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# Sublethal effect assessment of a low-power and dual-frequency anti-cyanobacterial ultrasound device on the common carp (*Cyprinus carpio*): a field study

Didier Techer <sup>a</sup>, Sylvain Milla <sup>a</sup>, Damien Banas <sup>a</sup>.

**a : Faculté des Sciences et Technologies de Nancy, URAFPA, Boulevard des Aiguillettes, Université de Lorraine, BP 70239, 54506 Vandœuvre-lès-Nancy, Cedex, France**

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## Keywords

Ultrasounds, biomarkers, common carp, pond.

## Abstract

The use of ultrasonication for cyanobacterial control in freshwater bodies has become increasingly popular during the last decades despite controversial efficiency on large scale application. Apart from that, little information is currently available regarding ultrasound toxicity potential towards non-target species. This work was designed to address this issue in the common carp using a low-power (7–9 W output) and dual-frequency (23 and 46 kHz) anti-cyanobacterial ultrasound device. Results showed that carps were unaffected by ultrasound exposure when exposed in floating cages in fish ponds over a 30-day period. The experiment duration was the main factor influencing all measured biological parameters in exposed and non-exposed organisms. Indeed, it was positively associated with an increase in fish condition factor. Cortisol level also tended to slightly increase over the number of days of experiment but its variation did not enable to sort out any ultrasound exposure-related stress. Moreover, an overall diminution along the experimental period of the expression level of a set of biomarkers could be reported, encompassing cellular antioxidant enzyme activities such as superoxide dismutase (SOD), glutathione peroxydase (GPx), catalase and glutathione S-transferase (GST), and lactate dehydrogenase activity. Subtle changes in these biomarkers were dependent of the type of enzyme activity and especially of the origin of fish (i.e., sampled pond) regardless of the presence of ultrasound equipment, reflecting thereby fish adaptation to local environmental conditions in each pond. In conclusion, this study does not provide indication that ultrasonication in the aforementioned conditions affects the welfare and physiological homeostasis of carps.

## Introduction

Cyanobacterial (blue-green algal) blooms frequently occur in many fish ponds, especially in fertilized ones where they can sometimes represent the major part of the total algal biomass (Sevrin-Reyssac and Pletikoscic 1990). Algal blooms usually start in summer and can last until autumn subsequently to the combined effects of water eutrophication and temperature warming as light intensity increases (Sevrin-Reyssac and Pletikoscic 1990). This phenomenon is often accompanied with the production of a wide range of toxins, i.e., cyanotoxins mainly in the form of intracellular organic molecules (Paerl and Tucker 1995; Sevrin-Reyssac and Pletikoscic 1990). These cyanotoxins maybe released in large quantities into surrounding water after cell lysis and pose a significant threat to the environment and human health (Paerl and Tucker 1995; Sevrin-Reyssac and Pletikoscic 1990). Various strategies for the control of harmful algal blooms including the

use of biological methods (e.g., biomanipulation, allelopathic treatments), engineering processes (e.g., sediment dredging, water mixing), and physico-chemical treatments (e.g., ultrasonic radiation, copper-based algacide application) have been proposed over the last decades (Ahn et al. 2003; Barrington et al. 2013; Jančula and Maršálek 2011). Recently, several laboratory-scale experiments have suggested biocidal ultrasound effects against cyanobacteria based on (i) the collapse (or implosion) of gas vesicles/vacuoles following cavitation phenomena, (ii) the occurrence of detrimental free radical reactions, (iii) the increase in photosynthetic pigment damages (particularly phycocyanin) with subsequent disruption of the photosynthetic activity, and finally the effective cyanobacterial growth inhibition (Rajasekhar et al. 2012). Consequently, numerous patents have been deposited worldwide and concomitant ultrasound devices

(some still in development) claiming to inhibit microalgae growth have been successively commercialized (Rajasekhar et al. 2012). Marketed products usually cover a wide range of acoustic power as well as ultrasound frequencies varying from 20 kHz to 1.7 MHz (Rajasekhar et al. 2012). Nevertheless, it is important to point out the inconsistencies in some studies regarding the ultrasound treatment efficiency against cyanobacterial blooms (Lüring and Tolman 2014). As a matter of fact, the lack of the system functioning details (usually protected by patents), the deficiency of a clear consensus for data format reporting often limit critical reviewing and comparisons between published results. Yet, it is usually assumed that longer exposure times to ultrasounds would result in significant cellular damages leading to cyanobacterial growth inhibition, which maybe partly due to the narrow area effectively covered by ultrasonication processes (Rajasekhar et al. 2012). Regardless the uncertainties surrounding the efficacy of the currently available anti-cyanobacterial devices in situ, the large scope of application for ultrasound-based water treatment technologies, that encompasses microbial control in water treatment plants, as well as in aquaculture facilities, reservoirs, and ornamental water bodies, requires a better knowledge of their potential (eco) toxicological impacts on non-target aquatic species such as fish (Colucci 2010; Doosti et al. 2012; Drabkova and 2007). Indeed, fish are the key component organisms of surface water ecosystems that maybe directly exposed to ultrasounds during sonication

treatments. Recently, ultrasonic hearing has been reported for a number of fish species belonging to the widespread clupeidae family (Astrup 1999; Popper et al. 2004). Moreover, fish exhibit physiological antioxidant system potentially sensitive to wave radiation and free radical reactions (Milowska et al. 2005).

This work primary aims to investigate the potential sublethal effects on the fish physiology of a long-term exposure to ultrasounds using the common carp fish species (*Cyprinus carpio*) as a model. This fish species is widespread in eutrophic lakes and reservoirs in Europe and Asia and is often found in most aquaculture ponds (Jeney and Jian 2009). Juvenile carps were chronically exposed to a low-power electric ultrasound device continuously emitting a dual-frequency of 23 and 46 kHz. After respectively 7 and 30 days of ultrasonication exposition, fish were sacrificed, condition factor indices were determined, and a panel of biochemical markers linked to fish physiological homeostasis were assessed, encompassing: (i) hepatic antioxidant enzyme biomarkers, i.e., total superoxide dismutase (total SOD), catalase (CAT), total glutathione peroxidase (total GPx), and glutathione S-transferase (GST) activities, (ii) lactate dehydrogenase activity related to cellular energetic metabolism, and (iii) circulating cortisol levels subsequent to stress.

## Material and Methods

### 2.1. Ethical statement

All applied treatments and procedures on fish were in compliance with the general guidelines of the Council of European Communities (European Union, No 2010/63/CEE) as well as the French Animal Care Guidelines

### 2.2. Ultrasound exposure system

The used ultrasound system consisted on a dual-frequency ultrasound device emitting continuous signals and powered by a solar floating platform. There were two transducers operating independently. They were immersed at a depth of around 0.5 m with one emitting at an average frequency of 23 kHz and the other at 46 kHz. The two frequencies are simultaneously transmitted using the two transducers mounted adjacent one another, with a distance of 10 cm between them. The supplied power to the transducers was initially fixed at 15 W. According to the manufacturer (SOFCHEM; TWIN-f® derived ultrasonic system), this apparatus has been especially developed for algae growth inhibition. Other technical specificities are proprietary information but the reader can refer to a related online technical documentation for more information, [http://www.sofchem.fr/antialgues\\_fluid\\_impact.htm](http://www.sofchem.fr/antialgues_fluid_impact.htm).

Moreover, details about the transmitters' directivity

(unidirectional), their emission level at 23 and 46 kHz, as well as the characterization of the theoretical signal resulting from the combination of [23/46 kHz] (as measured by SinapTec Ultrasonic Technology for SOFCHEM in an 8 m × 6 m × 6.5 m fresh water basin) are illustrated in Fig. 1. The

measurement of the resulting emitted acoustic signals in water was carried out using a Brüel & Kjær Type 8103 hydrophone. This kind of calibrated waterborne-sound transducers are omnidirectional over a wide frequency range from 0.1 Hz to 180 kHz with a receiving sensitivity of -211 dB re 1 V/μPa. They are generally used in a laboratory or industrial context, and they are known to be particularly suitable for acoustic investigations of marine mammals and cavitation study (more technical specificity available online at <https://www.bksv.com/en/products/transducers/acoustic/microphones/hydrophones/8103>).

A completed description of the receiving chain used for acoustic signal measurements with the Brüel & Kjær Type 8103 hydrophone is given in Fig. 2. Other technical specifications about the different electronic devices composing the receiving chain were as follows: converter [Brüel & Kjaer 2647 CCLD 1Mv PA Pc]; conditioner [1704-A-001 amplifier with acoustic filter mode CCLD ON X1]; multimeter [Fluke 287 true RMS]; and oscilloscope [Pico 3203D]. The oscilloscope was used on site whereas the BLogging Multimeter<sup>^</sup> was used in the laboratory. The sound pressure level (SPL in dB re 1 μPa) measured by the hydrophone could be determined from a knowledge of the hydrophone free field sensitivity, the gain in the measurement system, and the amount of voltage measured according to the equation (Au and Hastings 2008):

$$\text{SPL} = |Mh| - G + 20 \log (V)$$

where Mh is the free field voltage sensitivity of the hydrophone in dB re 1 V/μPa, considering that the hydrophone is

connected to an amplifier of gain  $G$  in dB, and  $V$  is the voltage read off the oscilloscope (or multimeter).

$$K = \left[ \left( \text{weight (g)} / \text{length (cm)}^3 \right) \times 100 \right]$$

### 2.3. Fish care and experimental design

Two-year-old common carps were procured from the fish farm-ing pond situated near the outdoor INRA facility of La Bouzule farm (Laneuvelotte, Meurthe-et-Moselle, France). Batches of no more than 50 fish were kept for a four-week acclimation-observation period into aquaculture polyester tanks previously filled with 2.8 m<sup>3</sup> of continuously aerated tap water. Considering that neither mortality nor abnormal behavior were reported through this period, fish were then transferred in experimental ponds for further ultrasound exposure study. Experimental ponds consisted on five formerly gravel pits currently used as fish ponds. Fish in two ponds were used as controls (without any sonication treatment), and in the three others were exposed to ultrasounds. The five ponds displayed quite similar physicochemical parameters in the range of pH [8.2–9.6], conductivity [177–480  $\mu\text{S cm}^{-1}$ ],  $\text{NO}_3^-$  [0–0.02 mg L<sup>-1</sup>],  $\text{PO}_4^{3-}$  [0–2.64 mg L<sup>-1</sup>],  $\text{SO}_4^{2-}$  [13.1– 39.9 mg L<sup>-1</sup>],  $\text{Ca}^{2+}$  [17.1–61.7 mg L<sup>-1</sup>],  $\text{Mg}^{2+}$  [5.7–9.9 mg L<sup>-1</sup>],  $\text{Na}^+$  [7.6–28.9 mg L<sup>-1</sup>],  $\text{K}^+$  [1.3–6.2 mg L<sup>-1</sup>]. In each pond, three 1 m<sup>3</sup> floating cages (1 m  $\times$  1 m  $\times$  1 m) with a flexible nylon netting (1 cm  $\times$  1 cm meshes) were assembled. For ponds concerned by ultrasonication, three cages were respectively at 1, 10, and 30 m away from the ultrasound devices. Three cages were also introduced into the control ponds, and they were spaced by the same distances as those fixed for ultrasound treated ponds, i.e., 9 m between the first and the second cage and 20 m between the second and the third cage. Twelve fishes were randomly distributed into each cage of the different ponds. They were allowed for an additional acclimation period of 13 days in each of these experimental ponds after which ultrasound exposure started. During the whole acclimation and exposure period, fish were daily fed at 2% of the total biomass at 8 A.M. using an automatic fish feeder (with commercial food pellet,  $\varnothing$  0.5 cm, Carpe extrudée-coul 3, Le Gouessant). Following the acclimation period, four fishes from each cage and at each distance of respectively 1, 10, and 30 m were sampled, corresponding to the "T0" sampling date. The same procedure was followed after 7 days (T+7) and 30 days (T+30) of ultrasound exposure; fish were also sampled accordingly in control ponds.

### 2.4. Fish sampling, morphometric measurements, and blood collection

At each sampling date, four fishes were randomly collected per cage, i.e.,  $n = 4$  fish per distance per pond. They were immediately anesthetized (on the inflatable boat used to reach each corresponding cage on the ponds) by immersion into tricaine methanesulfonate solution (MS-222, 0.3 g/L) buffered with 0.6 g/L  $\text{NaHCO}_3$  (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Length (cm) and weight (g) were determined in order to calculate condition factor indices according to Fulton's formula (Nash et al. 2006):

Blood was collected in a maximum delay of 3 minutes following fish net collection and stored on ice in heparinised microtubes before centrifugation (1700 g for 25 min) at the laboratory for plasma recovery. Aliquots of plasma were then stored at  $-80^\circ\text{C}$  until further measurement of cortisol levels by ELISA. Following blood sampling, fish were killed by a blow to the head prior to ventral dissection using a scalpel. Then, liver fragments ( $\sim 200$  mg) were collected, snap-frozen into liquid nitrogen on boat and stored at  $-80^\circ\text{C}$  at the laboratory until further processing.

### 2.5. Cortisol level measurement

Measurements of circulating cortisol levels were achieved directly onto plasma samples in coating buffers according to the manufacturer's instructions (DIASource). All measurements from each fish were assayed using commercial ELISA kits (Cortisol ELISA KAPDB270, DIASource), and results were calculated from means of duplicates for each sample

### 2.6. Tissue processing and biomarker determination

In order to investigate more thoroughly potential adverse welfare effect following fish exposure to ultrasounds, the assessment of LDH, total GPx, CAT, total SOD, and GST activities were conducted as current enzymatic biomarkers on fish livers collected from ultrasound treated and control ponds. All chemicals required for liver homogenization and enzymatic activity determination were procured from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and were of analytical grade. Liver homogenates were obtained after bead-beating homogenization (zirconium oxide beads equal to 2 $\times$  the mass of sample) in 2 mL screw-cap microcentrifuge tubes using a Bullet Blender (Hoyt 2009) in five volumes of 100 mM Tris buffer (with 1 mM EDTA, 150 mM KCl, pH 7.8, 4  $^\circ\text{C}$ ). The homogenates were centrifuged 10,000 $\times$  g, 15 min at 4  $^\circ\text{C}$ , and the resulting supernatants (S9 fractions) were used for further protein content and enzyme activity determination in microtiter plate format with a Labsystem Multiskan EX microplate reader (Techer et al. 2015). Protein content was determined on appropriate S9 fraction dilutions according to the method of (Smith et al. 1985) (Thermo Scientific Pierce bicinchoninic acid (BCA) Protein Assay kit, No. 23225) with bovine serum albumin as the standard. Absorbances were read at 595 nm after an incubation period of 0 min at 37  $^\circ\text{C}$ . Total SOD activity (EC 1.15.1.1) was measured by using Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) coupled with the xanthine oxidase activity (Sigma-Aldrich 19160 SOD determination kit) (Peskin and Winterbourn 2000). One unit of SOD was defined as the amount of enzyme required for inhibiting WST-1 oxidation by 50% per minute. CAT assay (EC 1.11.1.6) was performed in microtiter plates using with 10 mM hydrogen peroxide as substrate (Techer et al. 2015). One unit of CAT activity corresponded to the amount of  $\text{H}_2\text{O}_2$  (in  $\mu\text{moles}$ ) decomposed per minute (with  $\epsilon_{240} = 0.0394 \text{ cm}^2 \mu\text{mole}^{-1}$ ). Total GPx activity (EC 1.11.1.9) was measured by the indirect method of (Drotar et al. 1985) using cumene

hydroperoxide as substrate. One unit of GPx activity was defined as the amount of enzyme catalyzing the oxidation of 1 nmol of NADPH per minute. LDH activity (EC 1.1.1.27) was determined according to the protocol of (Jesus et al. 2013) with slight modifications as described in (Techer et al. 2015). One unit corresponded to the amount of enzyme hydrolyzing one nanomole of NADH substrate per minute. The activity of GST (EC 2.5.1.18) was measured following the method of (Habig and Jakoby 1981) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. One unit of GST activity was defined as the amount of enzyme required to conjugate 1 nmol of CDNB per minute. All enzyme activity determinations were performed at 25 °C, and the wavelengths used for total GPx, GST, and LDH assays were 340 nm, whereas for SOD and CAT it was respectively fixed at 450 and 240 nm. For a defined sample, each enzyme activity was determined in duplicate from two different aliquots of the same liver homogenate sample, and finally expressed as U/mg protein. The mean obtained from these measurements were then used for further calculations.

## 2.7. Data treatment and statistical analysis

### Results and Discussion

According to the literature, the use of ultrasonication for water treatment purposes presents the advantage of the mechanical effects (due to vibrational resonance), leading to the cavitation and to cellular membrane dislocation with biochemical alterations such as oxidative damages that can inhibit cyanobacterial growth (Rajasekhar et al. 2012; Shankar and Pagel 2016). To our knowledge, the present study is the first one addressing the issue of non-specific adverse health effects of an ultrasound device by measuring after long-term exposure to ultrasounds physiological indicators of stress (including condition factor and circulating cortisol level) as well as specific cellular biomarkers (namely, enzyme activity modulation) in a commonly farmed fish species.

### 3.1. In situ measurement of acoustic signals in ultrasound treated ponds

Data recovery from ultrasound devices at the end of experiments revealed that ultrasonication had actually occurred for an average of 22.5 h per day (continuously transmitted signals) in the exposed ponds owing to the specially engineered floating solar power-driven platforms. With a nominal power fixed at 15 W but reaching an effective level of 11 to 12 W in situ (depending on solar illumination), an average output power of 7 to 9 W (which was the effective electrical power provided to the transmitting transducers) could be recorded according to the on-board driver cards connected to both transducers. This power was sufficient for ultrasonication occurring at both frequencies of 23 and 46 kHz on average respectively in each pond. As reported in Table 1, peak to peak voltage dropped from  $145 \pm 18$  mV at a distance of 1 m in front of the transducers to about  $62 \pm 30$  mV at 10 m away from the emission source. Measured voltage kept on decreasing according to the distance from transducers, leading to a lowest value of  $18 \pm 9$  mV at 30 m (Table 1). Thus, depending on the distance from the ultrasound transmitters (Table 1), the observed decrease of the acoustic signal level was not always consistent with a spherical divergence law—i.e., the signal amplitude should have been

All data were expressed as mean  $\pm$  SE. Box-Cox power transformation was applied, and the assumption of normality and homogeneity of variance were checked using the Shapiro-Wilk test and the Browne-Forsythe test, respectively. Then, data from fish sampled from control and ultrasound-exposed ponds regarding the effects of the exposure period (0, 7, and 30 days of fish exposure in ponds), the distance (1, 10, and 30 m from ultrasound source), the ultrasound exposure scenario (presence/absence of ultrasonication), and the inherent environmental variability of ponds (ponds considered as a nested factor within the Ultrasound exposure^ factor) were analyzed using hierarchical (nested) ANOVA, followed by the Tukey multiple comparison procedure. Data were finally grouped according to the significance of factors as determined by nested ANOVA for a better clarity on histogram representation. All statistical analyses were carried out at an initial level of significance of  $p < 0.05$ , using Statistica v.10 software.

in first approximation always inversely proportional to the range to the source. Indeed, the decrease in peak to peak voltage between 10 and 30 m was observed with a ratio of 3 on amplitude (Table 1). Taking this into account, one should have expected values around 600 mV at 1 m (with a corresponding predicted SPL value of 215 dB, according to the equation from (Au and Hastings 2008)), whereas only  $145 \pm 18$  mV (and  $\sim 187$  dB SPL, Table 1) were reported at this distance from the ultrasound transducers (Table 1). A hypothesis maybe that the measurements were done outside the main radiation lobe: this risk decreases with range (for obvious geometrical reasons); hence, it leads to a better measurement accuracy at 10 and 30 m of distance from the emission source. Data transformation in terms of sound pressure levels also reflected a significant loss of acoustic pressure level along the distance gradient, with recorded values varying from 187.7 (highest measured value at 1 m) to 152.6 dB SPL (lowest measured value at 30 m) (Table 1). According to the literature, pressure level in the range of 180 dB re 1  $\mu$ Pa would not constitute a noticeable underwater noise pollution^ (Lurton and Antoine 2007). The observed diminution in peak to peak voltage as measured with the hydrophone may be largely due to the arrangement of the ultrasound devices in water and to the pond configuration. Indeed, such decreases may be explained by common physical phenomena which are (1) the divergence of the wave surface when propagation range increases, and (2) the absorption of sound waves inside the surrounding medium (visco-thermo-chemical effect). Regardless the diminution in sound pressure level, in the framework of an ecological risk assessment any potential disturbance in normal fish physiology has to be sorted out prior to any large scale use of such an ultrasound technology

### 3.2. Fish condition factor and cortisol levels

Fish are well-known to display a broad range of physiological, behavioral as well as biochemical responses and adaptation following repeated and long-term exposure to environmental stressors (Barton 2002; Reinert 1992). In a previous laboratory experiment, Zimba and Grimm (2008) reported

that channel catfish fingerlings exposure to continuously emitting ultrasound sources drastically altered their feeding behavior (without mentioning sonication details). Nevertheless, this phenomenon was reversed when ultrasound equipment was turned off, thereby suggesting a reversible stressful effect of ultrasonication in a natural setting. Thus, as a starting point for a whole-body stress response investigation, condition factor was determined for all ultrasound exposed and non-exposed organisms at each sampling date (Fig. 3). Results indicated that the exposure period was the only factor affecting fish growth and global health condition (Table 2). As illustrated in Fig. 3, longer exposure period was associated with higher fish condition indices regardless treatment modality (i.e., ultrasound treated ponds vs non-treated ones), leading to the assumption that carps were not deterred at all from feeding with the designed ultrasound devices. Moreover, concern variations of circulating cortisol levels (Table 2, Fig. 4), these latter were mainly affected by: (i) exposure period (Table 2) with an overall higher cortisol production concomitant to a longer fish rearing period into cages (Fig. 4a), and (ii) distance (Table 2) with more elevated plasma hormone concentrations in fish sampled in the first cage series (at 1 m from ultrasound transducers) followed by those sampled at 10 m away and finally at 30 m away regardless the presence of an ultrasound device (Table 2; Fig. 4a, a'). The analyses also indicated slight variations depending on the studied pond (Table 2) but multiple comparison procedure did not enable to sort out any significant difference among treated and non-treated ponds. Since carp biomass was kept on increasing along the experimental period in all ponds (as reflected by enhanced condition factors during the exposure period), and taking into account that fish biomass per cage was well below the recommended maximum density of 150 kg m<sup>-3</sup> for this species (Schmittou 1991), these observations would indicate that cortisol levels were altered by rearing conditions *stricto sensu*, notably by the confinement into the cages, rather than by environmental disturbances inherent to pond variability. As common carps are forage fish species, one may hypothesize that prolonged caging (up to 30 days in floating cages) would have been sufficient to stimulate stress-related hormone production in our experimental conditions. Indeed, cortisol is a major stressed-produced hormone invertebrates, and it is usually released proportionally to the magnitude and duration of the stressor (Barton 2002; Reinert 1992). However, it has to be pointed out that increases in plasma cortisol levels are an adaptive physiological response which is not systematically detrimental for the whole-animal performance and maintenance of the homeostatic mechanisms at long-term (Barton 2002), and this has been herein illustrated by better carp growth condition indices (Fig. 3). Moreover, the observation that overall cortisol levels were quite lower in fish sampled at 30 m could be associated with the fact that these individuals were usually the last ones being sampled. By this way and because plasma cortisol level can occur rapidly, i.e., in delay of 3 min (Nematollahi et al. 2009), one may easily hypothesize that fish from a longer distance of 30 m would have been already adapted to the stress consecutive to boat navigation onto water surface and sampling, leading to a readjustment of cortisol concentrations to lower basal values for these distances (Fig. 4)

### 3.3. Enzymatic biomarker evaluation

In order to clear out more thoroughly potential sublethal effects following long-term ultrasound exposure, enzymatic biomarkers related to oxidative stress regulation (SOD, GPx, CAT) as well as biotransformation (GST) and cellular energetic metabolism (LDH) were measured on liver homogenates of both control and exposed fish (Fig. 5). The use of such a panel of cellular biomarkers is especially convenient in order to detect early organism alteration before it affects higher levels of biological organization with potentially irreversible ecological consequences (Cossu-Leguille and Vasseur 2013; van der Oost et al. 2003). Moreover, assessing the evolution of biomarker variation over time allows for a better understanding of the way that exposed fish may respond and adapt to the environmental stressors (Cossu-Leguille and Vasseur 2013; van der Oost et al. 2003). As illustrated in Fig. 5a, b, c, d, e, the main factor influencing biomarker changes was the exposure period (Table 2: nested ANOVA,  $p < 0.001$  for SOD, GPx, CAT, GST, and LDH activities). Overall, enzymatic activities tended to decrease overtime and to stabilize at the end of experiments around an average value dependent of the type of enzyme assessed, regardless the application of an ultrasonication treatment (Fig. 5a, b, c, d, e and Table 2: nested ANOVA). The absence of any ultrasound effect was reinforced by the fact that the  $B_{distance}^{\wedge}$  factor (especially from ultrasound treated ponds) did not influence at all enzymatic activities variation (Table 2: nested ANOVA). Fish were affected to a lesser extent by the origin of the experimental pond itself (Table 2: nested ANOVA), meaning that the initial environmental variability occurring at each pond was the second main factor governing biomarker evolution over time. Moreover, as depicted in Fig. 5a', b', c', d', e' following the multiple post-hoc test procedures, no specific trend could be reported when comparing fish enzymatic activities from different ponds. In other words, the results dealing with fish from control and/or exposed ponds could significantly differ from each other depending on the measured enzymatic activity which could lead to a completely dissimilar kinetics of change when focusing the attention on one category of enzyme to another (Fig. 5a', b', c', d', e'). SOD, GPx, CAT, and GST are four major enzymes of the fish's antioxidant defense system which can give early warning on sublethal effects involving reactive oxygen species (ROS) formation (Cossu-Leguille and Vasseur 2013; Hermes-Lima 2005). LDH has been also shown to be useful as an indicative effect criterion in aquatic toxicology (Diamantino et al. 2001). Thus, these five categories of enzymes were chosen as biomarker of effects so as to give early warning about putative cell damages following ultrasound exposure. Indeed, ultrasounds are likely to produce thermal and mechanical effects, particularly in the focal area of the beam waves where they can lead to acoustic cavitation (Shankar and Pagel 2016). This latter may be roughly defined as the interaction of ultrasounds with gas bubbles which can cause rapid and important changes in bubble size, and is often accompanied with an increased local temperature (up to 1000 °C) as well as ROS formation (Rajasekhar et al. 2012; Shankar and Pagel 2016). Cavitation is favored at a long wavelength, i.e., at a low ultrasound frequency (Rajasekhar et al. 2012; Shankar and Pagel 2016). Within this context, our results would indicate that carps were preserved from oxidative stress, and most probably from any adverse health effect resulting from mechanical ultrasound processes utilizing the low-power dual frequency ultrasound equipment.

## Conclusion

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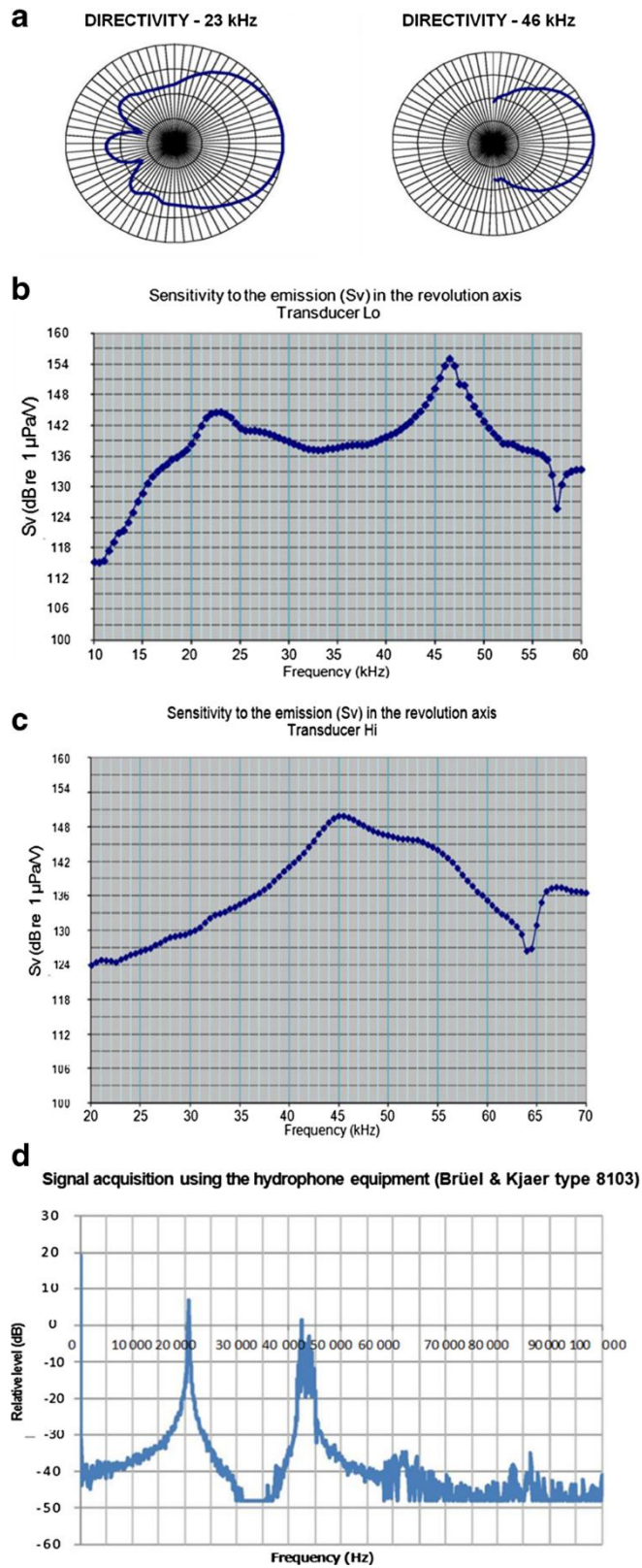
In this study, ultrasonic acoustic signal measurements suggested a rapid decrease of acoustic power in such an open environment like aquaculture pond. Moreover, results did not reveal any adverse health effect of concomitant fish ultrasound exposure to both 23 and 46 kHz frequencies over a 30-day period. Indeed, the determination of condition factor variations suggested a constant fish growth all over the experimental period. Besides, cortisol levels increases were affected by the duration of the experiment and distance between cages, which may be attributed to fish rearing and sampling conditions. The assessment of specific biochemical

markers of cellular effects in the form of antioxidant enzymes and lactate dehydrogenase activity showed a global decrease of enzyme activities over time, with slight and local variations depending on the considered pond, thus suggesting a fish adaptation to different environmental conditions in each pond regardless the exposure to ultrasounds. Further assessment of the sublethal effects of ultrasonication under various cyanobacterial bloom conditions (with potential cyanotoxin releases) would be required to completely warranty the eco-friendly properties of such a technology.

**Table 1. Acoustic signal level measurements in ultrasound treated ponds as a function of distance from transducers. Data are expressed as mean  $\pm$  SE for peak to peak voltage (in mV; read from a digital multimeter coupled to a hydrophone) whereas ranges of sound pressure level (SPL) are reported in decibel (dB SPL)**

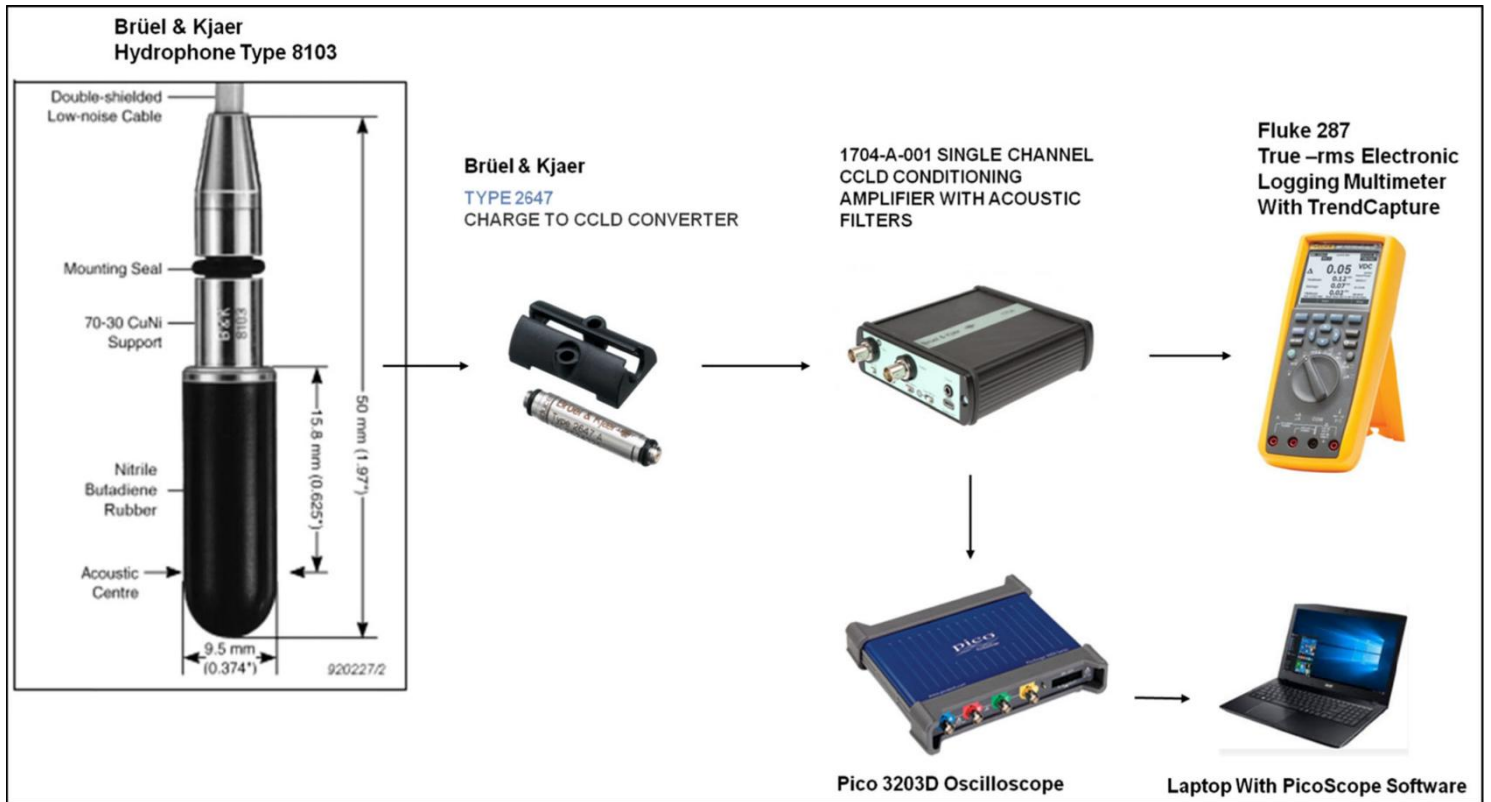
	Distance from ultrasound source (m)		
	1	10	30
Peak to peak voltage	145 $\pm$ 18	62 $\pm$ 30	18 $\pm$ 9
Sound pressure level range (dB SPL)	187.4–187.7	158.6–185.3	152.6–175.1





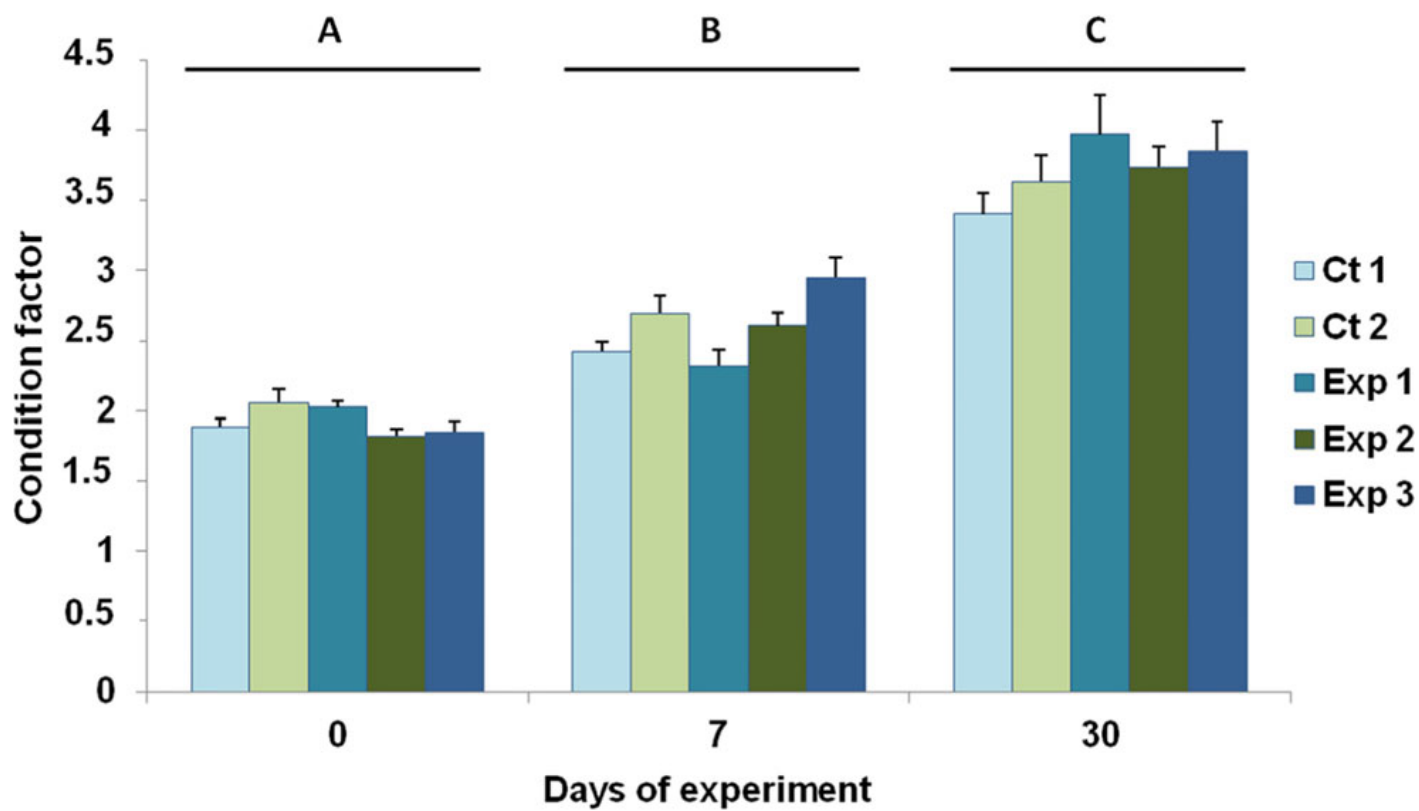
**Figure 1. Characterization of the ultrasonic sensor emission, as provided by SOFCHEM:**

a Directivity. b Sensitivity to the emission (transducer Lo). c Sensitivity to the emission (transducer Hi). d Signal acquisition using the hydrophone equipment (Brüel & Kjaer type 8103)



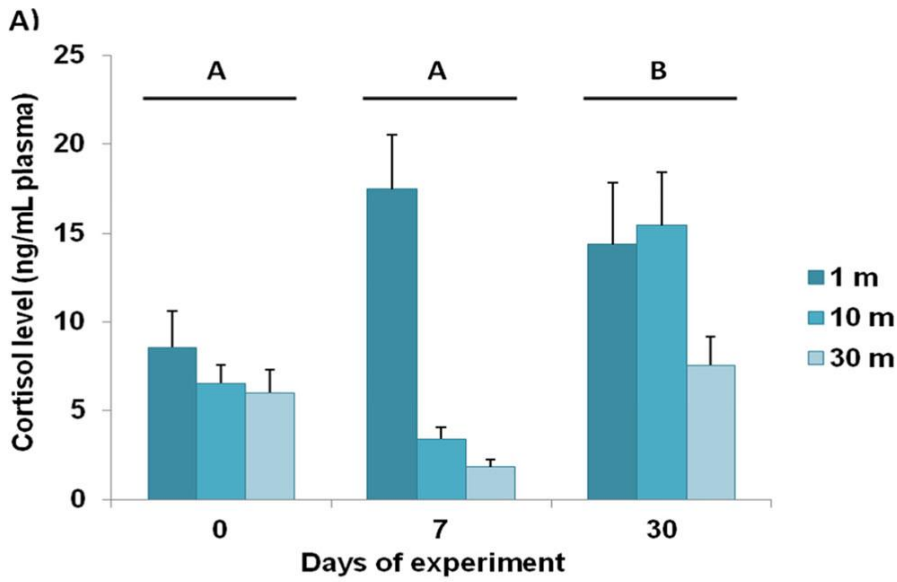
**Figure 1. Description of the receiving chain for acoustic signal measurement using the hydrophone equipment “Brüel & Kjaer type 8103”**

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**Figure 3. Condition factor assessment in fish at T<sub>0</sub>, T<sub>+7</sub>, and T<sub>+30</sub> days of experiment in control (Ct) and ultrasound-exposed (Exp) ponds.**

Ct 1: control pond 1; Ct 2: control pond 2; Exp 1: exposed pond 1; Exp 2: exposed pond 2; Exp 3: exposed pond 3. Data are expressed as mean ± SE. Differences between groups are indicated by different capital letters ( $p < 0.05$ )

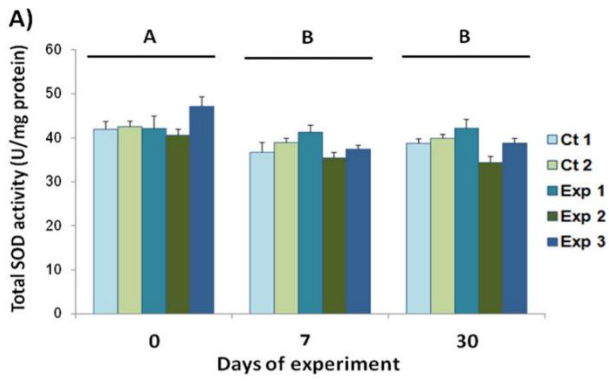


A')

	1 m	10 m	30 m
1 m		**	***
10 m	**		*
30 m	***	*	

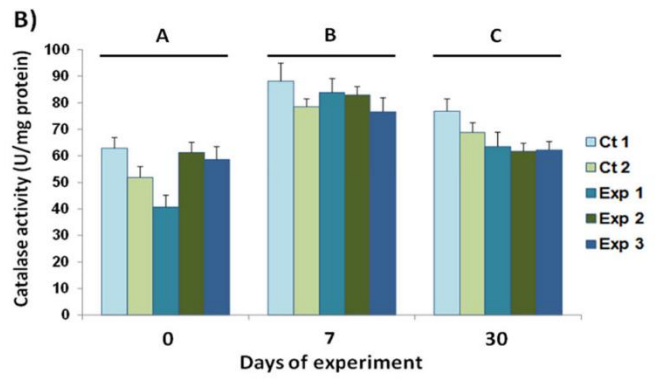
**Figure 4. Plasma cortisol levels in fish at T<sub>0</sub>, T<sub>+7</sub>, and T<sub>+30</sub> days of experiment according to exposure distance (1, 10, 30 m).**

Data are expressed as mean ± SE. Differences between groups are indicated by different capital letters ( $p < 0.05$ ). (A') Results from Tukey test indicating the overall significant differences between fish, all grouped according to the distance;  $p < 0.05$  (\*),  $p < 0.01$  (\*\*), and  $p < 0.001$  (\*\*\*)



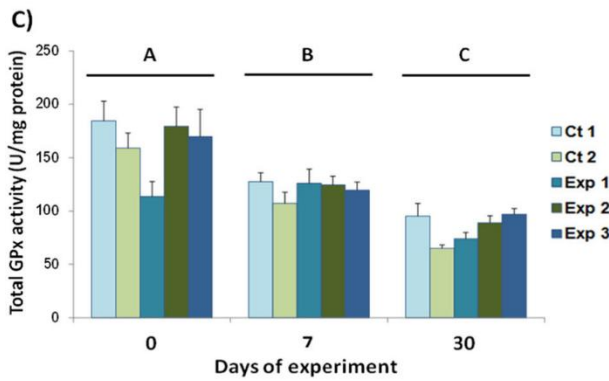
A')

	Ct 1	Ct 2	Exp 1	Exp 2	Exp 3
Ct 1		n.s	n.s	n.s	n.s
Ct 2	n.s		n.s	n.s	n.s
Exp 1	n.s	n.s		**	n.s
Exp 2	n.s	n.s	**		*
Exp 3	n.s	n.s	n.s	*	



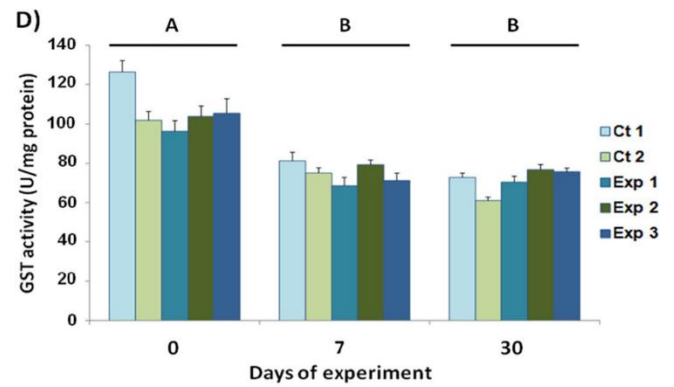
B')

	Ct 1	Ct 2	Exp 1	Exp 2	Exp 3
Ct 1		n.s	**	n.s	n.s
Ct 2	n.s		n.s	n.s	n.s
Exp 1	**	n.s			n.s
Exp 2	n.s	n.s	n.s		n.s
Exp 3	n.s	n.s	n.s	n.s	



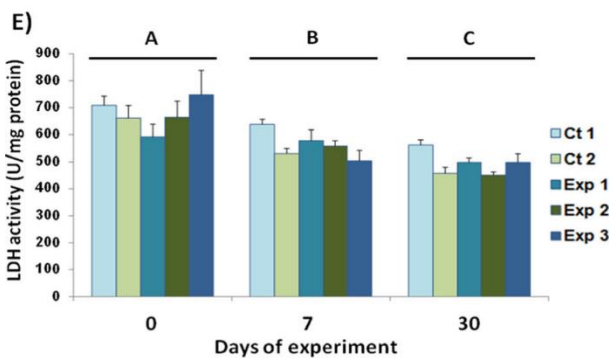
C')

	Ct 1	Ct 2	Exp 1	Exp 2	Exp 3
Ct 1		n.s	*	n.s	n.s
Ct 2	n.s		n.s	n.s	n.s
Exp 1	*	n.s		*	n.s
Exp 2	n.s	n.s	*		n.s
Exp 3	n.s	n.s	n.s	n.s	



D')

	Ct 1	Ct 2	Exp 1	Exp 2	Exp 3
Ct 1		**	***	n.s	n.s
Ct 2	**		n.s	n.s	n.s
Exp 1	***	n.s		*	n.s
Exp 2	n.s	n.s	*		n.s
Exp 3	n.s	n.s	n.s	n.s	



E')

	Ct 1	Ct 2	Exp 1	Exp 2	Exp 3
Ct 1		*	*	*	*
Ct 2	*		n.s	n.s	n.s
Exp 1	*	n.s		n.s	n.s
Exp 2	*	n.s	n.s		n.s
Exp 3	*	n.s	n.s	n.s	

**Figure 5. A, B, C, D, E: enzymatic activity assessment in fish livers at T<sub>0</sub>, T<sub>+7</sub>, and T<sub>+30</sub> days of experiment in control (Ct) and ultrasound-exposed (Exp) ponds.**

A SOD activity, B CAT activity, C GPx activity, D GST activity, E LDH activity. Ct 1 control pond 1, Ct 2 control pond 2, Exp 1 exposed pond 1, Exp 2 exposed pond 2, Exp 3 exposed pond 3. Data are expressed as mean  $\pm$  SE. Differences between groups are indicated by different capital letters ( $p < 0.05$ ). A', B', C', D', E': results from the Tukey test indicating the overall significant differences between fish, all grouped according to the experimental pond; Ct 1 control pond 1, Ct 2 control pond 2, Exp 1 exposed pond 1, Exp 2 exposed pond 2, Exp 3 exposed pond 3; levels of significance:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*), and  $p < 0.001$  (\*\*\*)

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