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NEW METHODS FOR STUDYING UNDERWATER NOISE EFFECTS ON MICROSCOPIC SESSILE MARINE INVERTEBRATES: CHALLENGES AND PERSPECTIVES

Lucia Manni [1*] **Giacomo Sabbadin** [1] **Simone Domenichi** [1]
Loriano Ballarin [1] **Valentina Zaffaroni-Caorsi** [2] **Giovanni Zambon** [2]
[1] Department of Biology, University of Padua, Padua, Italy
[2] Department of Earth and Environmental Sciences, University of Milano-Bicocca, Milan, Italy

ABSTRACT

The negative effects of underwater noise have been studied in a few marine invertebrate species. Scanty information is available on ascidians, filter-feeding tunicates, although they are dominant among epibenthic fauna. In past playback experiments in tanks, the closure rate of the oral siphon of solitary ascidians (about 10 cm long) was evaluated using videos. In a new study, we aimed at verifying the underwater noise effects on *Botryllus schlosseri*, an ascidian forming colonies of tiny, transparent animals about 1.5 mm long. Since videos were not useful due to the animal's small size, we adjusted and fine-tuned behavioral and physiological tests never used before for analyzing noise effects. We exposed colonies collected in the Venetian Lagoon to continuous noise (30 min; peak bands 63-125 Hz) mimicking the low frequency maritime traffic noise. We adjusted tests evaluating the responsiveness of two different mechanoreceptors of the oral siphon and the heartbeat frequency under the stereomicroscope, and developed an assay for studying the animal filtration rate. Preliminary results show that noise effects can be carefully measured and statistically analyzed. These methods represent new, valuable tools that could be translated in future to other filter-feeding, small and transparent animals or adjusted to large animals.

*Corresponding author: lucia.manni@unipd.it.

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1. INTRODUCTION

Although underwater noise is considered an emergent pollutant, its effects on marine invertebrates have been considered in a few zoological taxa, mainly crustaceans and mollusks. A minor number of studies regard other organisms, such as Bryozoa, Echinoderms, Cnidarians, Tunicates, and zooplankton [1-2]. These studies, although limited to a few species, evidenced that different aspects of animal life can be impacted by noise, such as embryonic development, behavior, physiology, and animal mortality. Since the invertebrate fauna represents the main component of marine ecosystems, these results underline the importance of intensifying the study of noise effects for preserving marine ecosystems.

Tunicates are a group of marine invertebrate chordates considered the sister group of vertebrates [3]. They include both pelagic and sessile animals. The latter are grouped in the Ascidiacea, a taxon of about 3000 species, both colonial and solitary, diffuse in shallow waters worldwide and among the dominant species of epibenthic fauna. Ascidians are barrel-like animals, living attached to the substrate, ranging from a few millimeters to some centimeters in size, furnished with two siphons (inhalant and exhalant) for water circulation inside the body, on which both respiration and feeding relay. As adults, therefore, they do not resemble vertebrates; however, their swimming larva, representing the motile phase for their dispersion in the environment, possesses features typical of the chordate body plan, such as a notochord as supporting structure and a dorsal neural tube,

which are shared with vertebrates. As sedentary and filter-feeding, adult ascidians perceive environmental stimuli mainly thanks to mechanoreceptors. Various sensory cells are in the oral siphon region, the most sensitive ascidian structure [4]. In particular, the oral siphon epidermis is rich in mechanosensory neurons, both grouped and isolated, sending their axon to the brain. These neurons are sensible to near-field vibrations and touch; their stimulation triggers the oral siphon closure. Moreover, the ring of oral tentacles at the base of the oral siphon is furnished with dedicated mechanoreceptor cells, the coronal cells, contacted at their base by neurites of brain sensory neurons controlling their activity. Coronal cells act as sentinels, intercepting potentially dangerous particles entering the pharynx via the oral siphon with the water inflow; their stimulation triggers the atrial siphon closure, a slight body wall contraction, and the consequent expulsion of seawater filling the pharynx. For their structure, function, and developmental features, coronal cells are considered homologous to hair cells of vertebrate ear and lateral line receptors, *i.e.* the mechanoreceptors perceiving noise and water movement.

To verify the effects of noise as a pollutant, only behavioral analyses were performed in a few ascidians. Individuals of the solitary ascidian *Styela plicata* were collected from two sites with different anthropogenic soundscapes and exposed to playback experiments in tanks (laboratory conditions). The experiments consisted of the emission of a boat motor signal, a song, and a water current to simulate turbulence [5]. Treated animals from both sites increased the frequency and duration of siphon closure events. In a subsequent study, *Styela plicata* and two additional solitary species, *Ciona intestinalis* and *Ascidiella aspersa*, were treated with ultrasounds at 30 and 35 kHz [6]. Animals exposed were able to perceive the stimuli and showed a frequency-dependent behavior that varied depending on the species and size of individuals. Data in both the studies referred to behavioral analysis collected from videos recorded during the trials (digital cameras attached to a support in front [5] or over [6] the animals located in tanks). The animal dimension (normally ranging from 8 to 20 cm in length) allowed recording videos of good resolution for the visual analyses of oral siphon behavior. No other methods have been developed so far for analyzing the effect of noise on ascidians.

The aim of this study is to present new assays specifically adapted or developed to assess the impact of anthropogenic underwater noise on the behavior and physiology of the colonial ascidian *Botryllus schlosseri* (Fig. 1). Colonies are composed of tiny, transparent adult animals (zooids), about 1.5 mm long. A colony grows thanks to the generation of

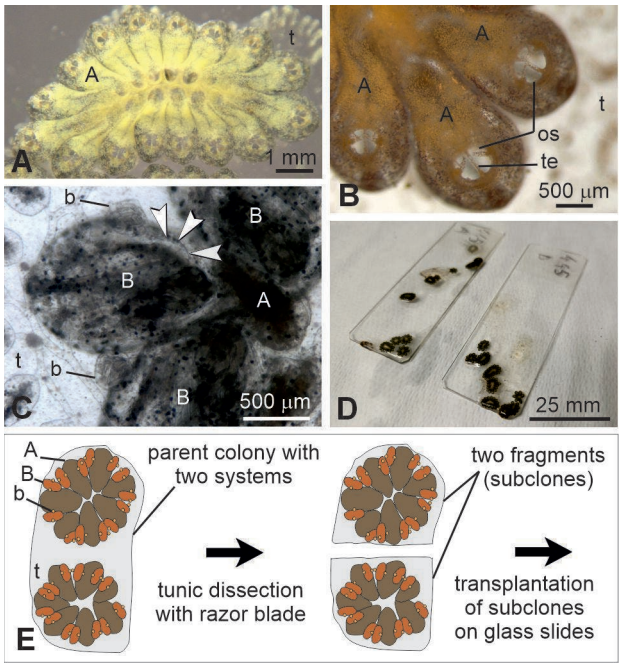

Figure 1. **A**. Colony of *Botryllus schlosseri* in dorsal view. A colony can be composed of hundreds of adult zooids organized in flower-like "systems". The system shown in the picture is composed of 16 adult zooids. Buds are not visible. **B**. Detail of the oral siphon of three adult zooids. The oral tentacles are recognizable inside the oral siphon. Dorsal view. **C**. Ventral view of three primary buds and a regressing adult zooid. Arrowheads point at the heart. **D-E**: Two slides (D) with several genetically identical colony fragments (subclones) derived from the same parent colony as shown in the illustration in E. A: adult zooid; B: primary bud; b: secondary bud; os: oral siphon; t: tunic; te: tentacle.

new individuals (buds) by asexual reproduction [7]. Since the zooid size does not allow the use of video recordings to accurately analyze animal behavior in tanks during noise treatment, we adapted three tests, originally developed to study other aspects of zooid life. In *B. schlosseri*, two tests were used to study aging and zooid regression during the asexual cycle [4]: the Siphon Stimulation Test (SST), testing the performance of the oral siphon epidermal neurons, and the Tentacle Stimulation Test (TST), testing the performance of coronal cells. Since these two tests evaluate mechanoreceptor cell performance, they can potentially provide information on noise impact. The third

test is a physiological test, so far used for studying *B. schlosseri* wellness: the Heartbeat Test (HBT), which quantifies the heartbeat frequency. In *B. schlosseri*, the average heartbeat at 21°C is 87 beats per minute [8] and stress impairs hemolymph flow [9]; we hypothesize the HBT can reveal if underwater noise induces stress on zooids. Lastly, we describe the development of the Filtration Rate Test (FRT). In the past, the filtration rate was measured in some ascidians, informing about the seawater filtered by animals per hour [10]. Since previous studies on the noise effect on ascidians showed that oral siphon behavior is altered by noise treatment, prolonging its closure frequency and duration [5-6], we hypothesize that this effect may impair the animal ability to inhale seawater, decreasing therefore the filtration rate. The FRT was never used as an index of animal wellness after noise treatment in ascidians, nor measured in *B. schlosseri* in normal conditions. Preliminary results show that our new experimental designs, exploiting tests that we adapted from previous uses or developed *de novo*, provide careful measurements of behavioral and physiological responses after noise treatments. These methodologies represent new, valuable tools that could be translated in future to other filter-feeding, small and transparent animals or adjusted to large animals.

2. MATERIALS AND METHODS

Colonies of *B. schlosseri* were collected in the Lagoon of Venice and reared in tanks filled with filtered seawater (FSW), in thermostatic rooms (17°C), at the Department of Biology, University of Padova. Colonies were fed every other day with *Tetraselmis* sp.. They were fragmented for creating clones (subclones; Fig. 1D-E) to be used in pairs as "control colony" and "treated colony" in experiments.
All the experiments were performed in three biological replicates at the rearing temperature in thermostatic rooms using colonies at the same phase of their asexual cycle (Fig. 2).

2.1 Source of noise and playback noise experiments

For noise treatment, plastic tanks containing 45 L of seawater, placed above a table equipped with an insulating sand layer, were set up as described in [11] (Fig. 2A). In summary, animals were treated for 30 min with a continuous synthetic noise (pink noise with two 1/3-octave bands amplified: 63-125 Hz), mimicking the underwater maritime traffic noise. Treated colonies were suspended in front of a loudspeaker located on the short side of the tank and were exposed to a noise of 160 dB SPL. In the meantime, control colonies were maintained in similar tanks but without noise (background noise present equal to 110 dB $Leq_{,30min}$).

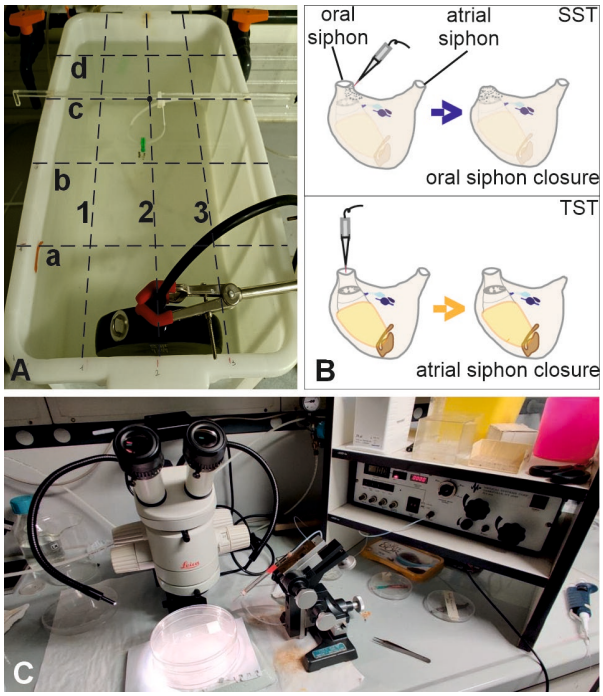

Figure 2. **A.** Noise exposure experimental setup. The loudspeaker is on the short side of the tank. Dotted lines a-d and 1-3 create a spatial coordinate system individuating the tank nodes used to map the tank noise levels. During noise treatment, colonies were suspended at the node c2 (evidenced by a dot). **B.** Illustration showing the animal response when a water jet emitted by a microcapillary stimulates the oral siphon epidermis in the SST (oral siphon closure, top) and the coronal cells of oral tentacles in the TST (atrial siphon closure, bottom). Image from [4], published under License 4.0 (CC BY-NC-ND). **C.** Setup for performing the SST and the TST. A colony of *B. schlosseri* on a glass slide is in a Petri dish filled with seawater under the stereomicroscope. A microcapillary, mounted on the micromanipulator is on its right, ready to be placed close to a zooid. The jet pressure of the microcapillary is controlled by the microinjector on the right.

2.2 Behavioral experiments: Siphon Stimulation Test and Tentacle Stimulation Test

We used a micromanipulator (Brinkmann MP II) leading a microcapillary connected to a microinjector (PLI-100 Pico-Injector, Medical Systems Corp) to elicit the mechanical stimulus (Fig. 2B-C). The microcapillary was prepared using a Narishige PD-5 horizontal capillary puller. The microcapillary was filled with a solution of phenol red (Phenol Red Solution, sigma P0290) 1/10 in seawater and was set so that, when working at 90 kPa for $6x10^3$ ms, it produced on a microscope slide a droplet with a diameter of 600 µm. Colonies were analyzed in dorsal view at the stereomicroscope Leika MZ6 during the tests. Once the microcapillary was placed close to the zooid tissue to test, the zooid was stimulated with the water jet to find the minimal jet pressure necessary to evoke the zooid response. This pressure value was recorded as an index of the zooid performance. The minimal pressure value was found using initially harmless pressure (20 kPa, injection time $10x10^3$ ms) and involved incremental steps of 5 kPa, with time increment of 1 min to avoid habituation.

2.3 Physiological experiments

2.3.1 The HearBeat Test

For HBT, colonies were analyzed in ventral view at the stereomicroscope and the heartbeat was counted for 30 s. The ascidian heart is a tubular structure, with an extremity opening in the subendostylar sinus, and the other opening toward the stomach lacuna [8]. Since the heart intermittently reverses the beat direction (approximately every 2 min in *B. schlosseri*), the heartbeat count was done after a few seconds from the beginning of heartbeat pushing the hemolymph flow toward the subendostylar sinus.

2.3.2 The Filtration Rate Test

For the FRT (Fig. 3), the protocol was adapted from [12]. Colonies to be tested were starved for one day and then immersed for 6 h in crystallizers with 500 mL of FSW containing the microalga *Tetraselmis* sp. at an initial known concentration (C_0). In the crystallizers, the microalgae were maintained in suspension by an aerator. The algal concentration was determined spectrophotometrically by measuring the absorbance at 640 nm. Previously, a calibration curve was built by plotting, in a graph, the absorbance of serial dilutions of algal suspensions *vs* the cell concentration measured using a Burker's hemocytometer after the fixation of the microalgae for 30 min in 1% glutaraldehyde and 1% sucrose in FSW at 4°C. The filtration rate was measured as the reduction in the concentration of microalgal cells as a function of time. At time intervals of 30 min from the colony immersion in the crystallizer, 1 mL of solution was collected, and its absorbance was measured; for each concentration, three absorbance measurements were performed. The filtration rate (FR) was determined using the formula

$$FR = [\text{Volume mL} / (n * \text{Time h})] * \ln(C_0 / C_t)$$

where C_0 and C_t are algal cell concentration at time 0 and t respectively, and n is the number of zooids per colony.

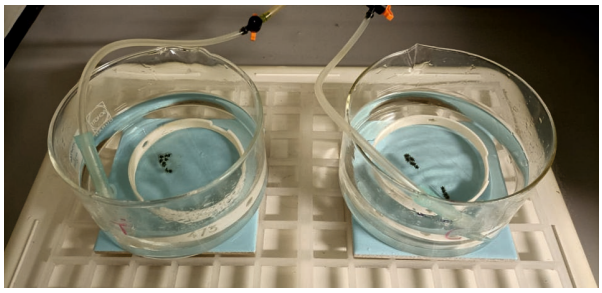

Figure 3. Setup for performing the FRT. Two slides supporting subclones coming from the same parent colony are in two different aerated crystallizers containing the microalgal solution. One of the subclone was previously treated with noise.

3. RESULTS

3.1 A new experimental design for studying noise effects on zooid mechano-sensitivity

To verify if underwater noise impairs the ability of zooids to respond to mechanical stimuli, we used both the SST and the TST (Fig. 4A). The new experimental setup was identical for the two tests. After preparing at least three pairs of subclones from three different colonies, we initially determined the zooid sensitivity by performing pre-treatment tests, *i.e.* the SST or the TTS, in at least 3 zooids per subclone. Then, we treated a subclone with noise for 30 min, whereas the control one was maintained in a different tank. We then determined again the zooid sensitivity by performing the same test (post-treatment test) on three zooids per subclone. Therefore, for each test (SST and TST), we collected data, in the form of minimal pressure values necessary to evoke the animal response, from 9 treated zooids. These data were compared with the 9 values obtained by the three control subclones in the post-

treatment phase, and with the value obtained by the pre-treatment tests.

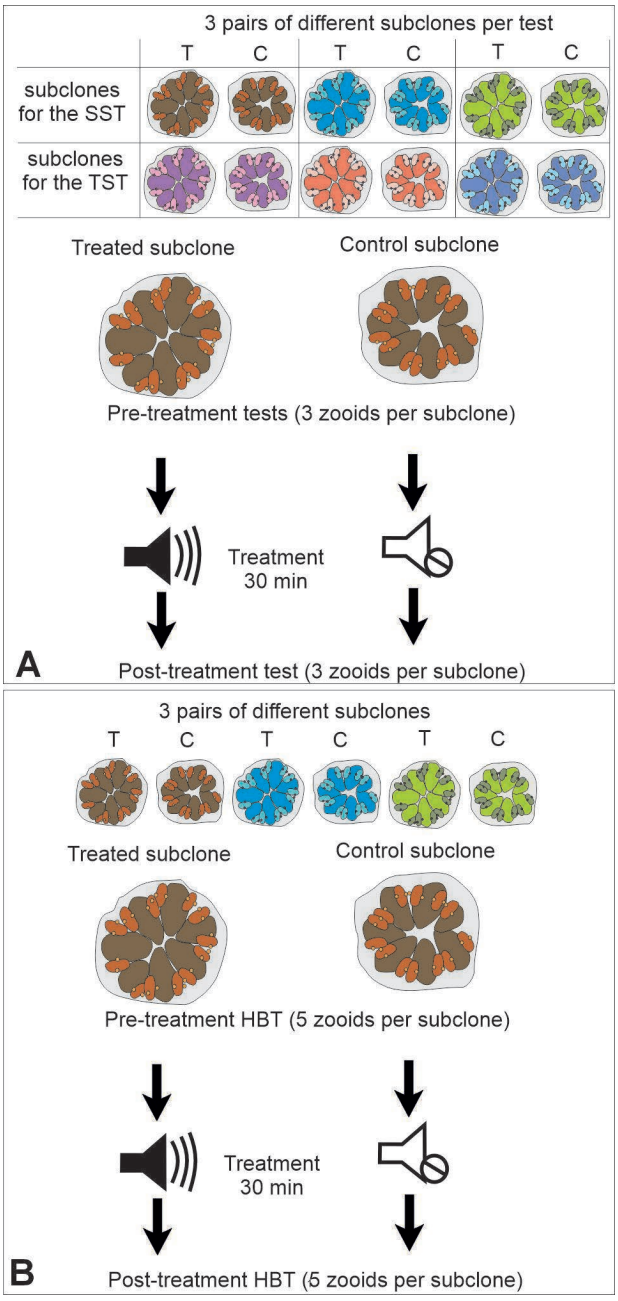

Figure 4. Test adapted to study noise effect in *B. schlosseri*. Experimental design for testing A. zooid mechano-sensitivity, and B. the heartbeat frequency after noise treatment. The procedure is shown for only one pair of subclones. See text for details. C: control subclone; T: treated subclone.

3.2 A new experimental design for studying noise effect on heartbeat frequency

The heartbeat frequency can be considered an index of zooid wellness, since in stressed animals it usually decreases. To determine if underwater noise impacts *B. schlosseri* heartbeat frequency, we designed the following new experiment: we prepared three pairs of subclones (the treated subclones and their twin control subclones) from three different colonies (Fig. 4B). We counted the heartbeats for 30 sec before (pre-treatment HBT) and after (post-treatment HBT) the noise exposure (30 min) in 5 different zooids per subclone. We obtained 30 values from the treated subclones (15 from the pre-treatment tests, 15 from the post-treatment tests) and the same number of values from the control subclones. The post-treatment HBT data from treated subclones were then compared with those obtained from the control subclones, and with those obtained from the pre-treatment HBTs.

3.3 A new experimental design for studying noise effect on filtration ability

Since noise treatment alters the oral siphon behavior [5-6], we hypothesized that noise, consequently, can alter the seawater amount entering the pharynx, so the filtration rate. For this reason, we developed *ex novo* the FRT (Fig. 5). Three pairs of subclones were prepared. The treated subclones underwent 1 h of noise treatment, while the control subclones were in a different tank. After treatment, the subclones were transferred into six crystallizers containing 0.5 L of microalgal solution for 6
h. Every 30 min, three samples of solution of 1 mL each were taken to measure their absorbance. The average absorbance level was calculated and used to infer the microalgal concentration, hence the filtration rate.

3.4 Noise treatment impairs *B. schlosseri* behavior and physiology

At the time of writing this article, the different types of tests have been carried out as shown in Figures 3-4 and in Table 1. Preliminary data shows that, when colonies are treated with a noise of 160 dB SPL, the zooid mechanosensitivity decreases: the pressure necessary to evoke the closure of the oral siphon in the SST

increases of about 30 KPa in treated colonies with respect to the control ones; similarly, in the TST, the closure of the atrial siphon is elicited when the pressure increases of about 25 KPa. It is to note that the higher the pressure necessary to stimulate the mechanoreceptors so to evoke the behavioral answer, the lower the animal sensitivity is. The HBT shows a decrease in heartbeat frequency after treatment of about 20%, considering that control colonies display about 85 beats/min. The FRT does not show any significant difference in control *vs* treated colonies figure.

Table 1. Test applied, parameters evaluated, and state of work in colonies treated with noise at 160.03 dB (IP: in progress; PR: preliminary results; <: decrease).

Test	Evaluated action	Evaluated parameter	Effect on treated colony	State of the work
SST	Oral siphon closure	Epidermal neuron sensitivity	<	PR
TST	Atrial siphon closure	Coronal cell sensitivity	<	PR
HBT	Heartbeat	Heartbeat number in 30 s	<	PR
FRT	Filtration	Filtration rate	None	IP

4. DISCUSSION

The small dimension of *B. schlosseri* represented a challenge for the study of noise effects. Indeed, differently from previous experiments on solitary ascidians [5-6], our preliminary recording videos, using a conventional underwater camera, had an insufficient resolution to collect data on noise effect on siphon behavior under treatment. This prompted us to find different methods for assessing noise effects on colonies. The methods described here require a more sophisticated setup and instrumentation with respect to video recording. Therefore, videos still represent a simple and cost-effective way to analyze solitary ascidians behavior, although a software for automatic video analyses

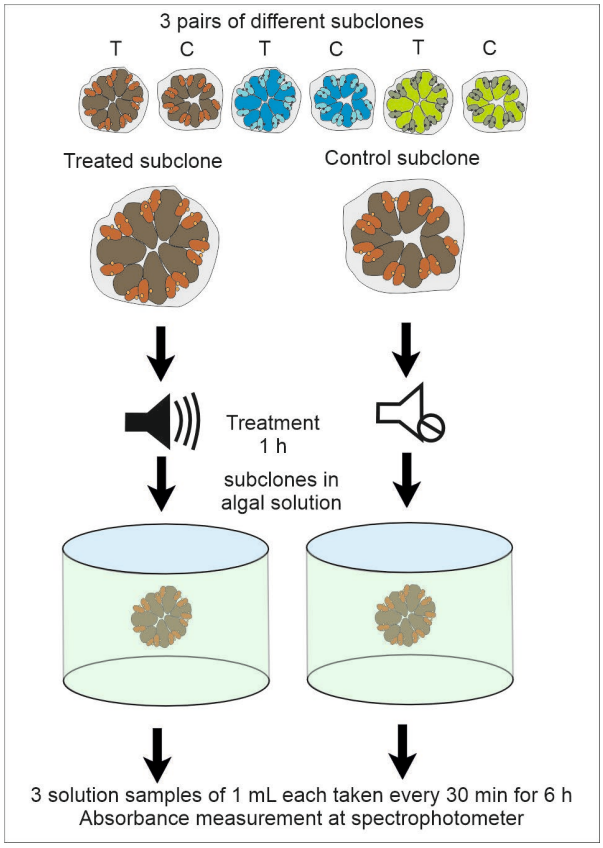

Figure 5. Test developed to study the noise effect on the filtration rate in B. schlosseri. The procedure is shown for only one pair of subclones. See text for details. C: control subclone; T: treated subclone.

is not available yet, making the experiments time consuming.

Our tests allow precise measurements of behavioral and physiological effects of noise treatments on *B. schlosseri*. They evaluate the sensibility of specific categories of sensory cells (sensory neurons on the oral on siphon epidermis, coronal cells on oral tentacles), and specific physiological parameters (heartbeat frequency, filtration ability). Moreover, considering that these tests are applied to clonal individuals, a robust statistical analysis can also be performed [13]. Requiring the zooid analyses under the stereomicroscope, the SST, TST, and HBT cannot be applied during noise stimulation. However, our data shows that these tests, applied immediately after noise stimulation in thermostatic rooms guaranteeing stable temperature conditions, are suitable for verifying noise effects. The SST

and TST reveal that noise impairs mechanoreception decreasing animal performance, and the HBT shows that noise decreases the heartbeat frequency, suggesting that animals are stressed by the pollutant. Our preliminary results on the FRT show that the filtration ability is not impaired by the noise treatment in *B. schlosseri*. This test provided physiological information on the negative noise impact in other marine invertebrates [14], however, different from the setup here proposed for *B. schlosseri*, in previous experiments the filtration rate was evaluated during the noise treatment, not after. Therefore, future experiments evaluating the filtration rate during noise during treatment in *B. schlosseri* will be necessary to inform us about noise effects on this physiological parameter.

The tests have the potential to be translated to other small-size, transparent aquatic animals. Nonetheless both the SST and the TST can be adjusted for large, not-transparent solitary ascidians. The use of a microcapillary for precise stimulation of the oral epidermis and oral tentacles needs the vision through a stereomicroscope, which is less easy when using large animals with respect to small ones. A continuous seawater flow should be also guaranteed to avoid animal stress, since solitary ascidians filter large seawater volumes [10]. Unfortunately, HBT cannot be applied to large solitary ascidians, since these animals are not transparent, and the heartbeat is not visible. The FRT requires a simple setup and can potentially be translated to other filter-feeding invertebrates, both of small and large size, both transparent and opaque. The test, evaluating the feeding capacity, allows consideration on animal fitness, therefore on long-term implications due to noise exposure.

In conclusion, the tests presented here offer new perspectives to study the effects of stress due to noise in various animal models, both small and transparent individuals, such as colonial ascidians or juveniles of solitary ascidians, and large individuals.

5. ACKNOWLEDGMENTS

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