AQUO

Achieve QUieter Oceans by shipping noise footprint reduction

FP7 - Collaborative Project n°314227

WP 4: Effects of ship noise on marine fauna
Task T4.2, Subtask T4.2.3

Cephalopod tolerance thresholds of low frequency sounds

Deliverable	Reference	Issue	Dissemination status
☒ yes ☐ no	D 4.4	Rev 3	PU
File name	AQUO_D4.4_V3.doc		

	Name	Organisation	Function	Date
Issued by	Marta Solé Michel André Mike van der Schaar	UPC	Task leaders	26/06/2015
Checked by	Michel André	UPC	WP4 leader	30/07/2015
Approved by	Christian Audoly	DCNS	Coordinator	27/08/2015

REVISION HISTORY

ISSUE	DATE	NAME	ORGANISATION	MODIFICATIONS
Rev 0	23/06/2015	Michel André	UPC	Comments
Rev 1	02/07/2015	Michel André	UPC	Revision
Rev 2	30/07/2015	Michel André	UPC	Final version
Rev 3	27/08/2015	Christian Audoly	DCNS	Reviewed version

CONTENTS

SUMMARY

This study has been realized in the scope of AQUO, a collaborative research project supported by the 7th Framework Programme through Grand Agreement N°314227, whose final goal is to provide to policy makers practical guidelines to mitigate underwater noise footprint due to shipping, in order to prevent adverse consequences to marine life. The present document is the deliverable D4.4 “Cephalopod tolerance thresholds of low frequency sounds”. This study has been realized in the scope of the work package N°4, which is dedicated to bioacoutics of marine fauna in relation with shipping underwater noise.

The extent to which ship noise in the sea impacts and affects marine life is a topic of considerable current interest both to the scientific community and to the general public. Cephalopods potentially represent a group of species whose ecology may be influenced by artificial noise that would have a direct consequence on the functionality and sensitivity of their sensory organs, the statocysts. These are responsible for their equilibrium and movements in the water column.

Controlled Exposure Experiments, including the use of a 50-400Hz sweep revealed lesions in the statocysts of four cephalopod species of the Mediterranean Sea, when exposed to low frequency sounds: (n=76) of *Sepia officinalis,* (n=4) *Octopus vulgaris,* (n=5) *Loligo vulgaris* and (n=2) *Illex condietii.* The analysis was performed through scanning (SEM) and transmission (TEM) electron microscopical techniques of the whole inner structure of the cephalopods’ statocyst, especially on the *macula* and *crista*.

All exposed individuals presented the same lesions and the same incremental effects over time, consistent with a massive acoustic trauma observed in other species that have been exposed to much higher intensities of sound: immediately after exposure, the damage was observed in the *macula statica princeps* (*msp*) and in the *crista* sensory epithelium. Kinocilia on hair cells were either missing or were bent or flaccid. A number of hair cells showed protruding apical poles and ruptured lateral plasma membranes, most probably resulting from the extrusion of cytoplasmic material. Hair cells were also partially ejected from the sensory epithelium, and spherical holes corresponding to missing hair cells were visible in the epithelium. The cytoplasmic content of the damaged hair cells showed obvious changes, including the presence of numerous vacuoles and electron dense inclusions not seen in the control animals. Those experiments were conducted in laboratory conditions.

Owing to a lack of available data on the pathological effects on cephalopod statocyst in wild conditions and on the onset mechanism of the acoustic trauma (in order to determine whether these animals are more sensitive to particle motion or acoustic pressure, or to a combination or both), we conducted offshore noise exposure comparative experiments on common cuttlefish (*Sepia officinalis*), in similar conditions as during the laboratory study, in terms of sound characteristics, received levels and time exposure. Particle motion measurements were also conducted, both in laboratory conditions, as well as at the same locations and depths where the individuals were exposed at sea.

Scanning electron microscopy (SEM) revealed similar injuries in the inner structure of the statocysts, as those found in cuttlefish in previous experiments. As for the particle motion data, the amplitudes (total sum of x, y and z components) were decreasing with increasing distance between the sensor. These findings support the validity of our previous results in laboratory conditions. Although the lesions were quantified versus received noise levels and

particle motion data, the analysis cannot yet determine threshold levels that would trigger the acoustic trauma. Acknowledging the validity of an experimental approach in laboratory conditions, this will constitute the next step of this research.

1. INTRODUCTION

Levels of introduced anthropogenic underwater sounds have increased significantly over the last century and anthropogenic noise is now recognized as a significant stressor for marine and freshwater fauna. While advances have been made in understanding the effects on marine mammals [1-5], and fishes [6-8], understanding the effects on marine invertebrates is a much more unfamiliar task. Much remains to be learned about the hearing or sound-producing capabilities of invertebrates and how they respond to, and are potentially affected by, man-made sounds. Several authors have addresses some studies on invertebrate sensitivity to noise and possible negative effects after sound exposure [9- 17]. A detailed literature review on these effects can be found in recent publications [18-20].

In a preliminary study [18-20], we showed that exposure to artificial noise had a direct consequence on the functionality and physiology of the statocyst, sensory organs, which are responsible for their equilibrium and movements in the water column. The statocyst morphology (Figure 1) and its functions have been extensively described elsewhere by different authors [21-27]. The statocysts are specialized balloon-shape bodies filled with endolymph that contain the sensory hair cells linning on the inside wall of the inner sac and are grouped into two main areas of the sensory epithelium: the *macula*-statolith system and the *crista-cupula* system. These systems have clear similarities to the analogous vertebrate vestibular [27, 28]. The cephalopods' statocyst hair cells carry kinocilia and are surrounded by microvilli forming elongated bundles. Adjacent accessory structures (statolith, statoconia, *cupula*) are responsible for sensory perception. When there is a stimulus, tiny deflations occur in the hair bundles, resulting in cell body depolarization and subsequent transmission of information to the sensory nervous system. The sensory input of the statocyst is used to regulate a wide range of behaviour, including locomotion, posture, control of eye movement and of the pattern of the body coloration, and are suspected to be responsible for the reception of the low frequency sound waves [24, 29, 30].

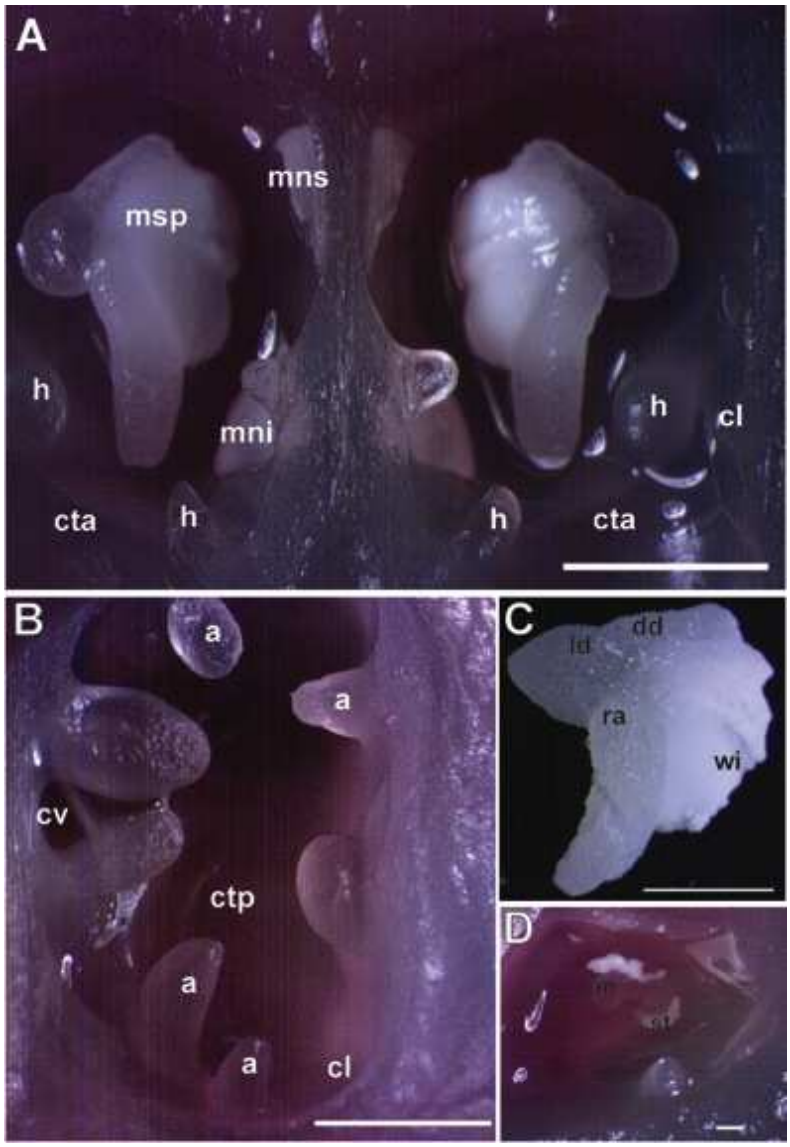


Figure 1. LM. *Sepia officinalis* statocyst structure. The statocyst cavities have been opened transversally. Photomicrographs. **A:** Anterior view. Each cavity show the three macula-statolith systems and two of the crista-cupula systems (cta, cl). **B:** Posterior view of one of the cavities. The two posterior crista segments (ctv, cv) are shown **C**: *S. officinalis* statolith showing its parts. **D:** The statolith has been removed and the *macula statica princeps* is visible into the cavity**. E:** In the *msp* epithelium some hair cells are displayed. Some statolith traces are visible (a: anticrista lobe, cl: *crista longitudinalis,* cta: *crista transversalis anterior*, ctp: *crista transversalis posterior*, cv*: crista verticalis*, dd: dorsal dome, h: hamuli lobe, hc: hair cells, ld: lateral dome, mni*: macula neglecta inferior*, mns: *macula neglecta superior*, msp*: macula statica princeps,* ra: rostral angle, ro: rostrum, wi: wing, st: statolith) **Scale bars**: **A, B** = 2 mm. **C** = 1 mm. **D** = 200 µm. **E** =100 µm.

Summing-up, although little is still known about sound perception mechanism in invertebrates [29-31, 33-35], recent behavioural and electrophysiological experiments conducted on cephalopods confirmed the species sensitivity to frequencies under 400Hz [30-32, 36] and the important role of statocysts in sound reception (18-20, 30, 31). While there is little uncertainty regarding the biological significance of particle motion sensitivity versus

acoustic pressure, the question is how particle motion is detected and transmitted to the statocysts from any acoustic source.

It was suggested that the particle motion can encompass the whole body of cephalopods and causes it to move with the same phase and amplitude: the statolith organs would be then stimulated by whole-body displacements [29, 35]. The statolith organs are inertial detectors in which a calcareous statocyst is attached to the sensory hair cells. When an animal accelerates, the statolith moves, bending the sensory hair cells. Thus, the statolith could serve as a receptor of kinetic sound components [29, 35]. However, to the best of our knowledge, there has been no study that showed that the whole body can vibrate when stimulated by underwater sound in aquatic invertebrates, or may be neither in aquatic vertebrates such as fishes.

2. LASER DOPPLER VIBROMETER ANALYSIS

The availability of novel laser Doppler vibrometer techniques has recently opened the possibility to measure whole body (distance, velocity and acceleration) vibration, as a direct stimulus eliciting statocyst response, offering the scientific community a new level of understanding of the marine invertebrate hearing mechanism. These techniques have already been applied to several species such as amphibians, reptiles and crustaceans [37, 38, 39]. Although, some preliminary experiments have been conducted using the laser Doppler techniques, no measurement of the whole body vibration induced by underwater sound was performed.

Under AQUO subtask 4.2.3, we conducted a series of measurements in controlled experimental conditions aiming at determining if cuttlefish and scallop bodies vibrate when stimulated with pure tone sounds. There was little doubt that marine invertebrates were sensitive to (i.e. perceive) low frequency sounds and that this sensitivity was not directly linked to sound pressure but particle motion. The missing component in the analysis was the demonstration that the statolith would act as a harmonic oscillator, excited when the whole animal body was vibrating when exposed to sound waves.

Figure 1. Laser Doppler Vibrometer Experimental Design. - Laser vibrometer (Polytec OFV-505 with OFV-5000 controller, water tank (80 x 30 x 40 cm), Agilent wave generator, loudspeaker and commercial amplifier.

The protocol included defining (and resolving) sources of uncertainties:

- influence of room and tank wall vibration
- choice of targets
- Laser Doppler Vibrometer calibration
- choice of frequencies
- distribution of acoustic pressure inside the tank
- position of the target

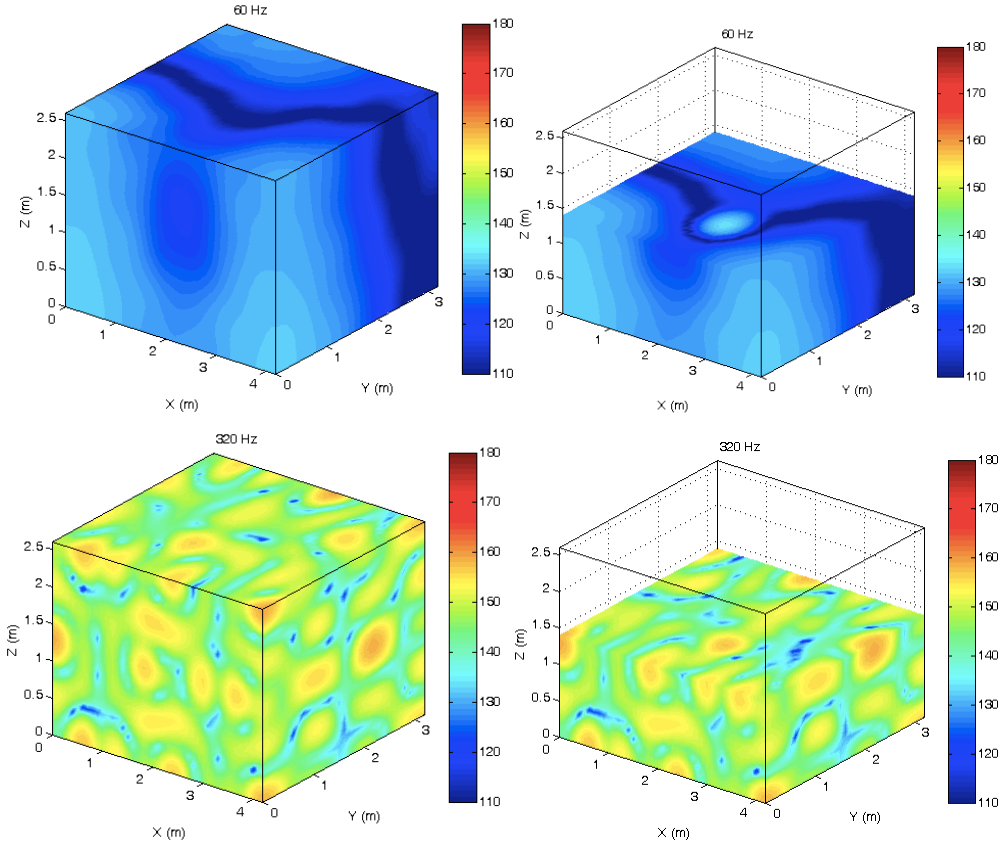


Figure 2. Sound field in the room where the experiments took place. The sound field in a closed space (i.e. room) with rigid walls is characterized by the normal modes obtained from the wave equation. Sound pressure in the enclosure (room) for the frequencies of 60 Hz and 320 Hz. Left plots: walls of the enclosure. Right plots: horizontal plane at the z position of the source.

2.1. The sound field inside the water tank

The water tank is again an enclosure but with one open wall. For the directions x and y there would be normal modes as we found in the room. However, since for the z direction there was no upper wall, in the direction z the condition of null particle velocity in this plane was not applicable. The sound field inside the tank was not only due to the propagation of the sound from the loudspeaker (direct field), and the modal field inside the room, but also due to the vibration of the walls of the tank.

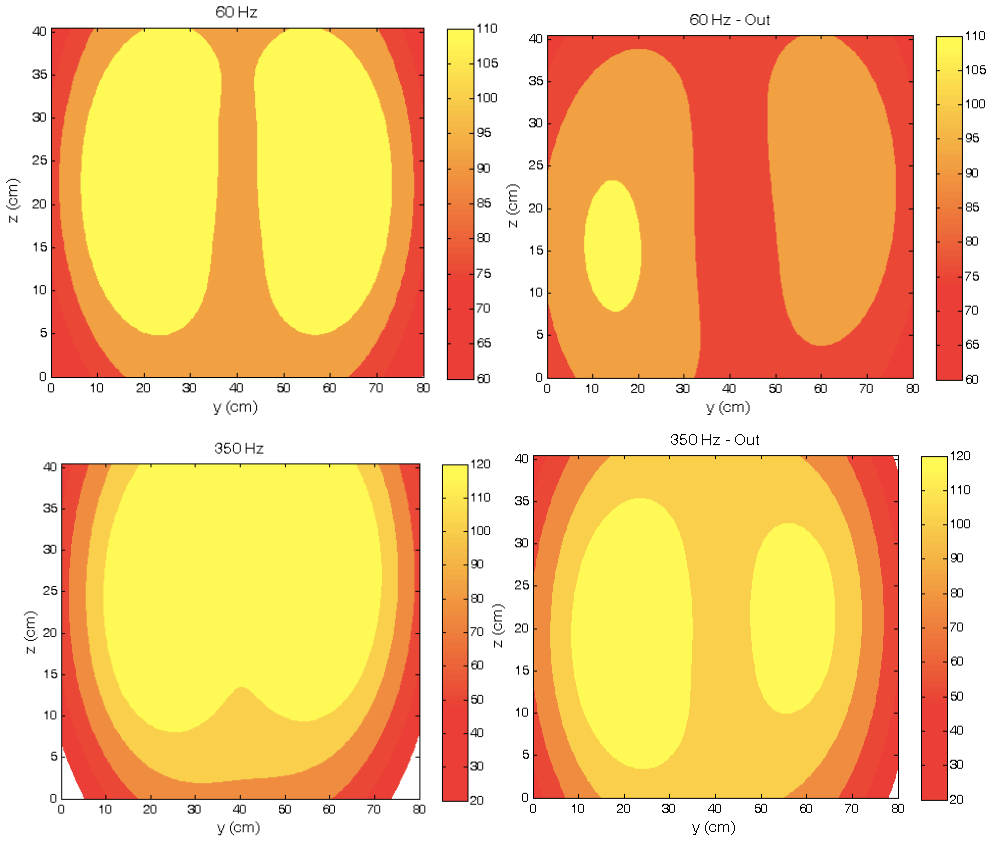
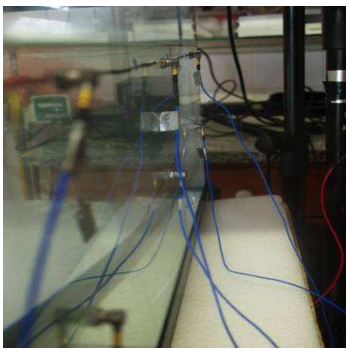


Figure 3. Acceleration of vibration in the front face of the water tank for the frequencies of 60 Hz and 350 Hz. Left plots: Loudspeaker inside the room. Right plots: Loudspeaker outside the room.



Measurements were taken at the walls of the tank to help visualize the vibration pattern of these walls at the frequencies of study and experimental conditions

2.2. Laser Doppler Vibrometer Calibration

Laser calibration was done using a vibration source working at 160 Hz and 9.8 m/s^2 (140 dB re 1e-6 m/s^2).

Vibration laser accelerometer		La (dB ref 1e-6 m/s^2)	
	60 Hz	120 Hz	320 Hz
x	70.2	92.0	104.7
y	70.9	76.8	97.7
z	82.4	96.4	113.1

Table 2-1: Laser calibration

The vibration measurement of a target was considered to be reliable if it was at least 10 dB higher than the vibration of the laser.

Hz	60	80	100	110	120	130	150	170	190	240	320	350	360
Bar	101	96	110	113	98	104	115	108	105	113	136	113	119
Scallop	100	97	109	113	96	102	109	118	95	110	136	114	115
Cuttlefish	103	100	114	114	106	106	116	112	107	115	138	108	115
Tape	106	104	116	118	109	107	114	112	108	119	140	115	117

Table 2-2: Acceleration (peak value) of targets at different frequencies in dB re 1e-6 m^2/s^4/Hz. Here, we chose 60Hz, 120Hz (MSFD) and 320Hz (higher resonance value) as the frequencies to be tested.

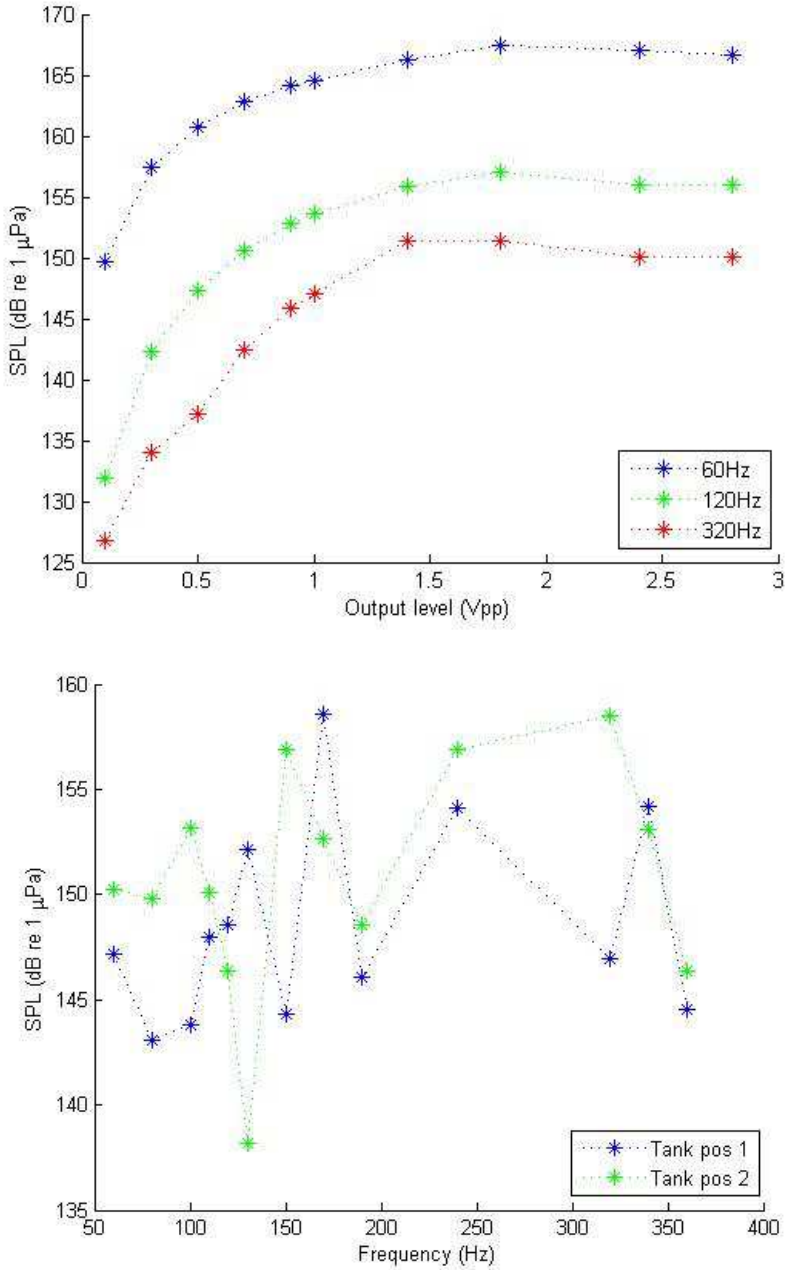


Figure 4. Sound pressure levels measured directly under the loudspeaker (pos 2) and the target position (pos 1) for all tested frequencies (up) and levels measured for different wave generator amplitudes at the target position (down).

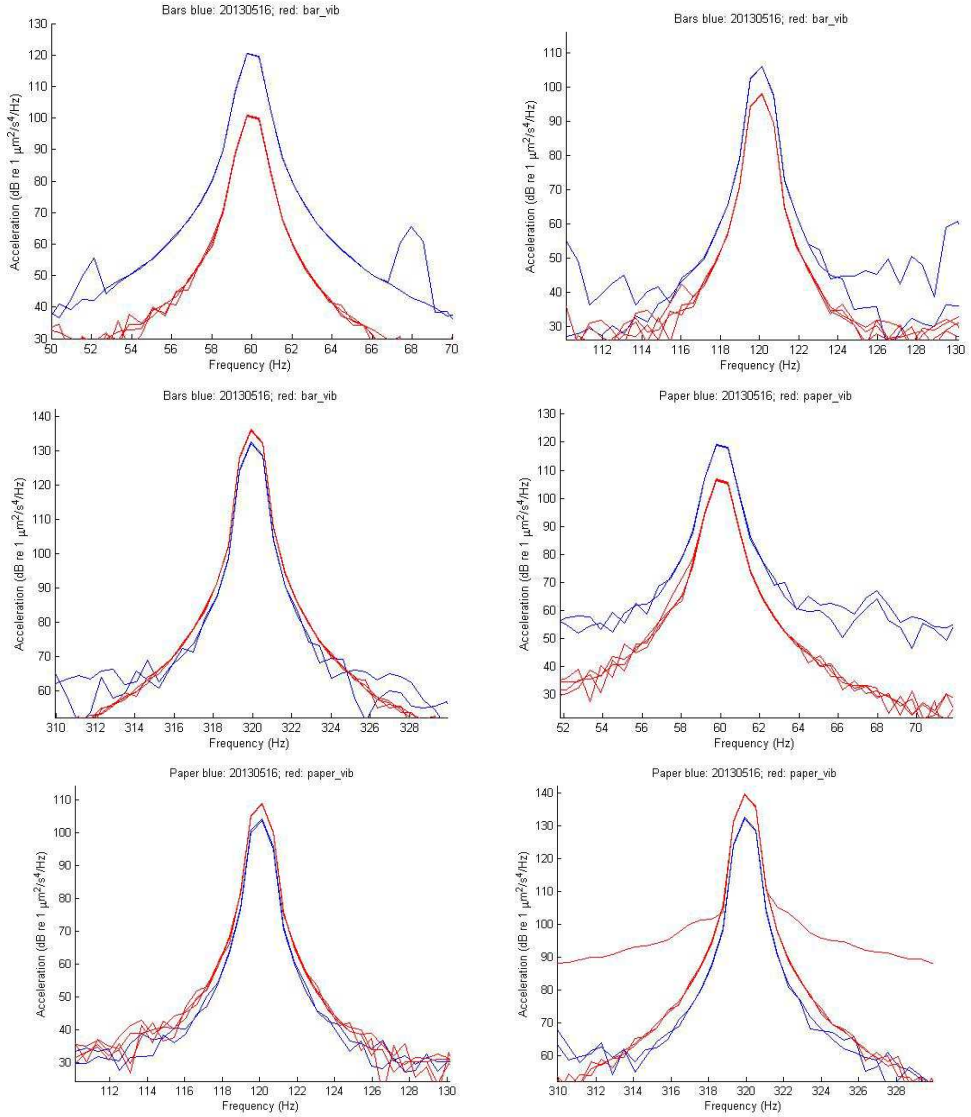


Figure 5. The consistency of the measurements and the effect of the precise position of the target were tested by measuring the inanimate targets at slightly different positions

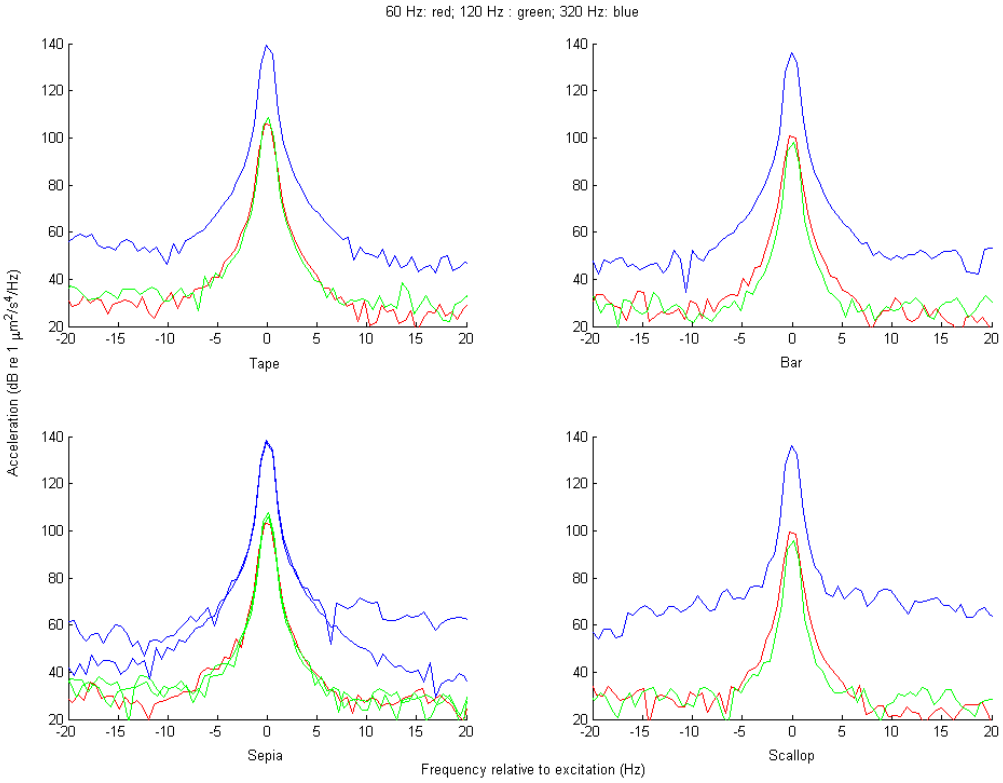


Figure 6. Measurement results of the four targets (reference targets and animals) at the three chosen frequencies, 60, 120 and 320 Hz

The differences of the laser Doppler acceleration measurements with the target vibration measurements allowed us to conclude that the exposure to sound has elicited the cephalopods and scallops whole body vibration, confirming the initial hypothesis that the particle motion can encompass the whole body of cephalopods and causes it to move with the same phase and amplitude [29, 35, 40]. It also consolidated the use of Laser Doppler techniques in underwater bioacoustics studies.

3. ANALYSIS OF LESIONS USING IMAGING TECHNIQUES

In our previous laboratory experiments, when exposed to relatively low intensity low frequency sounds, Controlled Exposure Experiments (CEE) revealed lesions which took place in the sensory epithelia of the statocysts' inner structures of four species of cephalopods: common Mediterranean cuttlefish (*S. officinalis),* common octopus (*Octopus vulgaris),* European squid (*Loligo vulgaris)* and southern shortfin squid (*Illex coindetii)* [18-20].

The aim of these experiments was to contribute to a better understanding of the onset mechanism of the acoustic trauma and the effects of noise, in sea conditions, on cephalopods by comparatively describing the ultrastructure of *S. officinalis* sensory epithelium after exposure to the same previous stimuli. The analysis uses imaging techniques.

3.1. Cephalopod specimens

Eighteen individuals from *S. officinalis* (mantle length 12-20 cm) were used immediately after been obtained from the Catalan coast (NW Mediterranean Sea). They were maintained a few hours in the gears for cephalopod collection used by the fishermen until the sound exposure in the sea.
Several specimens (see below) were used as controls and were kept in the same conditions as the experimental animals until being exposed to noise.

3.2. Sound Exposure Protocol

Sequential Controlled Exposure Experiments (CEE) were conducted on adult individuals. An additional set of 6 individuals was used as a control. The same sequential CEEs were conducted as with other cephalopods spp. studied in previous experiments [20, 21]. The difference here is that, since the results from the analysis with *S. officinalis* showed lesions immediately after noise exposure, and incremental effects up to 96 hours (longest period of observation), we concentrated the study on animals sacrificed immediately after and 48 hours after exposure, thus reducing the number of specimens used in the experiments**.**

The exposure consisted of a 50-400 Hz sinusoidal wave sweeps with 100% duty cycle and a 1-second sweep period for two hours. The sweep was produced and amplified through Lubell Transducer, while the received level was measured at each depth by a calibrated SMID hydrophone (Fig. 7). The cages with the animals were placed at 7m, 12m and 17m depth (Fig. 7) close to the OBSEA platform (also used in WP1) in front of our laboratory in the Vilanova i la Geltrú, Barcelona. Particle motion measurements were also conducted at the same locations and depths where the individuals were exposed. Some of the animals were used as controls and were kept in the same conditions as the experimental animals until the latter were exposed to noise, in an independent trap 2,5 km away from the site of experiments.

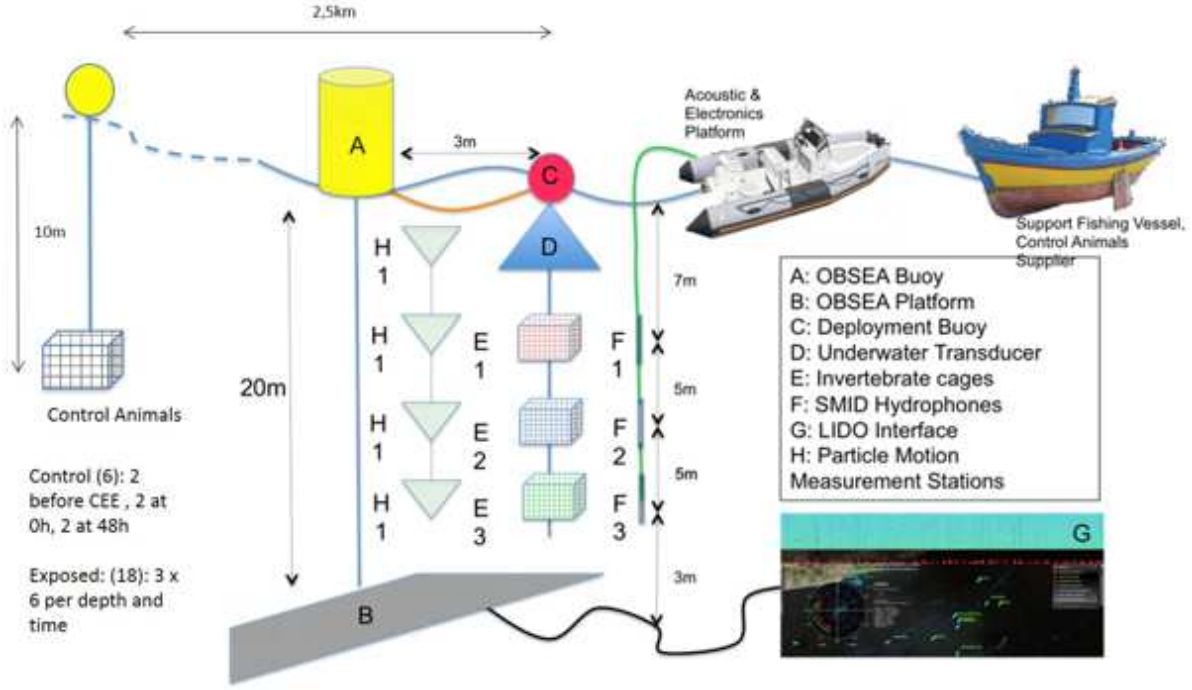


Figure 7. Drawing of experimental protocol

The sacrificing process was identical for controls and exposed animals. After the exposure, the individuals that were not immediately sacrificed were placed in traps in the same location in the sea where we had performed the experiments. The control animals were moved to traps in the same location of the exposed to sound animals that were not immediately sacrificed. Following exposure, the samples were obtained from the individuals (exposed and controls) at 0h and 48h after sound exposure. An additional set of 2 control animals were sacrificed before the experiments to obtain statocyst samples.

As stated in previous publications [18-20], it must be reiterated here that the experiment was not set up to find specific threshold levels, but designed to investigate if *S. officinalis* in offshore conditions would present similar acoustic lesions, as found in animals exposed to a similar stimulus in laboratory conditions.

3.3. Removal of statocysts

In all experiments, isolated head preparations were obtained by decapitation. The experimental protocol strictly followed the necessary precautions to comply with the current ethical and welfare considerations when dealing with cephalopods in scientific experimentation ([41], **DIRECTIVE 2010/63/EU** [42]**, REAL DECRETO 53/2013** [43]**, ORDEN ECC/566/2015** [44]).

This process was also carefully analyzed and approved by an external expert in Ethics (see AQUO report on Ethics). The statocysts with their surrounding cartilage were extracted and fixed for observation and analysis. For fixation, the statocyst cavity was opened and special care was taken to prevent mechanical damage to the inner tissues. The analysis was performed on tissues obtained from left and right statocysts.

3.4. Imaging Techniques

The same imaging techniques were used as in previous works [20, 21]: individuals were processed according to routine SEM procedures (Scanning Electron Microscopy).

3.4.1. Scanning electron microscopy

Forty-eight statocysts from twenty-four *S. officinalis* were used for this study. Fixation was performed in glutaraldehyde 2, 5 % for 24-48h at 4ºC. Statocysts were dehydrated in graded alcohol solutions and critical-point dried with liquid carbon dioxide in a Leica EmCPD030 unit (Leica Mycrosystems, Austria). The dried statocysts were cut open and flattened out to expose the statocyst structures and then mounted on specimen stubs with double-sided tape. The mounted tissues were gold-palladium coated with a Polaron SC500 sputter coated unit (Quorum Technologies, Ltd.) and viewed with a variable pressure Hitachi S3500N scanning electron microscope (Hitachi High-Technologies Co., Ltd, Japan) at an accelerating voltage of 5kV in the Institute of Marine Sciences of the Spanish Research Council (CSIC) facilities.

3.4.2. Quantification and Data analysis

We considered for the analysis the region comprising the whole sensory area of the macula *statica princeps*, the biggest subunit of the macula-statolith system. The length of the area comprising hair cells was determined for each sample, and 2500 μm^2 (50 x 50 µm) sampling squares were placed along the center length of the area at 5, 25, 50, 75 and 95% of the length axe of the macula *statica princeps* (Fig.8).

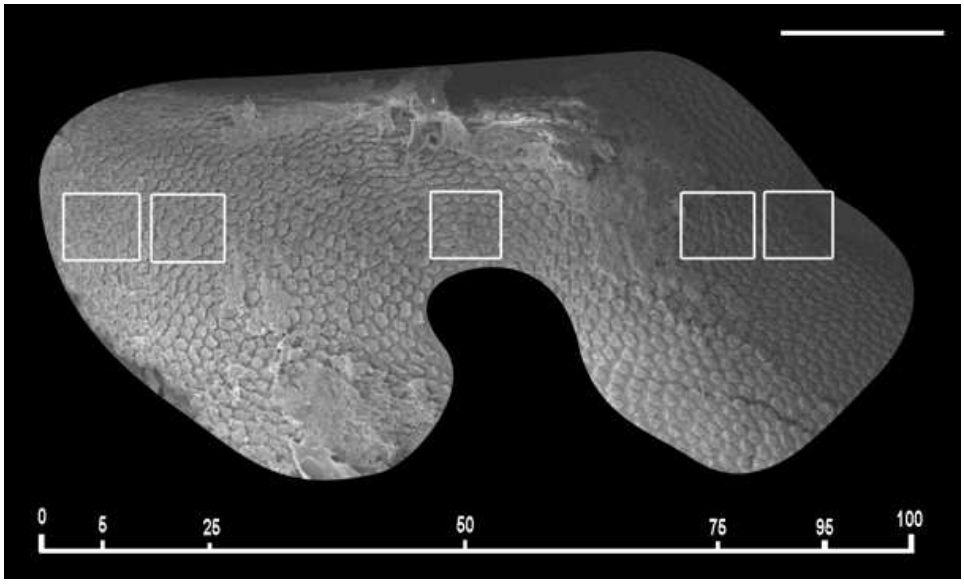


Figure 8. SEM. *Sepia officinalis* macula static princeps. Hair cell bundles count locations on the *S. officinalis* macula statica princeps. Hair cells counts were sampled at five predetermined locations: 5, 25, 50, 75 and 95% of the total macular length. A 2500 μm^2 box was placed at each sampling area and hair cells were counted within each box.
Scale bar = 100 µm.

We were able to observe some abnormal features on the surface of sound-exposed epithelia, as well as differences in hair cells appearance. Hair cell damage was analyzed by classifying the hair cells as intact (hair cell undamaged), damaged (bundle of kinocillia partially or entirely missing, bentor fussed), extruded (hair cell partially extruded of the epithelium) and missing (hole in the epithelium caused by the total extrusion of the hair cell).

Damage was quantified as the percentage of extruded and missing hair cells. The damaged category included a wide range of different types of lesions with different severities; this makes direct comparison between animals more difficult. The extruded and missing categories are well-defined easier to compare and the presence of extruded cells shows the limit of severe damage after sound exposure.

Damage due to sound exposure was tested using permutation tests. Data was summed over all regions. Damage was measured as the percentage of extruded and missing hair cells. Permutation tests were repeated multiple times with N=1000 (test groups: control animals vs exposed animals (0h all distances). The influence of distance to transducer was tested using Kruskal-Wallis analysis of variance test and permutation tests repeated multiple times with N=1000 (test groups: exposed animals 0h separated per distance).

Although we appreciated an evident increase in the extension and severity in the quantification of the lesions in the macula *statica princeps*, like in the previous laboratory experiments, we did not test the variables concerning animals sacrificed 48h after sound exposure because of the impossibility to control the environmental conditions effects the animals were exposed to.

3.4.3. Ultrastructural analysis of the Sepia officinalis macula statica princeps sensory epithelium

From right after until 48h after sound exposure (Figure 9 B-H) in comparison with the same tissues from control animals (Figure 9A), damage was observed on the *macula statica princeps* (*msp)* sensory epithelium, by SEM analysis.

For the analysis done just after the sound exposure:

- There were spherical holes on the base of the hair cells and a rupture of the plasma membrane, probably due to the extrusion of the internal cellular material (Figure 9D, arrowhead).
- Some hair cells had lost a number of kinocilia (Figure 9E, white asterisk) or showed bent and flaccid kinocilia (Figure 9B, arrowhead).
- The microvilli was bent flaccid and disorganized in all samples (Figure 9B, arrow).
- In some cases the bundle of kinocillia of the hair cells are completely fused (Figure 9C).
- The hair cells were partially (Figure 9F-H, black asterisks) or totally ejected (Figure 9F, white asterisk) from the sensory epithelium; in the late case the holes left on the epithelium are visible ejected (Figure 9F, white asterisk).

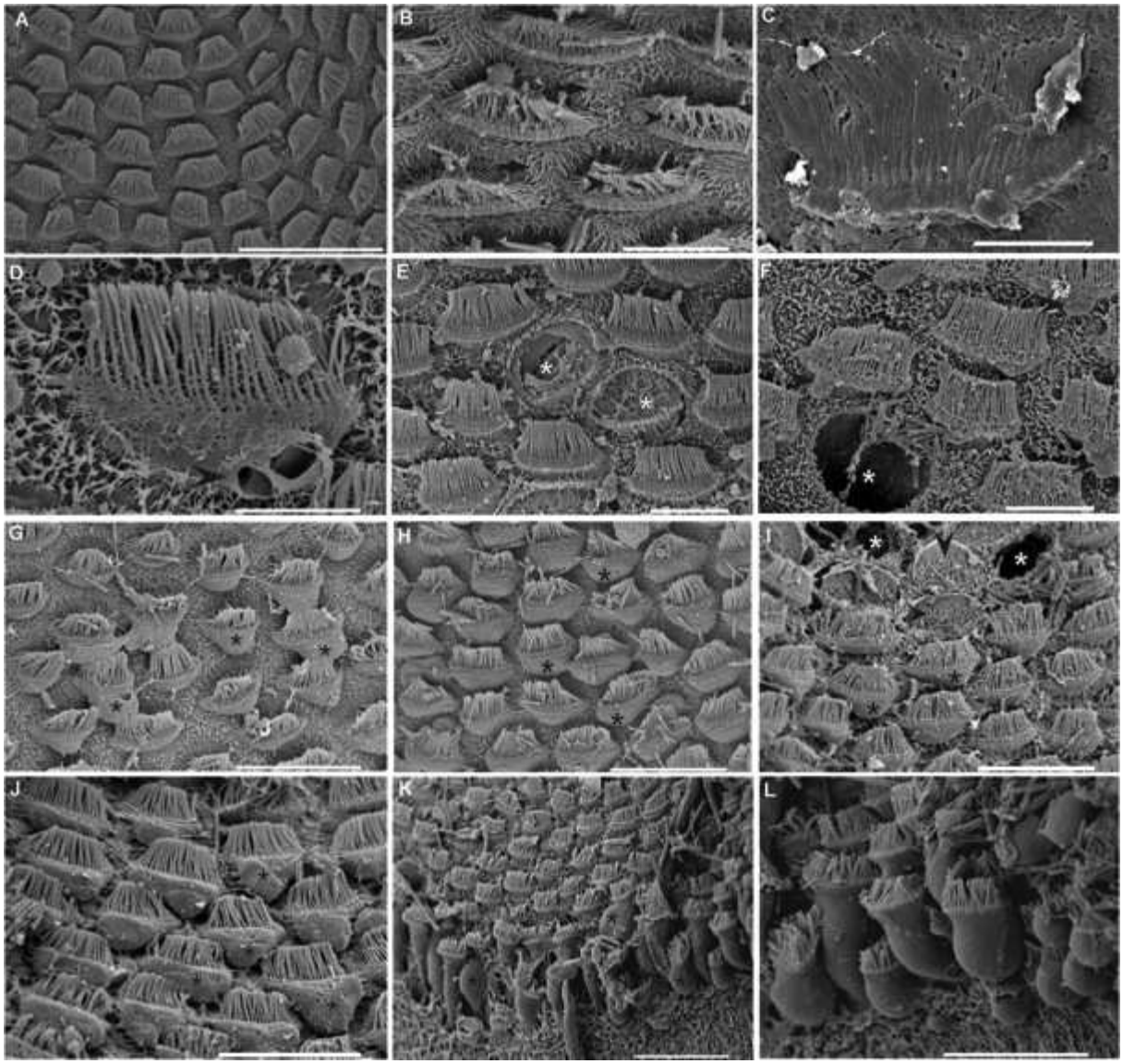


Figure 9. SEM. *S. officinalis* macula statica princeps (msp). A: control animals. B - H: immediately after sound exposure. I-L: 48h after sound exposure.

A: The arrangements of the kinociliary groups of the hair cells in regular lines following the epithelium shape are visible. **B**: Hair cells present bent and flaccid kinocillia (arrowhead) and disorganized microvilli (arrow). **C**: A hair cell presents its bundle of kinocillia totally fused.
D: A hair cell shows spherical holes on the base and rupture the plasma membrane (arrowhead). **E**: Some hair cells have lost the bundle of kinocillia (white asterisk).
F: A hole on the epithelium due to a hair cells extrusion is visible (white asterisk).
G, H: The apical poles of the hair cell extruded above the epithelium in the statocyst cavity are visible (black asterisk). In **G** some kinocillia of different hair cells are fused**. I**: The apical pole of some hair cells are extruded into the statocyst cavity (black asterisks). Some hair cells have been totally ejected lefting holes on the sensory epithelium (white asterisks). Arrowheads signs to some hair cells that have lost the bundle of kinocillia. **J:** A large section of sensory epithelia presents all its hair cells extruded above the epithelium (black asterisks). **K**: Almost all the cell body of hair cells is ejected from a large region of the sensory epithelium (black asterisks). **L**: Detail from K shows the cell body of the hair cells extruded. **Scale bars: A, K** = 30 µm. **B, E, F** = 10 µm. **C, D** = 5 µm. **G-J, L** = 20 µm

In animals sacrificed 48h after sound exposure (Fig. 9 I-L):

- The sensory epithelium of the msp presented the same lesions but the time after the sound exposure increased its gravity and extension.
- Hair cells partially or totally ejected from the sensory epithelium are visible (Fig. 9 I-H black asterisks), letting holes on the epithelium ejected (Figure 9 I, white asterisk).
- The apical ciliated apex and part of the cellular body were extruded above the sensory epithelium into the statocyst cavity (Fig. 9 I-H black asterisks).
- Some hair cells had totally, or in a considerable number, lost the kinocilia (Fig. 9 4I arrowheads) or exhibited bent kinocilia.
- In some regions almost the totally cellular body is visible in the process of extrusion of the sensory epithelium (Fig. 9K, L).

3.4.4. Image and Data analysis

The abnormal features we identified on the surface of sound-exposure epithelia included damaged hair cell (bundle of kinocillia partially or entirely missing or fussed), extruded (hair cell partially extruded of the epithelium) and missing hair cell (hole in the epithelium caused by the total extrusion of the hair cell). The number of damaged, extruded and missing was counted for each image. The number of damaged, extruded and missing hair cell increased with time and decrease with the distance to the transducer (Figure 10, 11).

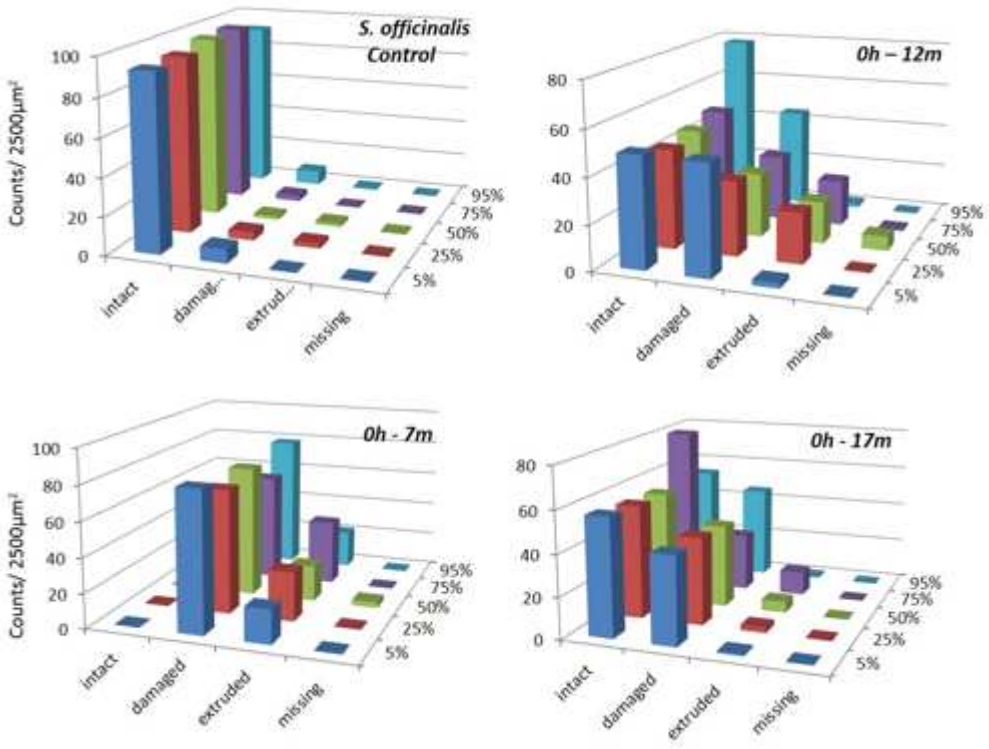


Figure 10. Mean intact hair cell, damaged hair cell, extruded hair cell and missing hair cell as a function of time after sound exposure at 5, 25, 50, 75 and 95% of the total length of macula statica princeps of *S. officinalis* (0h after sound exposure at 7m, 12m and 17m of depth versus control). Note the increase of damage, extruded and missing cells versus control animals with increase of time and with decrease of distance to transducer.

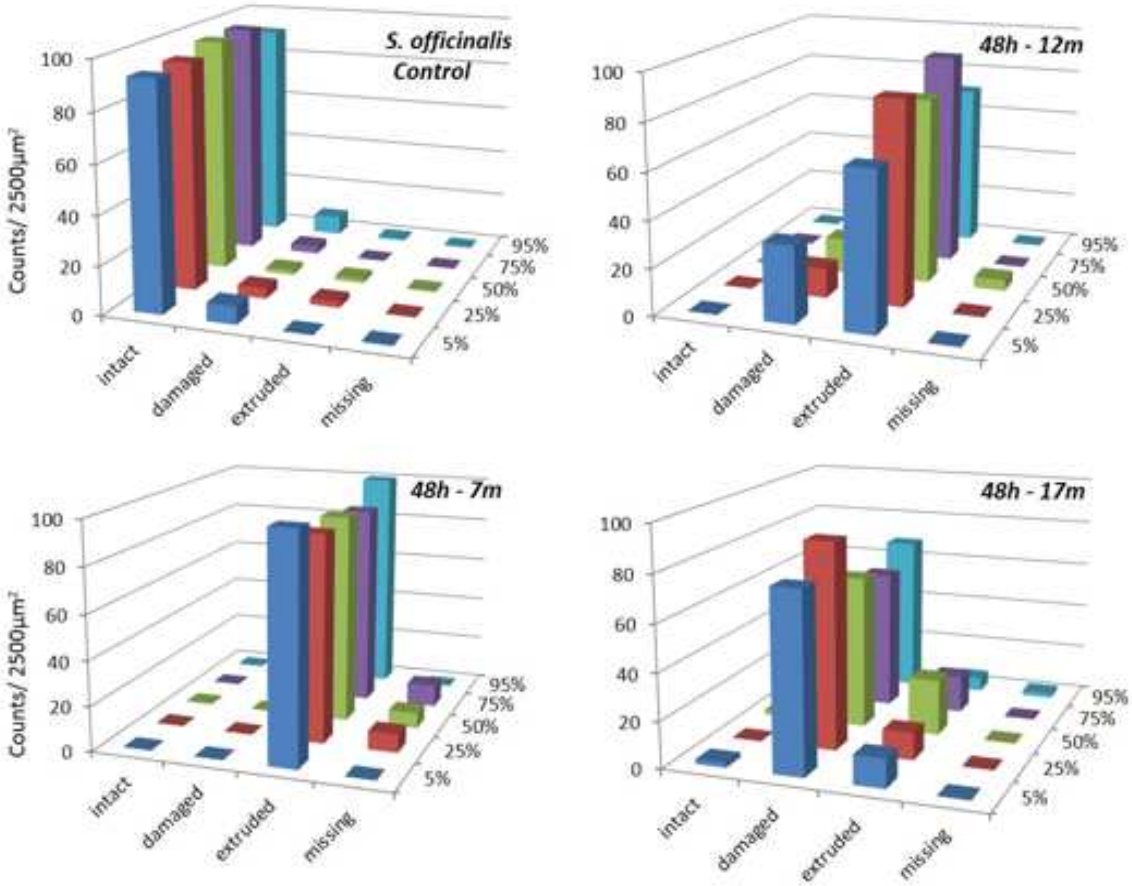


Figure 11. Mean intact hair cell, damaged hair cell, extruded hair cell and missing hair cell as a function of time after sound exposure at 5, 25, 50, 75 and 95% of the total length of macula statica princeps of *S. officinalis* (48h after sound exposure at 7m, 12m and 17m of depth versus control). Note the increase of damage, extruded and missing cells versus control animals with increase of time and with decrease of distance to transducer.

Damage was quantified as the percentage of extruded and missing hair cells because categories are well-defined, easier to compare and the presence of extruded cells show the limit of severe damage after sound exposure. After statistical test, the quantification shows that there were effects on macula static princeps ephitelium by exposure to sound (p= 0,002) (Figure 12A).

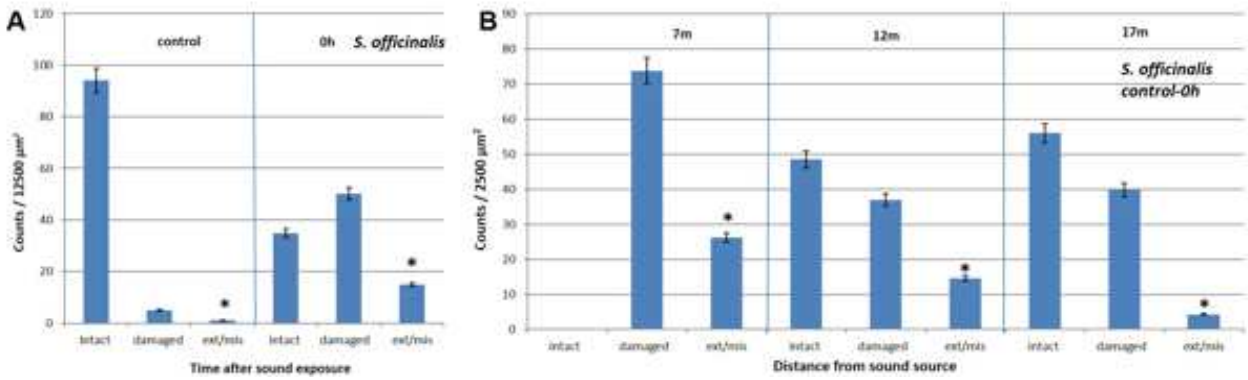


Figure 12. A: Mean (±SE) intact hair cell, damaged hair cell, extruded/missing hair cells on macula statica princeps ephitelium of *S. officinalis*, 0h after sound exposure versus control (*p = 0,002). **B**: Mean (±SE) intact hair cell, damaged hair cell, extruded/missing hair cells on macula statica princeps ephitelium of *S. officinalis*, 0h after sound exposure, in function of the distance to the transducer. Permutation test 7m-2m (*p = 0.005); permutation test 7m - 17m (*p= 0.005); permutation test 12m - 17m (*p = 0.001).

We concluded that the distance to the transducer time after exposure has also effects on the level of the lesions in *S. officinalis* macula static princeps epithelium (Figure 12B). The level of lesions quantified as number of extruded/missing hair cells at 0h after sound exposure decreased with distance. Kruskal-Wallis analysis of variance shows that the three groups (7m, 12m and 17m depth) do not share the same median (p= 0.007).

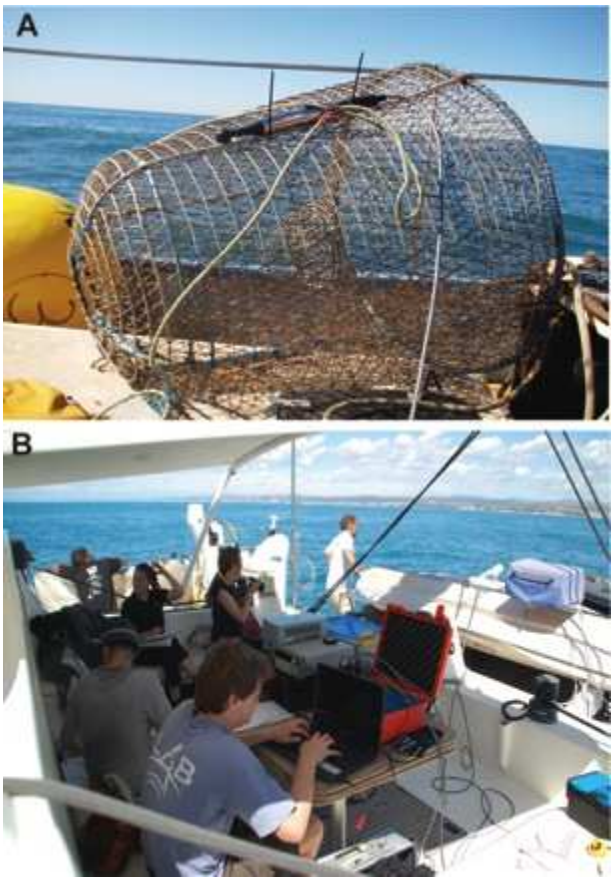


Figure 13. A: Position of the SMID hydrophone on the cage; **B**: ship platform from where the experiments were conducted

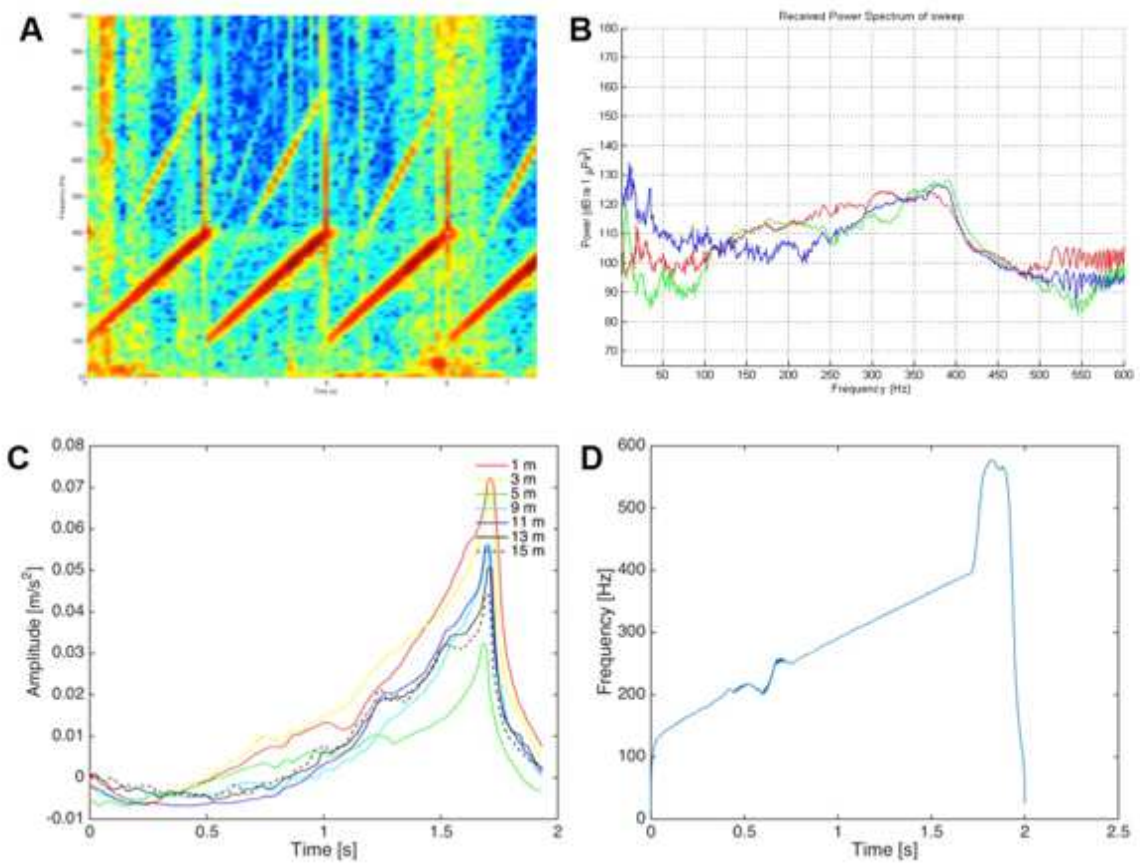


Figure 14. A: Spectrogram of the sweeps **B:** Power spectrum of the sweeps on all channels averaged over 8 seconds (4 sweeps) with a 1 Hz resolution **C, D:** The total amplitude and the frequency was calculated using Hilbert transform. The amplitudes (total sum of x, y and z component) are decreasing with increasing distance between the source and the sensor, except for 5 m, which was assumed to be due to some unknown error handling.

4. CONCLUSION

While concern about the effects on man-made noise on marine environment is increasing, there is an urgent need to define and quantify the added spatial-temporal variability of acoustic pollution from different sources and identify the resulting short, medium or long-term changes and effects on marine fauna. However, we face the relative lack of information on the sound processing and analysis mechanisms in marine organisms, particularly in cephalopods, and we still do not know enough about the important role they play in the balance and development of populations. In the context of noise pollution, and in particular ship noise, its potential and direct impacts on cephalopods are in many cases speculative. However, ship noise may potentially affect them.

The laboratory experiments conducted before the start of AQUO Project indicated the potential effects that sound exposure could have on cephalopods sensory organs: the statocysts. Many important questions were remaining, amongst them:

- what is the role of particle motion on cephalopod sound perception?
- how the sound vibration is transmitted to the statocysts?
- is the acoustic trauma found in laboratory exposed individuals repeatable in open sea conditions?
- can we establish threshold values that would trigger acoustic trauma in cephalopods?

The study undertaken within Subtask 4.2.3 has allowed answering most of these questions, although the latter still needs further research.

We conducted offshore noise exposure comparative experiments on common cuttlefish (*Sepia officinalis*), in similar conditions as during the laboratory study, in terms of sound characteristics, received levels and time exposure.

Particle motion measurements were also conducted, both in laboratory conditions, as well as at the same locations and depths where the individuals were exposed at sea.

Scanning electron microscopy (SEM) revealed similar injuries in the inner structure of the statocysts, as those found in cuttlefish in previous experiments.

As for the particle motion data, the amplitudes (total sum of x, y and z components) were decreasing with increasing distance between the sensor. These findings support the validity of our previous results in laboratory conditions.

Although the lesions were quantified versus received noise levels and particle motion data, the analysis cannot yet determine threshold levels that would trigger the acoustic trauma. Acknowledging the validity of an experimental approach in laboratory conditions, this will constitute the next step of this research.

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