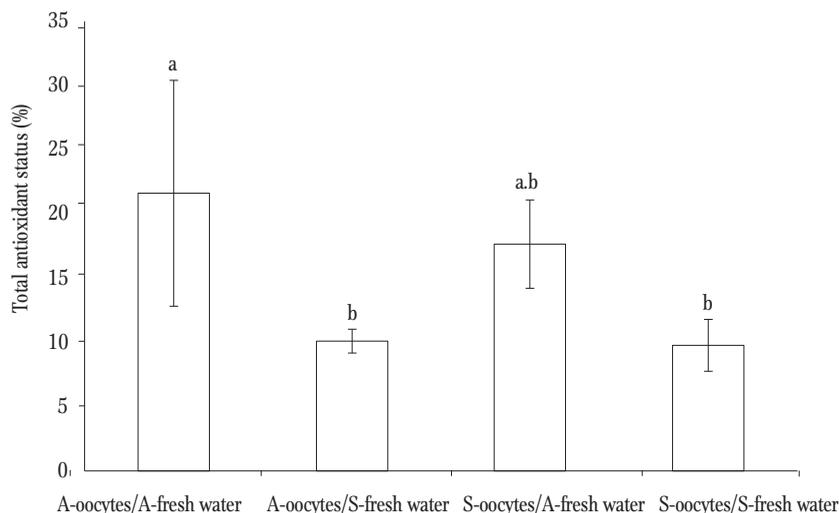


Figure 2. ABTS^{•+} scavenging activity of *B. callensis* oocytes from an unpolluted river activated with unpolluted fresh water (A-oocytes/A-fresh water), oocytes from an unpolluted river activated with polluted fresh water (A-oocytes/S-fresh water), oocytes from a polluted river activated with unpolluted fresh water (S-oocytes/A-fresh water), and oocytes from a polluted river activated with polluted fresh water (S-oocytes/S-fresh water). Values are expressed as mean \pm standard deviation ($n = 12$). Values with different letters are statistically different at $P < 0.05$.

The scavenging activity of oocytes collected from the Agrioun River and activated with unpolluted Agrioun fresh water (A-oocytes/A-fresh water) or polluted Soummam fresh water (A-oocytes/S-fresh water) was 21.64 and 10.14%, respectively. While, the scavenging activity of fish oocytes collected from the Soummam River and activated with unpolluted (S-oocytes/A-fresh water) or polluted fresh water (S-oocytes/S-fresh water) was 17.69 and 9.82%, respectively. It seems clear that the antioxidant activity of fish oocytes collected from the Agrioun River and activated with Agrioun fresh water is significantly higher than that of oocytes collected from the Agrioun River and treated with Soummam fresh water ($P < 0.05$). Likewise, the antioxidant activity of fish oocytes collected from the Soummam River and activated with unpolluted Agrioun fresh water presented important antioxidant activity when compared to the oocytes collected from the Soummam River and treated with polluted Soummam fresh water ($P < 0.05$).

It is well evidenced that cellular enzymic and nonenzymic antioxidants are interrelated systems that interact with each other to control ROS production and maintain physiological concentrations in biological systems (Al-Gubory 2014). Therefore, the evaluation of

total antioxidant power has the advantage of taking into account the interaction of different compounds, and this could be useful in practice since it is difficult to measure individual antioxidants (Niki 2010).

In the present study, the polluted fresh water significantly decreased the total antioxidant activity of the fish oocytes. It has been reported that antioxidant systems can be induced after exposure to pollutants reflecting an adaptation of the species to their environment; however, these systems can also be inhibited (Winston and Di Giulio 1991, Stegeman et al. 1992). The results suggest that toxicity induced by the xenobiotics contained in Soummam fresh water might have had significant negative effects on the antioxidant status of the living organisms. Previous studies demonstrated an up-regulation of ROS production in fish cells after treatment with chemical environmental contaminants (Hegazi et al. 2010, Zhang et al. 2012).

Catalase activity

Catalase is an important enzyme in the antioxidant defense system protecting animals from oxidative stress. The effects of fresh water on catalase activities

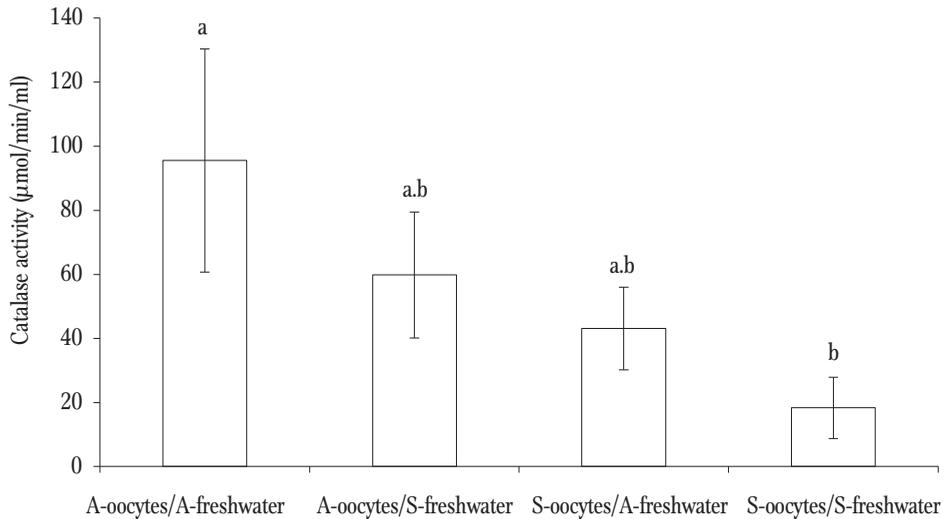


Figure 3. Catalase activity of *B. callensis* oocytes from an unpolluted river activated with unpolluted fresh water (A-oocytes/A-fresh water), oocytes from an unpolluted river activated with polluted fresh water (A-oocytes/S-fresh water), oocytes from a polluted river activated with unpolluted fresh water (S-oocytes/A-fresh water), and oocytes from a polluted river activated with polluted fresh water (S-oocytes/S-fresh water). Values are expressed as mean \pm standard deviation ($n = 12$). Values with different letters are statistically different at $P < 0.05$.

of the studied oocytes are illustrated in Figure 3. Catalase activity was significantly affected by sampling source and the quality of the fresh water activation. Fish oocytes collected from the Agrioun River and activated with its unpolluted fresh water revealed the best catalase activity ($95.56 \mu\text{mol}/\text{min}/\text{ml}$), while, fish oocytes collected from the Soummam River and activated with its polluted fresh water showed the lowest catalase activity ($18.41 \mu\text{mol}/\text{min}/\text{ml}$). Here too, the activation of fish oocytes (collected from the Soummam River) with unpolluted Agrioun fresh water improved catalase activity, whereas, the activation of fish oocytes (collected from the Agrioun River) with polluted Soummam fresh water decreased catalase activity. Accordingly, the level of catalase activity is positively correlated with the level of total antioxidant status (TAS).

The enzyme catalase catalyzes the decomposition of highly toxic hydrogen peroxide into water and molecular oxygen. It is one of the earliest antioxidant enzymes to be induced to protect organisms against ROS produced within cells. In this study, fish oocytes collected from the same area provided differential responses in terms of catalase (according to the source of the fresh water). Catalase activity was significantly

higher in oocytes activated with unpolluted Agrioun fresh water (Fig. 3.)

Many studies have reported that antioxidant enzyme activity can be induced in low concentrations of pollutants and damaged under higher concentrations (Madeira et al. 2013, Rama and Manjabhat 2014, Xie et al. 2015). At higher concentrations, chemicals can directly inhibit the activity of enzymes, or indirectly reduce the concentration of the enzymes by damaging cell organs (Brown et al. 2004, Jemec et al. 2007). The reduction of catalase activity can also result from the accumulation of H_2O_2 and other oxyradicals (Choi et al. 2010). In this study, catalase levels decreased significantly in fish oocytes that had been exposed to polluted fresh water compared to those treated with unpolluted fresh water. This decrease could be an indication that the production rate of hydrogen peroxide in exposed fish far exceeded the scavenging ability of catalase, and, consequently, this resulted in oxidative stress.

Low catalase activities are also reported in *M. cephalus* from a polluted estuary (Ennore Estuary) in India (Padmini et al. 2009) and in *D. labrax* from Aveiro Lagoon in Portugal (Maria et al. 2009). In contrast, previous studies on grey mullet and sardine,

Sardina pilachardus (Walbaum) report higher catalase and superoxide dismutase activities in fish from polluted areas (Rodriguez-Ariza et al. 1993, Peters et al. 1994). Other studies have shown that some metal pollutants result in oxidative damage in aquatic organisms (Pretto et al. 2010). Similarly, Atli et al. (2006) report that catalase activity decreased significantly in *O. niloticus* exposed to various toxic metals. Furthermore, Pruell and Engelhardt (1980) report a Cd-induced decrease in the catalase activity in the mangrove killifish, *Fundulus heteroclitus* (L.).

Antioxidant activity estimated by cell-free hemoglobin analysis

In the last few decades, various methods have been developed and applied in different systems for the assessment of oxidative status (Niki 2010). Giulivi et al. (1994) proposed a method to determine the intracellular rate of Hb autoxidation and the concentrations of H_2O_2 and O_2^- in red cells. It has been demonstrated that, under oxidative stress, Hb is oxidized, resulting in Hb denaturation and precipitation. In addition, hydrogen peroxide leads to an increase in the relative oxidation levels of Hb (Xiang et al. 2013).

Recently, we proposed a new approach, based on cell-free hemoglobin analysis, to evaluate antioxidant activity. It is based on the spectrophotometric measurement of Hb concentration at the specific wavelength of 412 nm. As hemoglobin is biological in origin, this molecule could express accurately antioxidant status, and it provides biologically relevant data. We demonstrated that isolated Hb is degraded under oxidative stress conditions, whereas, when sufficient antioxidants are present, Hb is protected from oxidative damage (Bellik and Iguer-Ouada 2015, Bellik and Iguer-Ouada 2016). In the present study, cell-free hemoglobin was treated with the intracytoplasmic contents of the fish oocytes. The results showed that Hb concentrations varied according to the fresh water activation (Fig. 4). Even if the difference was not statistically significant, the oocytes collected from the polluted Soummam River and activated with its fresh water showed the lowest values of Hb concentrations when compared to fish oocytes collected from the same river but activated with unpolluted Agrioun fresh water (Fig. 4). In fact, Hb was best preserved in oocytes collected from Agrioun River and activated with unpolluted Agrioun fresh water. The significant reduction of Hb concentrations treated with the

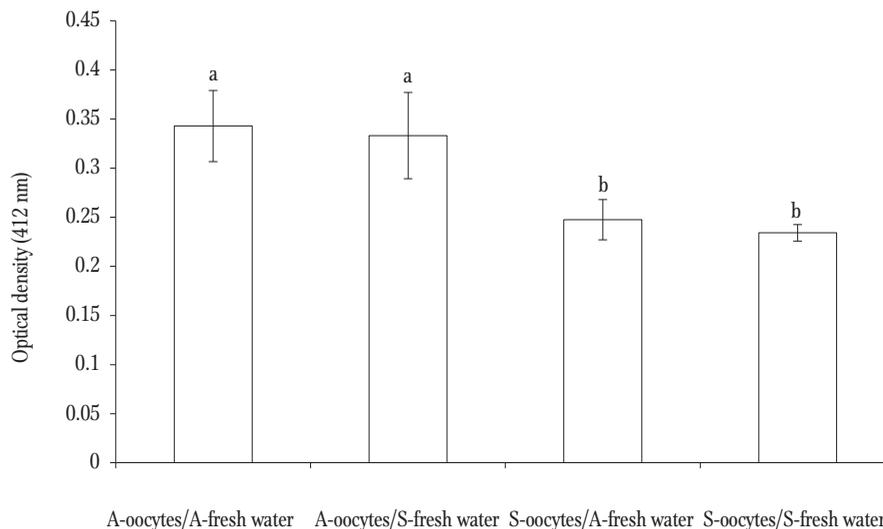


Figure 4. Hemoglobin breakdown exposed to 50 mM H_2O_2 in the presence of intracytoplasmic contents of *B. callensis* oocytes from an unpolluted river activated with unpolluted fresh water (A-oocytes/A-fresh water), oocytes from an unpolluted river activated with polluted fresh water (A-oocytes/S-fresh water), oocytes from a polluted river activated with unpolluted fresh water (S-oocytes/A-fresh water), and oocytes from a polluted river activated with polluted fresh water (S-oocytes/S-fresh water). Values are expressed as mean \pm standard deviation ($n = 12$). Values with different letters are statistically different at $P < 0.05$.

intracytoplasmic contents of polluted oocytes could be explained by the possible failure of the antioxidant systems in scavenging H_2O_2 stemming from low levels of antioxidant capacity and catalase activity.

Summary and conclusions

The present work aimed to investigate the effect of polluted and unpolluted fresh water on the oxidative status of *Barbus callensis* oocytes during the spawning period. The results show that oxidative biomarkers varied according to the quality of fresh-water activation and the sampling site. Total antioxidant capacity, catalase activity, and cell-free hemoglobin concentrations were lower in fish oocytes activated with polluted fresh water. Based on these results, it can be assumed that exposure to environmental pollutants overwhelms oocyte antioxidant protection with subsequent cellular oxidative damage and potential reproduction impairment.

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Author contributions. K.W. carried out the experimental work and wrote the manuscript, Y.B. conducted the experimental work, completed the preparation and correction of the manuscript, and submission procedure, M.I.-O. designed the experimental work and corrected the manuscript.

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