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# ***Contrasted effects of an anti-cyanobacterial ultrasound device on the non-target freshwater invertebrate species Gammarus roeseli***

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## **Keywords**

Ultrasound, blooms, water treatment, environmental remediation, *Gammarus*, biomarkers.

## **Abstract**

The aim of this work was to investigate the effects of an anti-cyanobacterial ultrasound device (supplied by an electrical power of 15 W and emitting at 23 and 46 kHz) on the widespread freshwater amphipod species *Gammarus roeseli*. First, laboratory scale experiments in 8-L glass tanks showed that an ultrasound exposure of 2 h and 40 min was sufficient to produce 50% mortality, along with a 6.5 °C water temperature increase. Avoiding excessive heating by using a water-cooling and recirculation system permitted an exposure time of 29 h for the same mortality rate. A potential relationship between temperature's rise and amphipod mortality was hence highlighted. Moreover, the use of plastic mesh bag (0.5 mm mesh size) as a physical barrier has not shown any lethal effects of ultrasound exposure. Furthermore, the induction of GPx or GST activity as oxidative stress biomarkers was not observed. This could be explained by reduced ultrasound intensity inside the mesh bags. Thus, according to these results, the tested ultrasound system is not expected to be acutely harmful in the field.

## **Introduction**

In the context of the global warming, cyanobacterial blooms are expected to occur on a more frequent basis in eutrophicated freshwater ecosystems (O'Neil et al. 2012; Paerl and Huisman 2008). In order to limit excessive microalgal growth and especially harmful algal blooms, a number of so-called "environmentally friendly" physico-chemical methods, including ultrasonication, have been developed over the last decades (Jančula and Maršálek 2011; Rajasekhar et al. 2012). The basic principles explaining the algacide properties of ultrasonication systems would rely notably on cyanobacteria gas vesicle disruption in conjunction with free radical production following aquatic exposure to ultrasound (Rajasekhar et al. 2012). Such sublethal effects may lead to reduced cell buoyancy and growth and ultimately to cell lysis with potential cyanotoxin release into the surrounding water (Rajasekhar et al. 2012). Hence, in order to evaluate the anti-cyanobacterial efficiency of some commercially available ultrasound transducers, several laboratory and field experiments—often contradictory—have been conducted (Wu et al. 2011). As an illustration, Inman (2004) observed a small but significant decrease in total chlorophyll a content during an in situ experiment using a floating low-powered (40 W) ultrasound

source operating at a frequency of 28 kHz in a sedimentation lagoon. On the contrary, Lüring and Tolman (2014a) did not find any effect of ultrasound (mix of 20, 28, and 44 kHz with an acoustic power of 0.7 W) on *Microcystis aeruginosa* (cyanobacteria) and *Scenedesmus obliquus* (green algae) under laboratory conditions. These authors have rather observed lethal effects on *Daphnia magna* Straus 1820, a freshwater microcrustacean which was used as a non-target species to assess the environmentally friendly feature of the ultrasound system (Lüring and Tolman 2014a). These results were of great concern since the loss of this zooplankton grazer during the experiments resulted in noticeable algal biomass rise (Lüring and Tolman 2014a). In fact, only few works (mainly focusing on *Daphnia*) have been conducted so far to assess the potential adverse biological effects of anti-cyanobacterial ultrasound devices on non-target freshwater organisms (Lüring and Tolman 2014a, b). To our knowledge, no experiments were conducted to In the context of the global warming, cyanobacterial blooms are expected to occur on a more frequent basis in eutrophicated freshwater ecosystems (O'Neil et al. 2012; Paerl and Huisman 2008). In order to limit excessive microalgal growth and especially harmful algal blooms, a number of so-called "environmentally friendly" physicochemical methods, including ultrasonication,

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The aim of this work was therefore to investigate the effects of a commercially available ultrasound device (emitting at 23 and 46 kHz) on the widespread amphipod species *Gammarus roeseli* as a model organism. To achieve this objective, a set of designed experiments were conducted on a laboratory scale. The effect of ultrasound on water temperature and subsequently *Gammarus* mortality as well as the use of fine mesh bags as physical barriers was thus investigated, revealing for the first time the importance of these latter in limiting ultrasound effects.

## Material and Methods

### 2.1. Ethical statement

All applied treatments on *G. roeseli* specimen were in compliance with the general guidelines of the Council of European Communities (European Union No /63/CEE 2010) and the French Animal Care Guidelines.

### 2.2. Ultrasound exposure system

Two transducers were placed adjacent one to another (~ 10 cm between them) emitting continuous signals at an average frequency of 23 and 46 kHz, respectively. They were connected to a function generator, which was used to drive the ultrasound transducers with a power of 15 W (Fig. 1). Details about the transducers' directivity (unidirectional), their emission level and the characterization of the theoretical signal resulting from the combination of [23/46 kHz] were previously described in Techer et al. (2017). According to the manufacturer, this ultrasound device has been designed especially for microalgae growth inhibition (SOFICHEM; TWIN-fi® Ultrasonic system). The measurement of the resulting emitted acoustic signals in water was carried out using a Brüel and Kjær Type 8103 hydrophone (omnidirectional, with a frequency range from 0.1.10<sup>-3</sup> to 180 kHz and a receiving sensitivity of – 211 dB re 1 V/μPa) connected to a digital multimeter (FLUKE Instruments) (Techer et al. 2017). A peak-to-peak voltage amplitude of sinusoidal signals output from the ultrasound devices could be measured in water, allowing for comparative evaluation of ultrasound frequency and intensity (recorded by the hydrophone coupled to the multimeter and expressed as voltage) depending on the hydrophone position from the transducers in the glass tanks.

### 2.3. Experimental design

Specimens of *Gammarus roeseli* were collected and handled according to the procedure described in Sornom et al. (2012). Briefly, the day before each set of experiments, specimens of around 15 mm (corresponding to intermediate-sized adults) were collected by handnets (5 mm mesh size) in vegetated areas from a reference site (Techer et al. 2016) in a non-polluted area (Bwild Moselle River, Tonnoy, 54,210, France). They were quickly brought to the laboratory (within 1 h of sampling) in 20 L opaque plastic containers filled with water taken from site. Some leaves and small branches were also added into each tank in order to minimize induced stress by handling and transport. In the laboratory, *G. roeseli* specimens were maintained in the dark at room temperature in those tanks in continuously aerated water (collected from the reference site) until the beginning of experiments (the next day). The invertebrates were thereafter gently transferred from plastic containers to exposure glass tanks using soft forceps with a blunt end (especially designed for handling small invertebrates). Test organisms were not fed during the exposures.

A set of three experiments involving different conditions was consecutively conducted: (i) uncontrolled physical parameter, (ü) the control of water temperature as a possible bias acting as an environmental stressor (other than ultrasound) on *Gammarus* mortality, and (üi) the use of an immersed plastic net container to mimic the potential effects of a physical barrier (Fig. 2):

- experiment #1 (Fig. 2a): 120 *G. roeseli* individuals were randomly and equally distributed into four glass tanks (28 ×

14.5 × 20.5 cm), each containing 8 L of clear water (from the reference site). Two tanks were exposed continuously and simultaneously to ultrasound (one transducer of 23 kHz and one of 46 kHz directly immersed into each tank), whereas two others were used as control (no ultrasound). All tanks were packed into polystyrene box to prevent from ultrasound propagation. This experiment lasted 6 h.

- experiment #2 (Fig. 2b): six glass tanks (with 20 *Gammarus* per tank) including a water-recirculating system between all the tanks (using aquarium pumps) were set up. Water running out of the tanks was collected into a big water supply where it was also regularly cooled (following the immersion of ice cube) before flowing back into the tanks. By this way, water temperature could be maintained not beyond 18.1 °C and was identical into ultrasound exposed as well as control tanks. This experiment ended up after 51h.

- experiment #3 (Fig. 2c): it involved the same recirculating water system, with the exception of two glasstanks per treatment, and all *Gammarus* (26 per tank) were previously encaged into synthetic bags (all together inside net bags) allowing for freely swimming and constructed from nylon mesh of 0.5 × 0.5 mm mesh size (0.025 mm<sup>2</sup>). This experiment finished after 7 days.

Each glass tank was inspected at the same time of water physicochemical parameter measurement. Moreover, the frequency of visual observations depended on invertebrate behavior. Dead *Gammarus* specimens were regularly counted during all exposure period for each experiment. Apparently dead individuals were observed under a binocular microscope, and mortality was defined as failure to respond to gentle mechanical stimulation (Taylor et al. 1991) in the absence of any other signs of physiological activity (ventilation or heartbeat).

## 2.4. Water physicochemical parameters

Physicochemical parameters, i.e., temperature, dissolved oxygen, pH, and conductivity were monitored at regular

intervals in each glass tank (in the same location between the different tanks) using appropriate digital probes directly immersed into water (PONSEL ODEON® Digital Handheld Multiparameter).

## 2.5. Sublethal effect assessment-biochemical analyses

Briefly, during the third set of experiments, six specimens per tank (n =12 per treatment, i.e., ultrasound vs control) were randomly collected (after 4 days of ultrasound exposure) and processed for total tissue homogenization (Techer et al. 2016) followed by total protein measurement (using the BCATM Protein Assay (PIERCE)), and enzymatic activity determination (total glutathione peroxidase (GPx) and glutathione S-transferase (GST)). GPx and GST activities were selected as potential biomarkers of sublethal effects such as oxidative stress after exposure to ultrasound (Techer et al. 2017). GPx and GST activities were estimated according to the protocol of (Paglia and Valentine 1967) and (Habig and Jakoby 1981), respectively, adapted to microtiter plate (Techer et al. 2016).

## 2.6. Statistical treatment

The mean lethal ultrasound exposure duration leading to 50% *Gammarus* mortality (here referred as LD50) was determined for each ultrasound exposure experiment. For this purpose, continuous counting of dead specimen during exposure was made for the set of the three laboratory tests. Mean LD50 values (expressed in hours ± 95% confidence intervals) were estimated using REGTOX macro Excel (v.7.0.6) based on non-linear regression (Hill model) (Vindimian 2012). For enzyme activity determination (experiment #3), all data were expressed as mean ± SE. Normality and homogeneity of variance were tested with the Kolmogorov-Smirnov test and Levene's test, respectively. Significant difference between treatments (ultrasound vs control) was checked by the Student t test. All statistical analyses were carried out with an overall significance level of 0.05 using Statistica v.10.

## Results and Discussion

During the first experiment (Fig. 1a), there was no water-recirculating system. Physicochemical parameters were constant in all exposure tanks except temperature. Indeed, in ultrasound tanks, temperature rose about 6.5 °C at the end of experiments (Table 1). A slight decrease in dissolved oxygen could be also reported in ultrasound treated tanks, presumably due to ultrasonication and concomitant lower oxygen solubility following the rise in temperature (Table 1). Nevertheless, water was always oxygen-saturated (Table 1). The LD50 in ultrasound exposed tanks was established at 161 min ([153; 167] min), i.e., ~ 2 h 40 min (Fig. 3a), whereas no mortality was reported in control tanks. The sharp and substantial temperature increase in tanks exposed to ultrasound during the experiment #1 may explain *Gammarus* mortality in those containers taking into account the species sensitivity to water temperature (Vincent 1985). Other hypotheses would rely on a direct lethal effect of ultrasound or on the interaction of ultrasound exposure and elevated temperature. Therefore, in order to avoid any bias related to environmental stressor other than ultrasound on *Gammarus* mortality, a water cooling and recirculating system was setup

to maintain temperature below 20 °C in exposed and control tanks for the rest of the experiments. As reported in the Table 1, water recirculation (and cooling) effectively limited the rise in temperature while leading to similar water temperature values in all experimental units, from 16.75 (experiment #2) to 18.1 °C (experiment #3), which was in accordance with the species thermal preferendum (Vincent 1985). As a consequence, the LD50 in ultrasound exposed tanks during the experiment #2 reached 29 h [26 h; 31 h] (Fig. 3b), clearly suggesting that the temperature increase was the main parameter directly influencing *Gammarus* mortality in the experiment #1 (LD50 of 2 h 40 min). According to this average delay before observing mortality (i.e., more than 1 day in the experiment #2), the tested ultrasound system could be considered as only slightly acutely harmful for the amphipods under these laboratory conditions. In field exposure conditions such as for instance in a fishpond, one may easily expect that the rise in temperature (as reported in the experiment #1) could be neglected, and the toxicity of ultrasound consequently lowered.

In order to test for the potential effects of a physical barrier mimicking in situ exposure conditions, the experiment #3 was conducted where *Gammarus* specimen was encaged into bags constructed from fine plastic mesh prior to ultrasound exposure (Fig. 2c). These latter resembled litterbags which are usually used in ecological monitoring studies (Gessner and Chauvet 2002). Since no mortality has been observed for ultrasound exposed *Gammarus* during the first 2 days of the experiment #3 (by contrast with the experiment #1 and #2 when dead individuals could be observed following the first hours of exposure), the experiment #3 duration was thus prolonged up to 7 days. Still, daily observations for a continuous 7-day exposure period to ultrasound did not permit to report any mortality (or signs of morbidity) for the ultrasound-exposed amphipods. These results were in contrast with those recently obtained by (Estévez-Calvar et al. 2017) who reported an overall higher effect of ultrasound (20–22 kHz, 200 W) on the mortality of sea water zooplanktonic species when placed into net containers made of a nylon mesh of 80  $\mu\text{m}$  (Estévez-Calvar et al. 2017). Moreover, in order to get insights into potentially more subtle biochemical changes following ultrasound exposure (in the experiment #3), GPx and GST activities were assessed as biomarkers of sublethal effects involving cellular oxidative stress (Techer et al. 2016). Total GPx levels of  $5.6 \pm 0.5$  U  $\text{mg}^{-1}$  protein and  $5.8 \pm 0.5$  U  $\text{mg}^{-1}$  protein were reported for non-exposed and exposed organisms, respectively (Fig. 4b), whereas GST activities reached  $75.6 \pm 7.1$  and  $86.6 \pm 9.1$  U  $\text{mg}^{-1}$  protein, respectively (also in non-exposed and exposed organisms) (Fig. 4c). Statistical analyses did not permit to sort out any significant difference between ultrasound-exposed organisms and control specimens (Fig. 4b, c). Therefore, these results suggest ultrasound-absorbing or attenuating properties of the mesh bags leading to no apparent biological effect on ultrasound-exposed *Gammarus* compared to non-exposed organisms.

These assumptions were reinforced by the measurement of the resulting emitted acoustic signals in ultrasound-exposed tanks using a hydrophone. Indeed, in the absence of any synthetic bag, sinusoidal signals corresponding to ultrasound

intensity measurement for a frequency of 46 kHz were in the range of + 475/– 515 mV at 8 cm in front of the transducer, whereas a range of + 11/– 15 mV was recorded with the 23 kHz transducer. By contrast, when using a plastic mesh similar to the one used for *Gammarus* bag confection, the ultrasound intensity for a frequency of 46 kHz was lowered to + 67/– 105 mV at 8 cm in front of the transducer, whereas only + 6/– 11 mV was obtained with the 23 kHz transducer (Fig. 4a). Despite recorded ultrasound frequencies of 23 and 46 kHz, their overall intensity seemed to be reduced from about two to seven times depending on the type of transducer in front of the synthetic nets. According to (Raišutis et al. 2007), the waveform of the ultrasound signal transmitted through a plastic object usually is distorted due to different factors, such as the ultrasound frequency dependent attenuation, phase velocity dispersion, and to some extent diffraction phenomenon, geometry of boundaries of the object etc. As an illustration, the use of some plastic materials such as polyvinylidene fluoride (PVDF) may result in important ultrasonic wave attenuation in relation with the material composition and structure (Raišutis et al. 2007). In this work, the composition of the plastic material used for *Gammarus* mesh bag confection was not provided by the supplier. Nevertheless, one could perhaps suggest that the used material of the plastic containers was a sort of polyvinyl (maybe fluoride-derived) polymeric material. Moreover, apart from the nature of the plastic container, the geometry of boundaries imposed by the grid of 0.5 mm squared of the synthetic mesh in the present work may have played a substantial role in ultrasound wave diffraction and/or attenuation. Indeed, potential waveform diffraction or distortion phenomenon due to the geometry of the mesh holes may have occurred resulting in intensity attenuation of ultrasound in the studied system (of 15 vs 200 W in the work of Estévez-Calvar et al. 2017). Further investigations concerning the ultrasound propagation and the spatial distribution of the acoustic pressure in presence of different kind of plastic materials may help in understanding the observed attenuation phenomenon.

## Conclusion and perspectives

The evaluation of the adverse effects of a dual-frequency ultrasound device on *Gammarus roeseli* through laboratory scale experiments revealed the rise of temperature as a major biological stressor. Consequently, particular attention must be given to the experimental conditions when evaluating the effects of such ultrasound devices on aquatic invertebrate species. Depending on our results, the tested

ultrasound device would not be acutely harmful to *Gammarus* under environmental conditions. Moreover, the use of a fine mesh bag (0.5  $\times$  0.5 mm mesh size) to mimic a physical barrier clearly attenuated the ultrasound intensity and consequently their adverse effects on the amphipods. Hence, in a context of field use, such an ultrasound device would not be expected to be harmful to freshwater macro-invertebrate species like *Gammarus*.

**Table 1. Measurement of water physicochemical parameters in glass tanks before experiments ( $T_0$ ) and at the end of experiments ( $T_f$ )**

		Experiment #1: no recirculating water, no cooling		Experiment #2: water recirculation and cooling		Experiment #3: water recirculation and cooling—plastic mesh bags	
		(-) US	(+) US	(-) US	(+) US	(-) US	(+) US
Temperature (°C)	T0	18.85 ± 0.05	18.50 ± 0.10	14.40 ± 0.00	14.40 ± 0.08	17.90 ± 0.00	17.90 ± 0.00
	Tf	18.95 ± 0.25	25.05 ± 0.35	18.03 ± 0.05	18.10 ± 0.00	16.75 ± 0.05	16.80 ± 0.00
O2 (mg/L)	T0	9.63 ± 0.02	9.43 ± 0.14	10.46 ± 0.01	10.46 ± 0.01	10.12 ± 0.00	10.52 ± 0.38
	Tf	9.20 ± 0.02	8.71 ± 0.09	9.84 ± 0.05	9.82 ± 0.04	10.15 ± 0.00	10.20 ± 0.03
O2 (%)	T0	104.7 ± 0.3	103.5 ± 1.1	103.5 ± 0.1	103.6 ± 0.1	107.8 ± 0.0	107.5 ± 0.5
	Tf	100.2 ± 0.1	103.7 ± 2.5	104.8 ± 1.0	105.2 ± 0.4	106.1 ± 0.0	106.4 ± 0.3
pH	T0	8.10 ± 0.00	8.10 ± 0.00	7.40 ± 0.00	7.43 ± 0.12	7.55 ± 0.05	7.55 ± 0.05
	Tf	8.10 ± 0.00	8.10 ± 0.00	7.87 ± 0.05	7.80 ± 0.00	7.65 ± 0.05	7.70 ± 0.10
Conductivity (µS/cm)	T0	333 ± 5	334 ± 1	192 ± 0	192 ± 0	205 ± 1	204 ± 0
	Tf	335 ± 3	344 ± 3	205 ± 0	205 ± 0	217 ± 1	217 ± 0

(-) *US* no ultrasound treatment, (+) *US* combined ultrasound treatment at 23 and 46 KHz frequencies. Data are expressed as mean ± SD

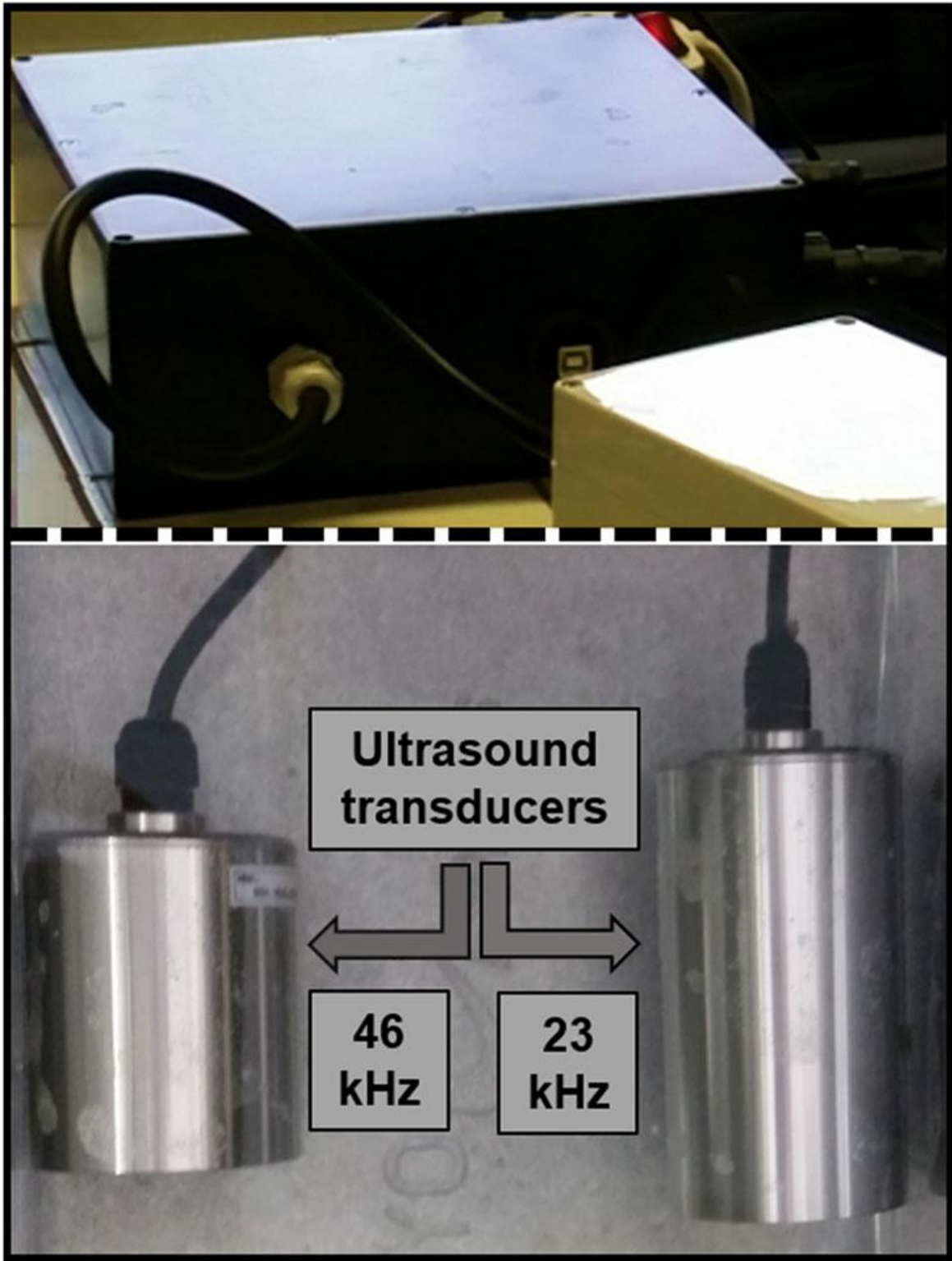
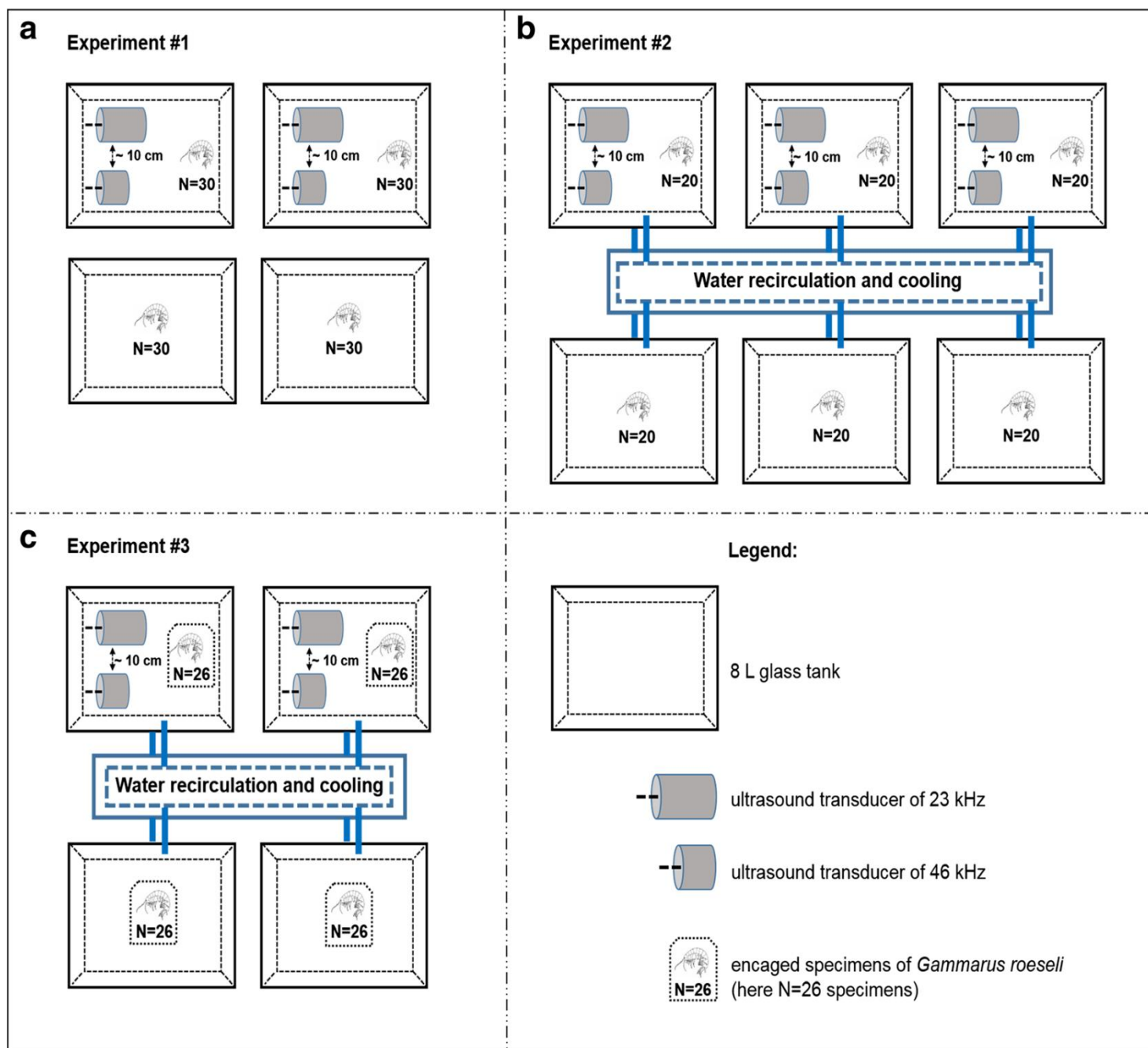


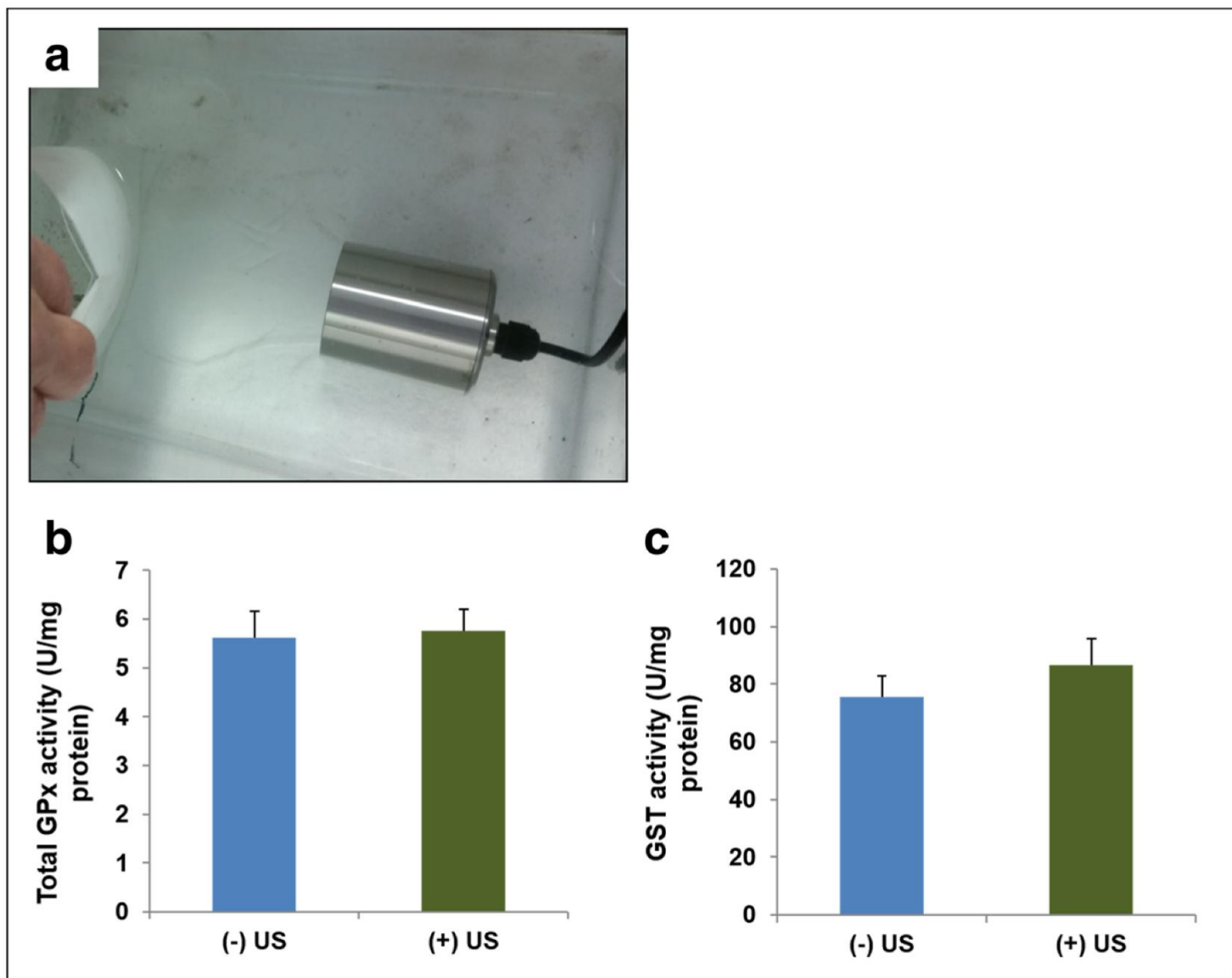
Figure 1. Picture of the ultrasound device used for the three set of experiments: a function generator driving the ultrasound transducers with a power of 15 W. b ultrasound transducers of respectively 46 and 23 kHz



**Figure 2. Ultrasonic experimental setup.**

Three experiments were performed: a experiment #1: two tanks continuously and simultaneously exposed to ultrasound vs two tanks non-exposed (control); all amphipods were freely swimming; b three tanks continuously and simultaneously exposed to ultrasound vs three tanks non-exposed (control); all amphipods were freely swimming, and a water cooling and recirculating system was set up; c two tanks continuously and simultaneously exposed to ultrasound vs two tanks non-exposed (control); all amphipods were encaged into 0.5 mm mesh plastic bags





**Figure 3.** a Illustration of the ultrasound frequency and intensity measurement behind the plastic mesh of 0.5 × 0.5 mm (immersed hydrophone) during emission from the 23 kHz transducer; b total GPx activity comparison in control and exposed organisms in the experiment #3; c total GST activity comparison in control and exposed organisms in the experiment #3. Data are expressed as mean ± SE (n = 12). Significant differences were checked by ANOVA ( $p < 0.05$ )

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