TECHNICAL REPORT

Lab experimental setting for analysis of *Mytilus galloprovincialis*' behaviour after wave-generated sounds exposure

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ABSTRACT

The present paper proposes an experimental lab setting and an innovative method in order to evaluate the behavioural reactions of Mediterranean mussels (*Mytilus galloprovincialis*) exposed to different acoustic stimuli. Ninety mussels were exposed for 30 minutes to one of five acoustic tones repeated in continuous or to the control condition. The tones lasted 1 s and differed in frequency band: Low frequencies-sweep (L, 0.1 - 5 kHz); Mid-Low frequencies-sweep (ML, 5 - 10 kHz); Mid frequencies-sweep (M, 10 - 20 kHz); Mid-High frequencies-sweep (MH, 20 - 40 kHz); High frequencies-sweep (H, 40 - 60 kHz). Experimental test sweeps were produced using a signal generator connected to underwater loudspeakers and a calibrated hydrophone was used to obtain acoustic recordings of sweeps and experimental tank background noise. An underwater camera, placed overhead the animals, recorded the mussels' behaviours. A continuous visual sampling procedure, performed by an observatory, was adopted to detect the mussel gape rate and the outpouching of the muscular foot while an automatic analysis was performed using EthoVision XT 9.0 software. The behavioural parameters assessed automatically included changes in mobility status, movement, velocity and turn angle that are usually estimated in vertebrates and higher invertebrates.

1. BACKGROUND

Sound detection is considered a primary sensory modality, an important component of vital intraspecific interactions (Webster et al., 1992; Fay and Popper, 1998; Au et al., 2000) and a key way to detect the surrounding environment mainly for vertebrates. However, the ability of marine invertebrates to detect and potentially use sound is poorly understood (Budelmann, 1992a, b; Mooney et al., 2012). Yet a growing body of literature suggests that marine invertebrates respond to sound in a variety of ways (Vermeij et al., 2010; Simpson et al., 2011; Filiciotto et al., 2014, Celi et al., 2013, 2015). For example, clams, *Donax variabilis*, responded to sound stimuli in a laboratory aquarium by jumping out of the sand, lying on the sand for several seconds, and digging in again (Ellers, 1995). Despite a burgeoning literature, there is a poor understanding of the frequencies and levels of sounds that generate functional responses in most invertebrates and thresholds have rarely been established. Until now there are no evidences about the mussel sound detection and/or behavioural reactions. Moreover, there are no lab techniques and methods to evaluate mussel behaviour and only Robson et al. (2007) proposed a new method for quantifying bivalve behaviour in *Mytilus edulis*. In particular, they employed a novel technique to quantify how blue mussels react to predation risk in their environment by quantifying mussel gape using a Hall sensor attached to one shell valve reacting to a magnet attached to the other.

The class Bivalvia is one of six classes of molluscs and comprises animals enclosed in two shell valves. Examples are mussels, oysters, scallops and clams. The Mediterranean mussel *(Mytilus galloprovincialis*) is one of the principal species which collectively are widely distributed from temperate to subarctic coasts of both the Northern and Southern Hemispheres.

The Mediterranean mussel generally lives in infralittoral areas, from the top of the intertidal zone to depths of few meters, attached to hard materials (rocks or piles) or substrates relatively movable (ropes) which adheres by sing fine linen tending to congregate each other and forming colonies also very numerous. Ambient noise could represent a very important external factor able to affect some biological activities of this species.

1.1. Objectives

The present work aims to propose an experimental lab setting and an innovative method in order to evaluate the behavioural reactions of Mediterranean mussels (*M. galloprovincialis*) exposed to different acoustic stimuli.

Mussel behaviour was evaluated by estimating changes in mobility status, movement, velocity and

turn angle that are usually assessed in vertebrates and higher invertebrates, after 30 minutes of exposure to five sweep tones of different frequency range. Moreover, the mussel gape rate and the outpouching of the muscular foot were assessed using a continuous visual sampling procedure.
The experiment set up presented here wants to give a new approach to measure indirectly the sound detection ability of mussels and the potential impact of acoustic exposure on some biological aspects in this species.

2. MATERIALS AND METHODS

2.1. Animals and husbandry

The present study was carried out at the Institute for Marine and Coastal Environment of the National Research Council (CNR-IAMC) facilities of Messina between April and June 2015.
Ninety mussels (*Mytilus galloprovincialis*) of 44.2 ± 6.99 g in weight (mean ± SD), 8.39 ± 0.49 cm in length of valve maximum axes (mean ± SD) and 3.87 ± 0.29 cm in length of valve minimum axes (mean ± SD) were used for the experiments. Animals were collected from a local farm and raised at the Marine and Coastal Environment Institute of the National Research Council (IAMC-CNR) of Messina, Italy.
Prior to the experiment, mussel specimens were maintained in a 5 m^3 (2.3 m diameter, flat bottom, 1.15 m depth) circular fibreglass tank. The tank was equipped with a flow-through system (with a complete water renewal each hour) of sea water. The water quality parameters were maintained in a range that was suitable for gilthead sea bream (an ambient water temperature of 20.06 ± 2.01 °C (mean ± SD) and a salinity of 38.31 ± 0.22 ‰ (mean ± SD) were maintained over the course of the husbandry.

2.2. Experimental set-up and protocol

The same basic experimental set-up was used for all trials (Fig. 1).

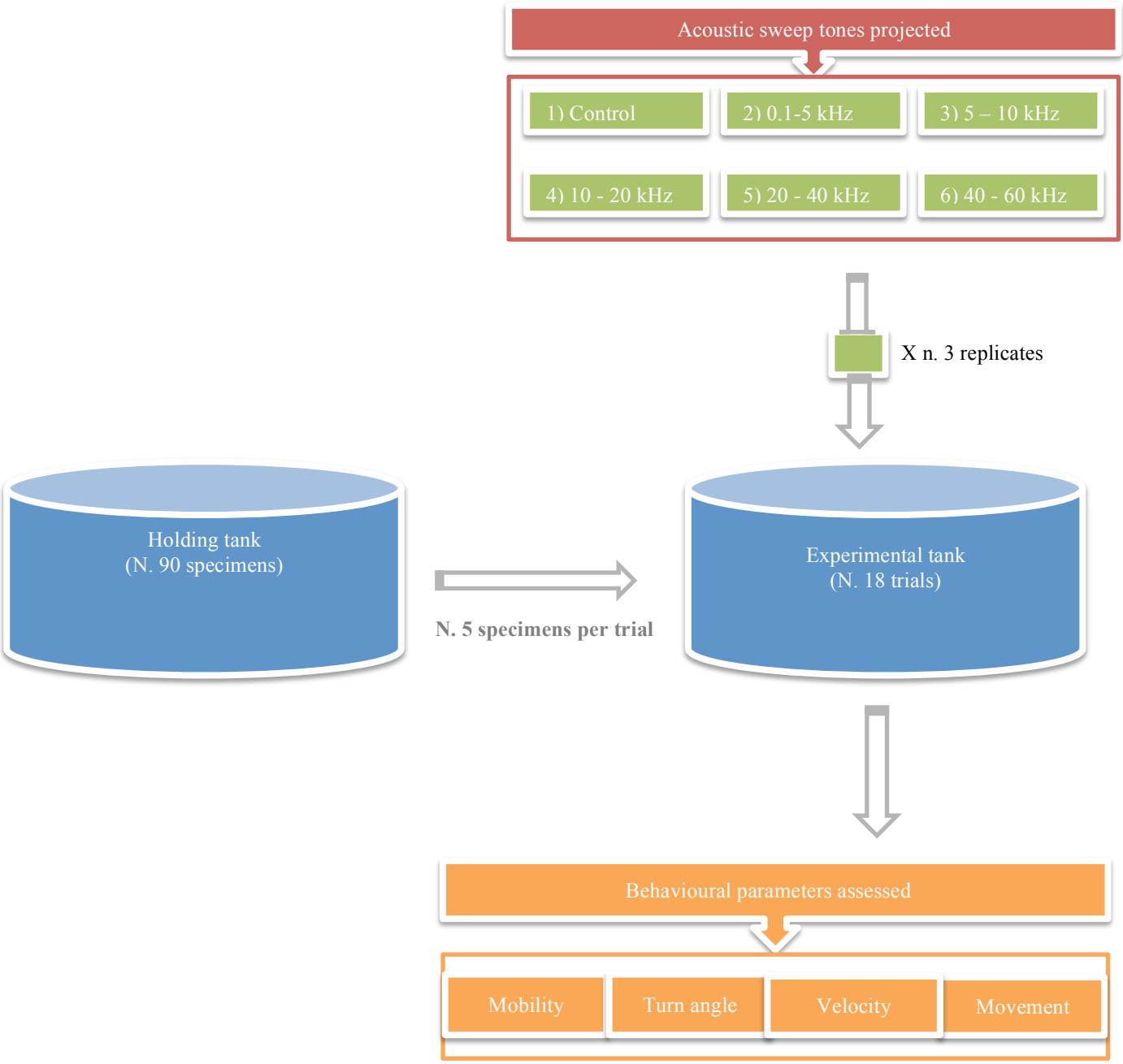


Figure 1. Schematic view of the experimental trial design.

Experimental trials took place in a fiberglass tank that was identical in size, shape and water composition to the holding tanks.

After the acclimation period, the mussels were collected randomly, one by one, from the holding tank and assigned to the trial. Five mussels were assigned to each experimental trial. A total of 18 trials were performed to test 6 experimental acoustic conditions; each acoustic condition was replicated in 3 trials.

Mussels were released in the experimental tank bottom on a plastic support equipped with a net in order to allow the anchorage of animals (Figure 2).

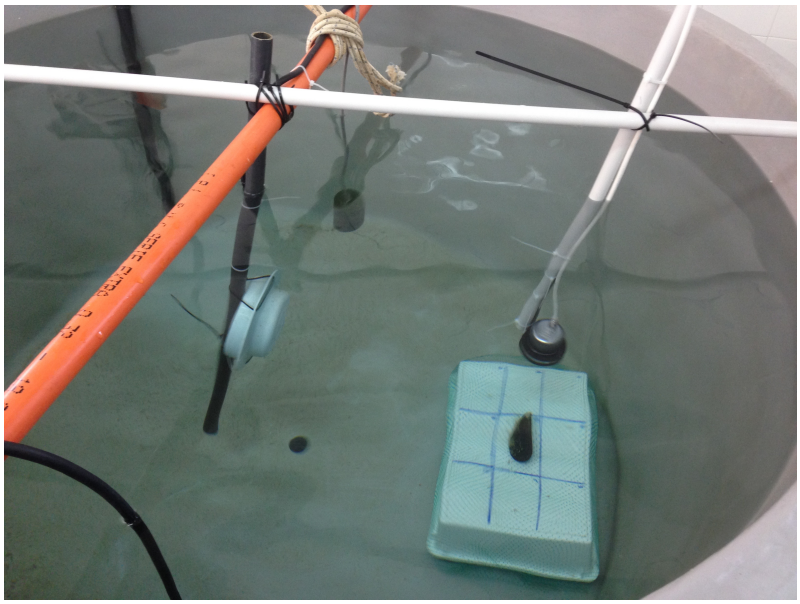


Figure 2. Plastic support covered by a net in order to allow the anchorage of mussels.

The total duration of each trial was 60 minutes; the experimental phase included pre-test (30 minutes) and during-test (30 minutes) periods.

Throughout the during-test phase, the mussels were exposed to one of the five acoustic tones repeated in continuous or to the control condition. The tones lasted 1 s and differed in frequency band: Low frequencies-sweep (L, 0.1 - 5 kHz); Mid-Low frequencies-sweep (ML, 5 - 10 kHz); Mid frequencies-sweep (M, 10 - 20 kHz); Mid-High frequencies-sweep (MH, 20 - 40 kHz); High frequencies-sweep (H, 40 - 60 kHz). Figure 3 shows the spectrograms of the different projected acoustic sweep tones.

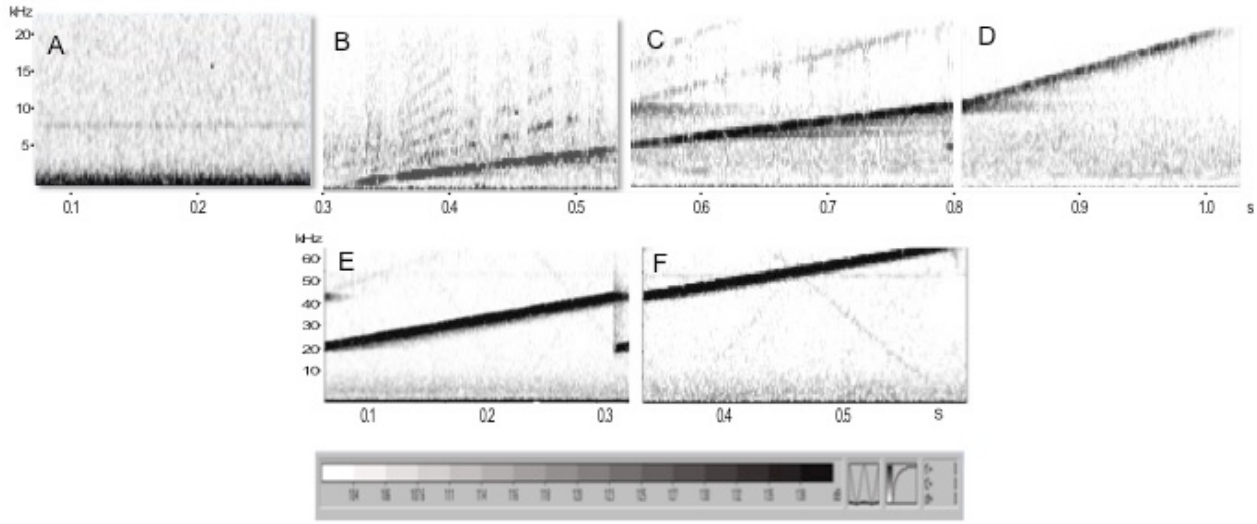


Figure 3. Spectrogram of the different sweep tones and the experimental tank background noise: (A) Experimental tank background noise; (B) sweep tone L in the frequency range 0.1 – 5 kHz; (C) sweep tone ML in the frequency range 5 – 10 kHz; (D) sweep tone M in the frequency range 10 –

20 kHz; (E) sweep tone MH in the frequency range 20 – 40 kHz; (F) sweep tone H in the frequency range 40 – 60 kHz.
Frequency (kHz) vs. time (s). The intensity is reflected by a grey scale (dB re 1 lParms, 1024-sample FlatDown window, sampling frequency 300 kHz).

The order in which the mussel groups were tested to a specific frequency-sound level combination was randomized every day, with the condition all trials should be carried out both in the morning and in the afternoon.
All trials were audio and video recorded using an acoustic generation/acquisition system and an underwater camera. To avoid disturbing the animals inside the experimental tank, a laboratory enclosure was placed 2 m away from the tank, and the audio-video equipment was installed there.
The aquaculture experimental plant is authorized by the Ministry of Health with the decree number 105/2014-A to use animals for experimental or other scientific purposes.

2.3. Sweep tones generation and acoustic recording system

Experimental test sweeps were produced using a signal wave generator (model 33220, Agilent Technologies, Santa Clara, CA, United States) connected to underwater loudspeakers. The loudspeaker UW30 (Lubell, Columbus, Ohio, USA) was employed for L, ML and M frequency sweeps projection, and a cylindrical fiberglass loudspeaker, assembled by the Bioacoustics Group of CNR-IAMC of Capo Granitola, was used for the MH and H frequency sweeps.
A calibrated hydrophone (model 8104, Brüel & Kjær, Nærum, Denmark) with a sensitivity of 205.6 ± 4.0 dB re 1V/µPa in the 0.1 Hz and 80 kHz frequency bands was used to obtain the acoustic recordings of sweeps and the experimental tank background noise. The hydrophone was used with a preamplifier (VP1000, Reson) with a 1-MHz bandwidth single-ended voltage that had a high-pass filter set at 10 Hz and a 32-dB gain. The equipment was connected to a digital acquisition card (USGH416HB, Avisoft Bioacoustics, set with no gain) managed by the Avisoft Recorder USGH software (Avisoft Bioacoustics). Signals were acquired at 300 kilosamples s^{-1} at 16 bits and visualized by the Avisoft-SASLab Pro software (Avisoft Bioacoustics).
The acoustic projection system arrangement is showed in Figure 4.

Figure 4. The equipment used for the acoustic stimuli projection in tank. The loudspeaker UW30 (left) was employed for L, ML and M frequency sweeps projection, and the cylindrical fiberglass loudspeaker (right) was used for the MH and H frequency sweeps.

2.4. Video monitoring system and analysis

Videos for the purpose of behavioural monitoring were recorded with an underwater camera (Skynet Italia s.r.l.) placed overhead the animals as showed in Figure 2. The camera was linked to a 4Channel LCD-DVR With 7 Inch Screen - H.264 (Fig. 5).

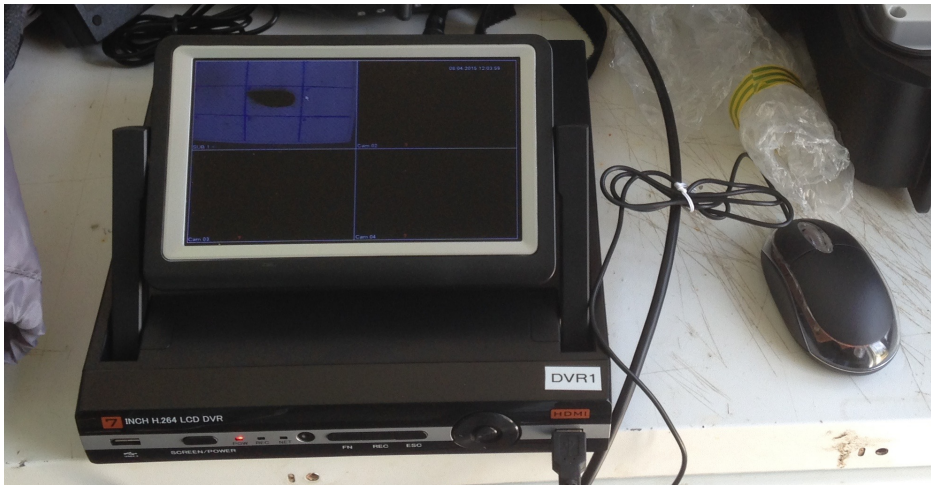


Figure 5. LCD-DVR system connected to underwater camera adopted for the video acquisition and storing.

A continuous visual sampling procedure (each event observed was annotated in a sampling table) performed by an observatory was adopted to detect the mussel gape rate and the outpouching of the muscular foot.

After the manual analysis of videos, an automatic analysis were performed using EthoVision XT 9.0 (Noldus Information Technology Inc., Wageningen, The Netherlands), which is software that distinguishes the object from the background on the basis of grey scale/brightness values, and extracts the coordinates of the geometric centre and surface area for each object per frame (see Figure 6).

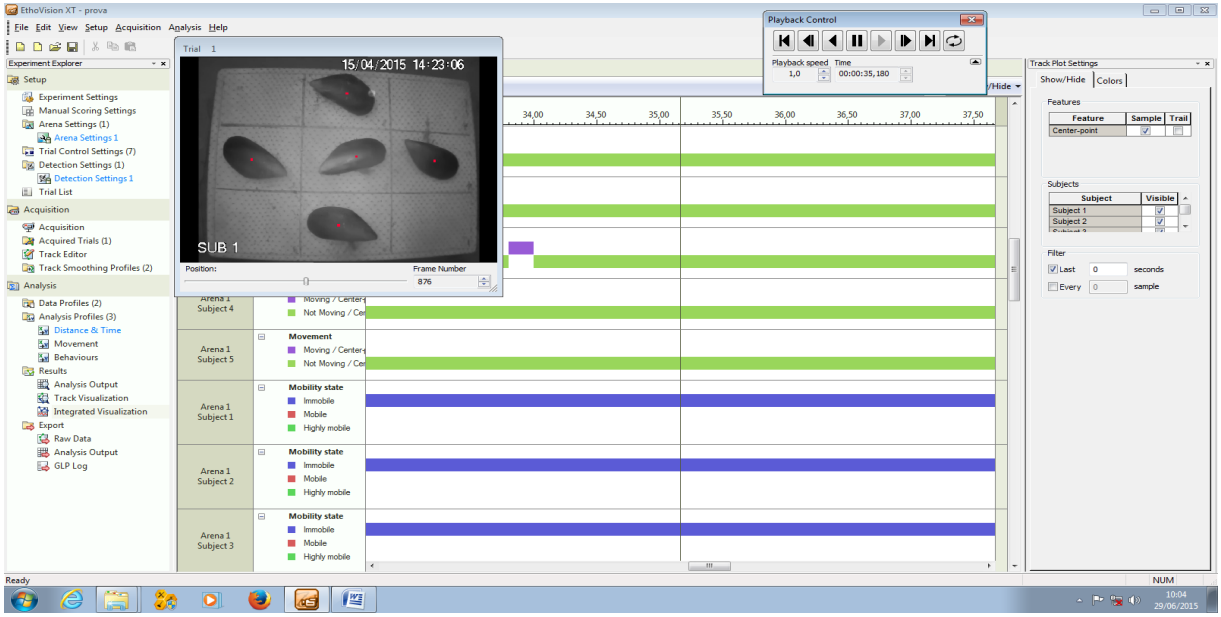


Figure 6. The mussels are distinguished from the background on the basis of grey scale/brightness values.

The experimental area was calibrated by inserting the measures across the plastic support inside the experimental tank, and the calibration axes were placed to designate the origin (0, 0) at the support centre (Figure 7).

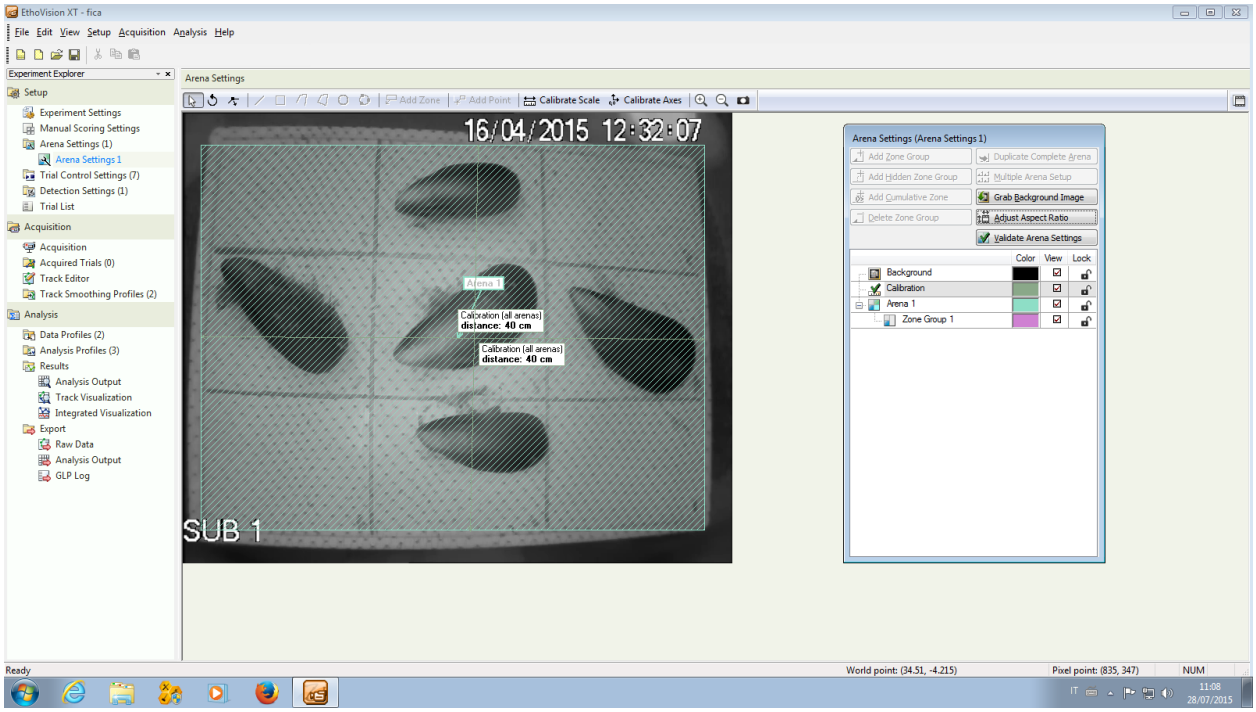


Figure 7. Setting of the experimental area with EthoVision XT 9.0

The mussels were identified individually by using a color marker tool of EthoVision XT 9.0 as showed in Figure 8.

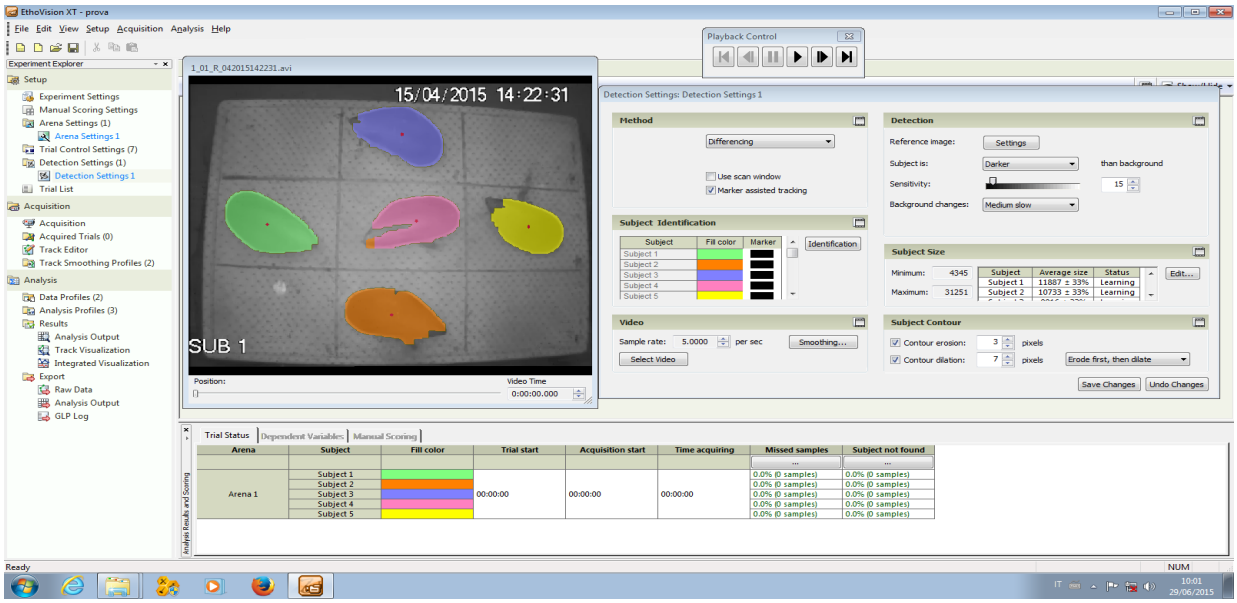
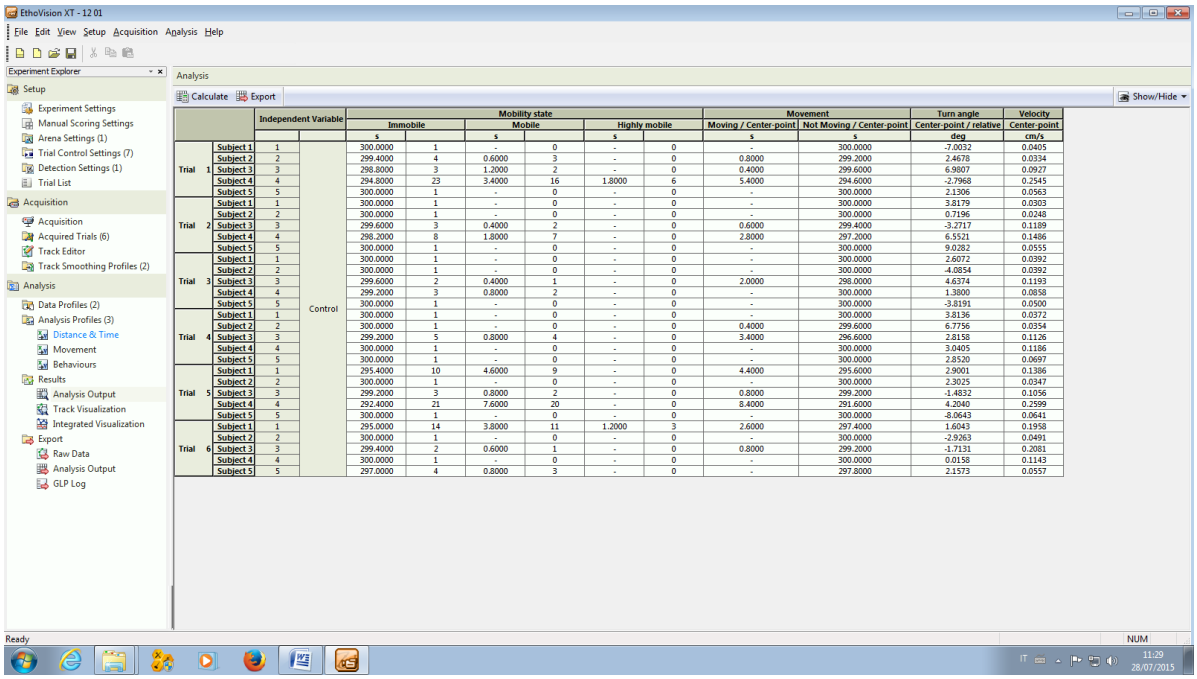


Figure 8. The mussels are identified individually with the color marker tool of EthoVision XT 9.0.

Usually, the software analysis is performed with a high frame resolution (25 per second) to distinguish the animals from the background on the basis of their grey scale/brightness values. Here,

considering the very slow movement of mussels, the setting analysis was changed in 5 frames per second.

The behavioural data were exported to Excel to generate 5-minute means of all of the extracted data (Fig. 9).



		Independent Variable	Mobility state						Movement		Turn angle	Velocity
			Immobile		Mobile		Highly mobile		Moving / Center-point	Not Moving / Center-point	Center-point / relative	Center-point
			s		s		s		s	s	deg	cm/s
Trial 1	Subject 1	1	300.0000	1	-	0	-	0	-	300.0000	-7.0032	0.0405
	Subject 2	2	299.4000	4	0.6000	3	-	0	0.8000	299.2000	2.4678	0.0334
	Subject 3	3	298.8000	3	1.2000	2	-	0	0.4000	299.6000	6.9807	0.0927
	Subject 4	4	294.8000	23	3.4000	16	1.8000	6	5.4000	294.6000	-2.7968	0.2545
	Subject 5	5	300.0000	1	-	0	-	0	-	300.0000	2.1306	0.0563
Trial 2	Subject 1	1	300.0000	1	-	0	-	0	-	300.0000	3.8179	0.0303
	Subject 2	2	300.0000	1	-	0	-	0	-	300.0000	0.7196	0.0248
	Subject 3	3	299.6000	3	0.4000	2	-	0	0.6000	299.4000	-3.2717	0.1189
	Subject 4	4	298.2000	8	1.8000	7	-	0	2.8000	297.2000	6.5521	0.1486
	Subject 5	5	300.0000	1	-	0	-	0	-	300.0000	9.0282	0.0555
Trial 3	Subject 1	1	300.0000	1	-	0	-	0	-	300.0000	2.6072	0.0392
	Subject 2	2	300.0000	1	-	0	-	0	-	300.0000	-4.0854	0.0392
	Subject 3	3	299.6000	2	0.4000	1	-	0	2.0000	298.0000	4.6374	0.1193
	Subject 4	4	299.2000	3	0.8000	2	-	0	-	300.0000	1.3800	0.0858
	Subject 5	5	300.0000	1	-	0	-	0	-	300.0000	-3.8191	0.0500
Trial 4	Subject 1	1	300.0000	1	-	0	-	0	-	300.0000	3.8136	0.0372
	Subject 2	2	300.0000	1	-	0	-	0	0.4000	299.6000	6.7756	0.0354
	Subject 3	3	299.2000	5	0.8000	4	-	0	3.4000	296.6000	2.8158	0.1126
	Subject 4	4	300.0000	1	-	0	-	0	-	300.0000	3.0405	0.1186
	Subject 5	5	300.0000	1	-	0	-	0	-	300.0000	2.8520	0.0697
Trial 5	Subject 1	1	295.4000	10	4.6000	9	-	0	4.4000	295.6000	2.9001	0.1386
	Subject 2	2	300.0000	1	-	0	-	0	-	300.0000	2.3025	0.0347
	Subject 3	3	299.2000	3	0.8000	2	-	0	0.8000	299.2000	-1.4832	0.1056
	Subject 4	4	292.4000	21	7.6000	20	-	0	8.4000	291.6000	4.2040	0.2599
	Subject 5	5	300.0000	1	-	0	-	0	-	300.0000	-8.0643	0.0641
Trial 6	Subject 1	1	295.0000	14	3.8000	11	1.2000	3	2.6000	297.4000	1.6043	0.1958
	Subject 2	2	300.0000	1	-	0	-	0	-	300.0000	-2.9263	0.0491
	Subject 3	3	299.4000	2	0.6000	1	-	0	0.8000	299.2000	-1.7131	0.2081
	Subject 4	4	300.0000	1	-	0	-	0	-	300.0000	0.0158	0.1143
	Subject 5	5	297.0000	4	0.8000	3	-	0	-	297.8000	2.1573	0.0557

Control

Figure 9. Data of the behavioural parameters analyzed are exported to Excel.

The automatic measurements involved the following behaviors: Mobility state, Movement, Velocity and Turn angle. Below is reported a complete description of the behavioural parameters analyzed with software:

- Mobility state: Calculates the cumulative duration for which the complete area detected as animal is changing, even if the center point remains the same; the parameter is measured in seconds.
- Movement: Moving if the subject running mean velocity exceeds the start velocity of 0.90 cm/s, the state then becomes Not Moving if the subject running average velocity set below of 0.70 cm/s; the parameter is measured in centimeters/seconds.
- Velocity: Mean distance moved by the center point of the subject per unit time; the parameter is measured in centimeters/seconds.
- Turn angle: the change in direction of the centre direction line between two consecutive samples; the parameter is measured in angular degree.

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