PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVII and XXXVIII cycles)

Name of PhD student: Domenico Sacco

Title of PhD research: Development of new experimental approaches for the study of the responses of marine organisms to multiple anthropogenic impacts

Name of PhD supervisor: Prof.ssa Silvia Bianchelli
Research lab name: Laboratory of Marine Biology and Ecology

[ ] XXXVI
[ ] XXXVII
[X] XXXVIII

PhD Curriculum::
[X] Marine biology and ecology
[ ] Biomolecular Sciences
[ ] Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:
[X] Actea Mobile Laboratory
[ ] Advanced Instrumentation lab
[X] Aquarium
[ ] MassSpec lab
[ ] MaSBiC
[ ] Simulation/informatics lab
[ ] Other. Please, indicate: .....

ABSTRACT (1000 characters, including spaces):

My PhD project focuses on the development of new processes and approaches for experimental studies in marine ecology and is divided into five main branches. They are all based on the use of aquaria, mesocosms and field activities (also integrated) for experiments.
In the first year of my PhD, I focused on new approaches of marine restoration of *Gongolaria barbata*. This approach is based on the use of stranded individuals onshore as an opportunity to obtain new recruits *ex situ* to be outplanted at sea. The first part of the experiment was conducted in aquarium. The second part was carried out at sea, initially by placing the structures in the ocean, and subsequently by monitoring the growth rate and coverage of juveniles.
In the meantime, the experimental setting for the second year' experiments has been projected.

Part 1. Scientific case of the PhD Research (2 to 3 pages, including figures)

- BACKGROUND

Understanding the basic biological functions of organisms is essential not only to comprehend their roles in ecosystems but also to manage and protect their populations. Studying biological processes such as growth, reproduction, and physiology, which can be addressed either in situ or by collecting specimens and raising them in aquariums, is particularly challenging for deep-sea organisms. Therefore, currently, the best ways to obtain foundational biological information about these organisms are (1) working with collected samples and analyzing them post-mortem and/or (2) maintaining them in aquariums to monitor biological processes and investigate behavior and physiological responses under different experimental treatments.

It is evident that aquarium experiments cannot perfectly replicate the real environmental and trophic conditions where these organisms are found. However, (1) in most cases, we do not have the opportunity to obtain equivalent in situ information because species at depths beyond a certain point are difficult to observe for extended periods, and (2) even with limitations, they yield valuable insights into the biological constraints of the species, which is particularly valuable when considering potential future scenarios of environmental restoration and climate change.

- SCIENTIFIC AIMS

The research is divided into 5 parts, with the common factor being the use of aquaria, mesocosms and field activities (also integrated) to develop new technological processes for the study of marine ecology. The controlled environment provided by aquaria, although artificial, allows for extended observation of marine organisms and enhances our understanding of certain aspects of their life cycles that are valuable for future marine restoration projects. The first fundamental step is to improve long-term maintenance while ensuring the highest standards of animal welfare

In this framework the scientific aims are to:

1) improve the technological and experimental infrastructure in marine ecological research and understand how integrated research, through the use of aquaria and mesocosms in the field, can answer questions related to multi-stress and multi-impact conditions and what are the future needs. Environmental conditions are also changing in deep sea, which requires technological development allowing the maintenance of organisms of deep and extreme environments;
2) evaluate multiple anthropogenic impacts, including cumulative and synergistic impacts due to changes in T and pH, as well as identify new model organisms to evaluate responses to impacts. Through the development of mesocosms, the effect of contaminants on food webs (*Cystoseira sp.*, filter feeders, crustaceans) in different areas of the Central Adriatic will be evaluated;
3) develop technological experimental systems for the maintenance of new models for scientific research with particular reference to ascidians;
4) test experimental systems for the upscaling of restoration interventions (macroalgae and seagrass) and date mussel;
5) develop experimental systems and technologies for the maintenance and manipulation of species in deep and extreme environments.

- WORKPLAN AND RESEARCH ACTIVITIES

The workplan is divided into 5 Workpackages (WPs), following the 5 main aims of the research.

Workpackge 1. Improve the technological and experimental infrastructure in marine ecological research.

Workpackage 2. Evaluation of multiple anthropogenic impacts.

Workpackage 3. develop technological experimental systems for the maintenance of new models for scientific research.

Workpackage 4. test experimental systems and approaches for the upscaling of restoration interventions.

Workpackage 5. develop experimental systems and technologies for the maintenance and manipulation of species in deep and extreme environments.

The WP1 is functional to the others.

WP 1. Objective. In agreement with EU biodiversity Strategy 2030, which requires to protect and restore the 30% of marine habitats by 2030. In the context of the project Marine Ecosystem Restoration (MARES, Spoke 2, Activity 2) funded by the National Recovery and Resilience Plan (PNRR), I have focused on improving the breeding techniques of *Gongolaria barbata* in an aquarium.

Methods

<u>In the aquarium</u>

Through preliminary observations we selected a donor site (DS), currently hosting a healthy population of *G. barbata*. We took few fertile apices to avoid impact on the donor population. The fertile apices were transferred to the aquarium facilities to allow the massive production of new juveniles. To guarantee the highest standards in the maintenance of marine organisms in a controlled environment, we will use LSS (Life Support System – Figure 1)

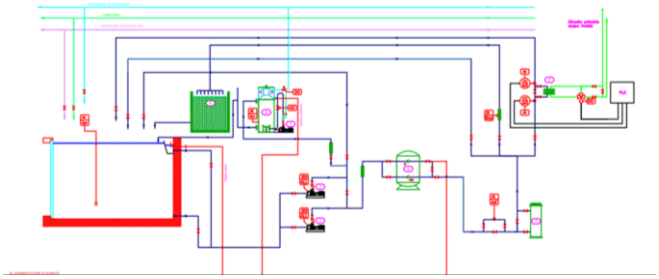

Figure 1 – LSS

On the 1° of June, 120 fertile apices of *G. barbata* were collected at the donor site of Scalaccia (43.607, 13.546)

The apices were stored for 24 h in dark and cold conditions (6°C)
Then, they were cleaned in filtered seawater to remove epiphytes
Finally, apices were placed into small bags (Figure 2)

Figure 2 - Small Bags

<u>Setting of the aquaria:</u>
Temperature = 20°C
Photoperiod = 15L:9D
Light intensity = 50-90 µmol photons m−2 s−1
Methodology = Aquarium Life Support System
Culture medium = Von Stosch + germanium dioxide
A total of 119 clay and 35 slides were placed in 7 tanks of 40 L, the bags with the apices were suspended in the aquaria to enhance zygotes'

During the first month, the early stages of *G. barbata* were checked on the slides to evaluate the development of zygotes and embryos
Evaluation of:
- length
- % coverage (Braun-Blanquet scale – Figure 3)

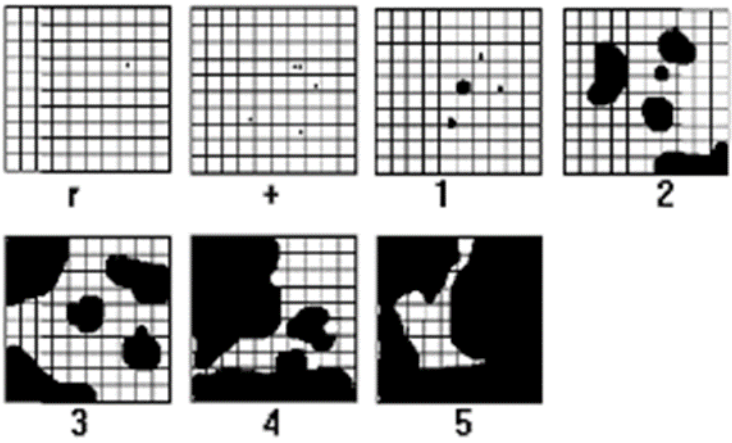

Figure 3 – Braun Blanquet scale

In the aquaria water exchanges were carried out twice a week and the following parameters were checked:
- temperature
- salinity
- pH
- dissolved oxygen

In the Aquarium after one month, when the juveniles were visible to the naked eye, it was measured the length using a ruler (through imageJ) and evaluated the % coverage on the tiles.

<u>In the field</u>

On the 25° of July, when the juveniles reached 1-2 mm in length, 54 tiles were selected to carry out the restoration intervention.
3 aluminium structures (Figure 4) were built for the anchoring of the tiles on the sea bottom (Figure 5). The selected restoration site was La Vela (43.559, 13.609).
Tiles were anchored to the seabed though an underwater drill.
The structures were fixed at depths ranging from 1 to 2 meters.

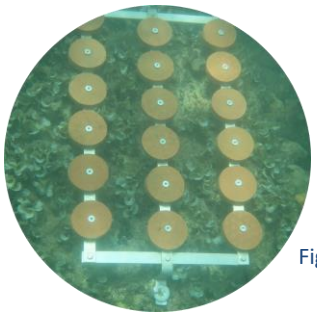
Figure 5 - Structures with tiles

Tiles were monitored once a month.
The following parameters were measured:
- length of the main axis and % coverage of juveniles on the tiles


Figure 4 – Aluminium structures

Expected/Obtained Results.

After the first month in the field, it was observed an increase in the length and % coverage of juveniles on the tiles.

Compared to juveniles in the aquaria, the juveniles in field showed a double greater growth rate.

Future Step

- Continuous monitoring throughout the year
- Collection of data on the associated biodiversity: epiphytic flora and associated biodiversity
- Comparison of length and % coverage with juveniles in the aquaria
- Comparison of associated biodiversity with natural populations

WP4. Objective. A "ping-pong" protocol was developed, based on the collection of stranded macroalgae, the use of their fertile apices to produce healthy zygotes and embryos, and the return of the juveniles back to the rocks (through ad hoc substrates) once reached a sufficient size. This demonstrates that recruits obtained from stranded specimens and maintained in mesocosms survive and grow vigorously in few weeks at rates similar to those collected in situ natural populations. The "ping-pong" protocol has the double advantage of obtained recruits without any contact or damage to the natural populations, and re-cover to a second life the stranded macroalgae that would be lost after stranding.

Methods

On 5th of April, along Sassonia beach (43,84° N – 13,02° E) adjacent to the Fano Marine Center (FMC) and on 13th of April, at two sites along the Fano beach (Lido 1 and Lido 2; 43,85° N - 13,01° E) (Fig. 1A and B) located in the city of Fano (Northern Adriatic Sea) fertile thalli of *Gongolaria barbata* were found stranded along the shore. Once collected, the thalli were immediately brought to the laboratory and stored in an aquarium tank of 40 L at the FMC. Some receptacles were taken from these thalli and observed at the stereomicroscope. Longitudinal and transversal sections were made through a razor blade to verify their fertility. The sections were observed using light microscopy.

Figure 4: A) Location of the study area in the Adriatic Sea; B) sites where the storm-detached specimens of Gongolaria barbata were collected.

Mesocosms' set-up for G. barbata *maintenance and reproduction*

The specimens were transported to the aquarium facility at the Polytechnic University of Marche (Ancona, Adriatic Sea) and were acclimatized before the positioning in the mesocosms. This process consisted of a slow mixing between the sea water used for the transport, rehydration of the algae and the water present in the tanks. This phase lasted two hours. Three mesocosms were used, one for each sampling site. At the beginning of the experiment the environmental data were: pH 8.37, temperature 16°C, salinity 37. The thalli were acclimated to 20 °C (i.e., the seawater temperature of April at the collection site). For determining the efficiency of beached reproductive thalli in releasing zygotes, inside each tank four clay tiles were located under the fertile specimens in order to capture as many zygotes as possible.

To guarantee the highest standards in the maintenance of algae in the mesocosms, we used the LSS (Life Support System). This system consists of three mesocosms of 50 L, a reserve in which there are 3 socks of 100 µm for mechanical filtration, immersed razor clams for biological filtration, Teco TK 500 cooler for maintaining the temperature. The light intensity was generated by two LED lamps (SilverMoon Marine 10 thousand Kelvin and SilverMoon Universal 6.5 thousand Kelvin) 40 cm away from the water head. Irradiance was measured with Photometer of the apogee Model MQ-500.

Light intensity and photoperiod were selected to reflect typical seasonal conditions during the reproductive phase of *G. barbata*. The photoperiod was set to a 15:9 h light:dark cycle, and light intensities

were chosen is 80-100 µmol photons m^{-2} s^{-1}. The cultivation medium was Von Stosch's enriched filtered seawater with addition of germanium dioxide, renewed every two weeks.
The main parameters (temperature, salinity, pH and light intensity) were monitored once a week. Furthermore, for routine maintenance of the system, water loading and unloading, lights, movement pumps, cooler, any water leaks at the pipe joints were checked. Moreover, to assure the sterilization of the system, once a week the socks were washed, tubs were siphoned to remove organic debris and it was done a water change of about 10% per week.

After two weeks, when the recruits were visible at the stereomicroscope, the clay tiles were observed once a week for 11 weeks, to follow the germlings' development, in term of length and coverage' class frequency. Germlings' length was measured to mm accuracy and the coverage of recruits was estimated considering the areas covered by germlings on 4 areas of each tile, observed at the stereomicroscope with magnification 6.4x.

Statistical analyses

The recruitment in controlled mesocosms was monitored for 11 weeks. Data on the juveniles' length (mm) and their percent coverage (expressed as 5 coverage classes: 1 = 5%; 2 = 5-25%; 3 = 25-50%; 4 = 50-75%; 5 = 75-100%) on the tiles were taken through an Olympus TG-6 camera and processed through ImageJ. Two-way repeated measuresTwo-way ANOVA design was applied to test for possible differences in the juveniles' length, considering the factors "tank" (corresponding to the sites Lido1, Lido2 and Sassonia) and "time" (from t1 to t11). Verification of the assumptions of normality (Shapiro-Wilk) were checked prior to conducting the analyses. Post-hoc comparisons on significant terms ($p < 0.05$) were performed by Tukey test. The Chi-Square ($\chi 2$) test of independence was used to check whether there was a significant association between the tank (Lido1, Lido2 and Sassonia) and the coverage classes' (1, 2, 3, 4 and 5) frequency. Statistical analyses were performed through the software jamovi 2.3 (jamovi project, 2022).

Results

Collected specimens reproductive traits

The collected specimens of *G. barbata* measured from approximately 20 to 50 cm in length (Fig. 2A). The receptacles are brought on terminal branchlets (Fig. 3B). They can be either solitary or branched (Fig. 2C). Their sizes range from 0.7 to just over 3 cm. The aerocystes, located at the base of the branchlets, can be single or two in chain, and measure 4.6 ± 0.5 mm (Fig. 2B).

In longitudinal section, inside the receptacles the number of conceptacles varies from 10 to 15, arranged in parallel rows (Fig. 2D). In cross-section, in the conceptacle there are oogonia and antheridia. The oogonia are dark and oval in shape, measuring on average 80 ± 20 µm in length. They are located in groups on the floor of the conceptacle. The antheridia, pigmented and poorly branched, measure approximately 20 ± 9 µm in length and are sited on the roof of the conceptacle near the ostiole (Fig. 2E).

Figure 5: A) Stranded specimen of G. barbata*; B) receptacles on terminal branchlets; C) variability of receptacles.*

Figure 6: Longitudinal section of a receptacle showing the conceptacles arranged in parallel rows.

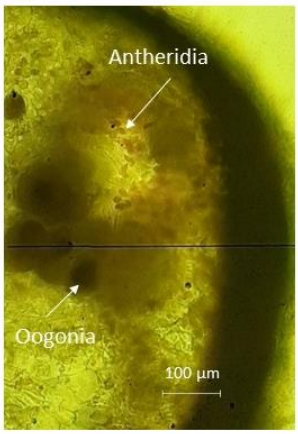

Figure 7: Cross section of a receptacle showing oogonia and antheridia located in the conceptacle.

Recruits' length

ANOVA analysis showed that the factors tanks, time and tank x time had a significant effect on recruits' length (Table 1). The average lengths were 2.1 ± 1.13 mm, 2.06 ± 0.95 mm and 1.79 ± 0.87 mm, respectively for recruits deriving from Lido1, Lido2 and Sassonia (Fig. 3).

Table 1: Two-way repeated measures ANOVA of juveniles' length according to time and tank.

	df	Sum of squares	Mean square	F	p
tank	2	1.90	0.948	6.56	0.003
time	10	67.25	6.725	19.2	<0.001
tank x time	20	15.86	0.793	5.49	<0.001

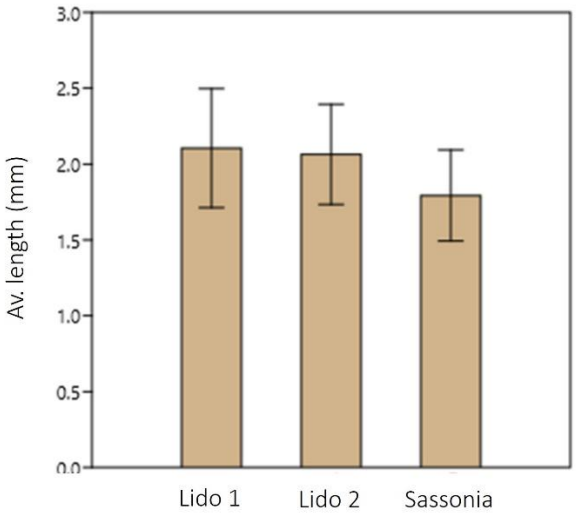

Figure 3: Average length of recruits in the three tanks.

Higher growth rates were observed in the last weeks (from t9 to t11) for Lido1, while for the tank Lido2 juveniles showed higher lengths at t6 and t8. Finally, for the tank Sassonia the highest length was only reported at t10, but with lower values than the other tanks (Fig. 4).

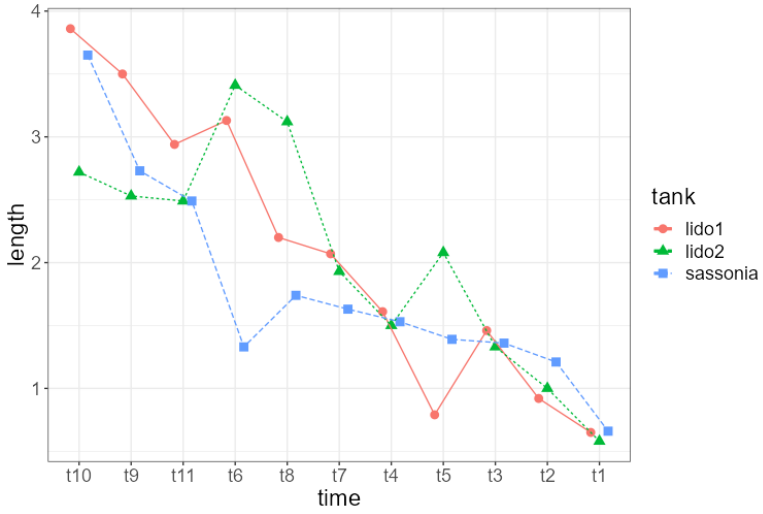

Figure 4: Length of the recruits in the three tanks during the experiment (from t1 to t11).

Coverage' classes frequency

The X'-Square test revealed that the coverage classes' frequency was significantly correlated with the tank ($p < 0.05$) (Table 2). After 11 weeks, the most frequent coverage class for the juveniles of Lido1 was class 3 (25-50%), while for Lido2 and Sassonia was class 2 (5-25%). Contrary to Lido1 and Lido2, the juveniles from Sassonia displayed a coverage class of 5 (75-100%), even if with the lowest frequency. Overall, juveniles from Lido1 displayed a higher frequency of class 4 (50-75%) than the juveniles from the other tanks. On the other hand, juveniles from Sassonia showed a greater frequency in the coverage class 1 (5%) than the juveniles from the other tanks (Fig. 5).

Table 2: Chi-Square test of the coverage classes' frequency

	Value	df	p
χ^2	21.4	8	0.006
N	99		

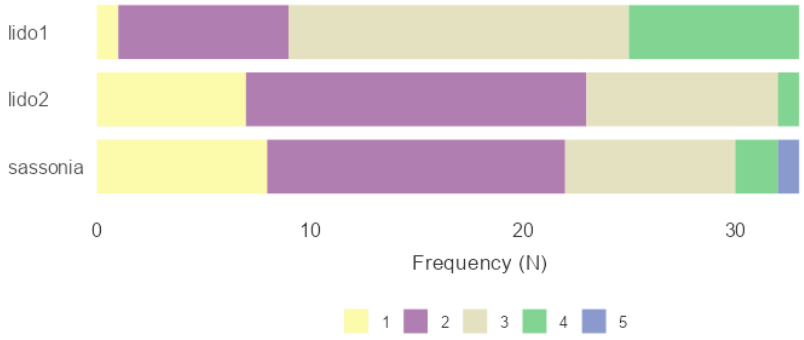

Figure 5: Coverage classes' frequency in the three tanks (coverage classes: 1 = 5%; 2 = 5-25%; 3 = 25-50%; 4 = 50-75%; 5 = 75-100%).

Throughout the time-course experiment, it was observed a different trend in the coverage classes' frequency among the three tanks. Indeed, from t9 to t11, the recruits from Lido 1 showed a higher coverage in the classes 3 and 4, while the recruits from Lido 2 in the classes 2 and 3 and the recruits from Sassonia in the classes 1 and 2. Overall, recruits from Lido 1 and Lido 2 presented low coverage classes (1 and 2) at the beginning of the experiment, and higher coverage classes (3 and 4) from t9 to t11 Contrary, for Sassonia, the highest coverage classes (4 and 5) were detected at the beginning of the experiment (Fig. 6).

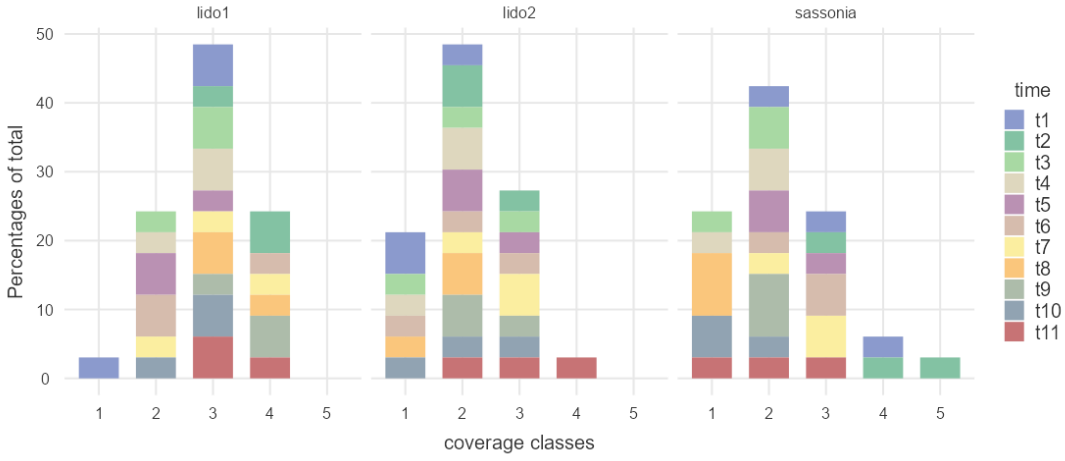

Figure 6: Coverage classes' frequency in the three tanks according to the time of the experiment.

Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

1. Innovazione e trasferimento tecnologico (didattica comune – 2 cfu)

2. Progettare la ricerca: i progetti europei (didattica comune – 2 cfu)

3. Elements of Marine Policy (Lezione frontale – 1cfu)

4. Microbial-mediated processes in aquatic ecosystems: from basic to applied research toward solving environmental problems (Lezione frontale – 1cfu)

5. Formazione specifica salute e sicurezza sul lavoro - RISCHIO MEDIO.

List of periods spent abroad

None.

List of conferences/workshops attended and of contributions eventually presented

1. FORESCUE (Biodiversa+, project number Biodiversa2021-134) kick off meeting, on 5th-6th June 2023, at Portonovo, Ancona, Italy.

Part 3. PhD student information on publications

List of publications on international journals

J1. Marletta G., Sacco D., Danovaro R., Bianchelli S. *Gongolaria barbata* stranded onshore: an opportunity for algal forest restoration. (in preparation, D. Sacco equally contributed as first author).

List of publications on conference proceedings

None.

List of other publications (books, book chapters, patents)

None.

14 Ottobre 2023

Student signature

Domenico Sacco

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Data: 2023.10.15 00:21:09 +02'00'

Supervisor signature

Silvia Bianchelli