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Innovative methods for marine research and automated culture of aquatic model organisms

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General introduction

The research of scientific knowledge refers to the process of discovering new information, understanding phenomena, and developing theories through the use of scientific methods (Edquist, 2004). This process involves observing, measuring, and experimenting to collect data, analysing that data to draw conclusions, and testing those conclusions through further experimentation and observation (Edquist, 2004; Hubbell et al., 2018). Scientific research is often conducted by scientists in universities, government agencies, and private companies, with the goal of expanding human knowledge and understanding the natural world (Beck et al., 2022; Emerson, 2018). This process is often iterative and collaborative, with scientists building on previous knowledge and working with peers to validate and improve upon their findings. Moreover, in the context where technology and knowledge are interconnected, an economy of expanding knowledge emerges, reinterpreting it as a resource as valuable as traditional productive factors such as property, labour, and capital (Emerson, 2018). Knowledge becomes a concrete resource that can be mobilized to gain a competitive edge (Devenport and Prusak, 1998); acquiring an economic value that makes it objective and marketable. Consequently, new issues constantly arise as: i) how to improve the tools to expand it; ii) how to use and reuse acquired knowledge; iii) how knowledge production occurs, and how scientific communities create their own objects of knowledge within their scientific production fields (Gherardi, Nicolini, 2004).

With the term “approach” in scientific research we refer to the methodology and strategy used to conduct research in order to gain new insights and understanding. So the scientific method is a systematic and logical approach to investigate a phenomenon, acquiring new knowledge, or correcting and integrating previous knowledge (Asrizal et al., 2018).

Scientific approach is widely considered as a reliable method for gaining new insights and understanding, but it can have several limitations. One of the main limitations is the so called “bias”, as the researcher's own biases and preconceptions can influence the design of the study, the interpretation of the data, and the conclusions drawn (Rosli et al., 2021). Additionally, scientific research is often focused on a specific question or problem, and the results may not be generalizable to other populations or situations. The replicability of scientific findings is also an issue, as scientific findings are not always easily replicated, which can call into question the validity of the original results (Pinto, 2019; Schiffer, 2005). Furthermore, scientific research can be highly *time-consuming and expensive*: not all the questions can be answered, sometimes due to lack of right resources (Schiffer, 2005). Moreover, even when scientific research has been conducted, the understanding of a subject might still be limited by the current state of knowledge, or the methods used. This can occur for several reasons. For example, the current state of knowledge in a particular field may not be advanced enough to fully understand a complex phenomenon. Additionally, the methods used in scientific research are not always able to provide a complete understanding of the subject at hand. Additionally, the limitations of the available technology or equipment may also affect the accuracy or precision of the measurements. Furthermore, certain research methods have limitations in terms of what they can reveal about a subject. For example, observational studies can provide information about correlations between variables, but cannot establish causality. As well, some research methods are better suited for certain types of questions than others (Schiffer, 2005).

It's important to note that the scientific approach is continuously evolving and improving, and scientists are constantly working to address these limitations and improve the quality of research (Milojevic, 2014). In fact, it has undergone significant changes in recent years,

driven by advances in approaches, technology and changes in funding and policy (Hubbell et al., 2018). Consequently, by combining different methods and techniques, and by incorporating new technologies, scientists are able to gain a more complete understanding of the subject. This is where innovation in scientific research can play a key role, as they can lead to new discoveries and a deeper understanding of the studied subject (Beck et al., 2022). New and innovative approaches can also lead to the development of new technologies and methodologies, having a significant impact in a range of fields and can help researchers overcome obstacles and limitations in their work, opening up new areas of research that were previously impossible. They can take many forms over technological and methodological improvements such as multidisciplinary approach, the use of large data sets and machine learning, or adopting Open Science (Hemminger et al., 2015). For example, new technologies such as CRISPR gene editing or super-resolution microscopy can enable researchers to conduct experiments and make observations that were not previously possible (Kalajdzic & Schetelig, 2017). Researchers from different fields can collaborate to tackle problems that cannot be solved by a single discipline, using techniques or theories from one field to make new discoveries in another field. By using large data sets and machine learning, scientists can analyse and make predictions from large amounts of data, which can speed up scientific discoveries (Qiu et al., 2016). Additionally, by sharing data, methods and results more openly, researchers can increase efficiency, trust, and reproducibility in the scientific process. In conclusion, innovation is important because it can open up new areas of investigation and can lead to new and unexpected discoveries that can have a significant impact on our understanding of the world (Djeflat, 2011; Kirkman, 1996).

Mainly, scientific research relies on traditional protocols. They involve the use of established methods and techniques within a single field of study to investigate a research

question. These methods are typically well-established and have been validated through previous studies (Maul, 2017). A key aspect of traditional protocols is replication, which is the process of repeating a study using the same methods to confirm or disprove the original findings. Nonetheless, traditional protocols can act as limits in a number of ways (Maul, 2017; Payne, 2006). They may limit the types of questions that can be asked or the methods that can be used to answer those questions. They may also limit the flexibility of the research design and not allow for adjustments based on new information or unexpected findings (Kira & A. Rendell, 2017). This can make it difficult to adapt to changing circumstances and can result in missed opportunities for important discoveries. Additionally, traditional protocols can create biases in the research by favoring certain types of studies or outcomes over others (Dwan et al., 2008). Studies that are more likely to produce statistically significant results may be given more weight than those that do not. This can skew the overall findings of the research and lead to inaccurate conclusions (Robinson-Cimpian, 2014). Several potential solutions reduce the limitations linked to traditional research protocols. Some of these include adopting a more flexible research design based on innovative methods.

To encourage interdisciplinary collaboration, bringing together ideas and approaches from different fields, can help as well to break down the limitations of traditional protocols and explore new and innovative approaches (Hedesan & Tendler, 2017). Moreover, replication must be promoted by reproducing previous studies, not only with the well-established protocols but also integrating new methods, to confirm or refute previous findings and build a more robust body of evidence. Another potential solution could be promoting diversity in the research community, to help to minimize the potential for bias and increase the diversity of perspectives and ideas in the field. It is worth

mentioning that many of these solutions are already in place or in progress (Chang & Chen, 2004).

Multidisciplinary, in particular, is a solution increasingly popular in the modern scientific research. Innovative multidisciplinary approaches involve the collaboration of researchers from multiple fields of study to investigate a research question. This approach allows for the integration of different perspectives and methods, which can lead to a more comprehensive understanding of the research question. Multidisciplinary research can also lead to the development of new and innovative methods and techniques (Edquist, 2004).

The integration of different perspectives, methods, and techniques can also help to validate findings and increase the robustness of the research. Multidisciplinary research can be applied in almost every scientific field, and it often leads to more comprehensive, in-depth and complex research projects, opening new areas of research and new opportunities for discovery (Mohs & Greig, 2017). This approach can also help to address complex, real-world problems that cannot be effectively addressed by one discipline, and it can also lead to more effective and efficient use of resources.

Several new multidisciplinary approaches in biology research are gaining popularity (Bowes & Jaffee, 2013). An example is the systems biology, which aims at understanding the interactions between different parts of a biological system, such as a cell or organism, by studying the system as a whole (Chuang et al., 2010). This approach involves the use of mathematical and computational methods to model complex biological systems and analyse large amounts of data (Oussous et al., 2018). Another possible approach is represented by synthetic biology, which involves the design new devices and systems that do not exist in nature. This field combines the principles of engineering and biology to

create new biological tools and systems for a variety of applications, such as medicine and biotechnology (Endy, 2005).

An additional new multidisciplinary approach is the field of bioinformatics which uses computational methods to analyse and interpret biological data(Wieser et al., 2011). This field combines biology, computer science, and statistics to analyse and understand biological data, such as DNA sequencing data, gene expression data, and protein structure data (Zrimec et al., 2021). It plays important roles in understanding the complexity of genome and other omics data in biology (Grigoriev et al., 2021). This is a growing method that combines engineering methods and advanced technologies to investigate biological systems.(Day, 2005)

New methods and approaches to improve marine research

Marine research is a prime example of the diverse and complex nature of scientific research, as it encompasses a wide range of disciplines and is critical for understanding the ocean and its impact on the planet (Muller-Karger et al., 2018). In fact, ocean cover more than 70% of the Earth's surface and play a vital role in regulating the Earth's climate, providing food and resources for human populations (Penesyan et al., 2010). Marine ecosystems are vital to the health of the planet and play a crucial role in maintaining the Earth's biodiversity (Lacroix et al., 2016). However, the study of these ecosystems has been facing significant challenges due to the vastness and complexity of the oceans, as well as the difficulties in accessing and observing marine organisms and habitats (Lacroix et al., 2016).

In recent years, innovative methods and technology improvements have played a crucial role in advancing our understanding of marine ecosystems, including advances in underwater imaging, remote sensing, and sensor technology, automated culture systems

as well as the applications of these technologies in various fields of marine research (Glaviano et al., 2022). Underwater imaging technology has been applied in the study of population dynamics, ecosystem function, and conservation of marine organisms, such as coral reef fishes and invertebrates, as well as in the study of deep-sea habitats, such as hydrothermal vents and cold seeps (Chen et al., 2017).

Remote sensing technology has been applied in the study of oceanographic processes, like ocean currents and temperature, as well as the distribution and abundance of marine organisms, such as phytoplankton and zooplankton which are important components of marine food webs(Tilstone et al., 2012).

Sensor technology has also been an important tool, particularly for long-term monitoring of oceanographic dynamics. Autonomous underwater gliders and moorings have been used to collect long-term data sets on physical and biological oceanographic variables (Franks et al., 2013).

Moreover, automated culture systems have revolutionized the way aquatic model organisms are cultured, allowing for precise control of environmental conditions and high-throughput experimentation (Horinouchi et al., 2014). This technology has been particularly useful for aquaculture, but also for the culture of aquatic model organisms for scientific purposes. Model organisms are species that have been chosen for their particular genetic, physiological, or behavioural characteristics that make them useful tools for scientific research (Baldridge et al., 2021). These organisms are used to study biological processes that are common to other species, including humans. By studying a model organism, scientists can gain insight into the underlying mechanisms of a wide range of biological phenomena, including development, genetics, disease, and evolution (Brenner, 2003). For this purpose, automated culture systems can provide precise control of physical and chemical parameters such as temperature, light, and nutrients, which

allows for the accurate simulation of in situ conditions (Mutalipassi, Maibam, et al., 2018).

Recent developments in technology have also enabled the creation of more compact culture systems, which are particularly useful for the culture of small aquatic organisms, such as planktonic larvae. These miniaturized systems reduce the need for large amounts of culture media and provide greater control over the culture environment, resulting in more precise and efficient experimentation (Mutalipassi, Maibam, et al., 2018).

Aims and structure of the thesis

According to all these considerations, two main approaches for promoting innovation in scientific research were attempted in this thesis: i) Optimizing innovative protocols within multidisciplinary studies, and ii) Implementing automatic culture techniques for aquatic model organisms.

On the overall, the primary objective of this thesis was to address the following research question: “How can innovative methods and automated culture techniques be used to improve the efficiency, accuracy, and reproducibility of marine research?”

Consequently, the specific purposes of this thesis were:

- A) To explore the possibilities of incorporating new techniques and a multi-disciplinary approach by bringing together different fields of engineering, pharmacology, and molecular sciences to face research questions as previously approached.
- B) To develop the use of automatic culture techniques for aquatic model organisms to improve the efficiency and reproducibility of research experiments.

In recent years, there has been a growing interest in the use of smart monitoring and automation in the management and sustainable exploitation of marine environments (Glaviano et al., 2022). This is driven by the need to respond promptly to the frequent caused by drilling stations and intense transportation of dangerous materials over ocean routes, as well as the changing coastal ecosystems due to increasing human activities, climate change, tourism, industrial impacts, and conservation practices (Magris et al., 2019). For these reasons, the current state of the art in terms of the technology and

methods used in smart monitoring and automation was reviewed in the Section 1, as well as the challenges and the opportunities associated with this approach.

The use of smart monitoring and automation in marine environments and model organism culture (such as crustaceans) has the potential to revolutionize the way we manage and exploit these resources (Mutalipassi, di Natale, et al., 2018a). By providing accurate and precise measurements and automating tasks such as feeding and monitoring, we can improve efficiency, reduce costs, and decrease the need for personnel. However, further research is needed to fully realize the potential of these technologies and to address the challenges and opportunities associated with their use.

The studies focused on research questions that had already previously approached and that have shown potential for the introduction of a new innovative approaches were presented in the Section 2.

Paracentrotus lividus, is a species of sea urchin economically relevant for the seafood market and plays a crucial role in the ecology of Mediterranean coastal ecosystems (Boudouresque & Verlaque, 2001). Moreover, it is commonly used as a model organism for studying a wide range of biological processes. In fact, research on *P. lividus* has led to many key discoveries in developmental biology, including the identification of genes involved in the control of cell division and differentiation, and the understanding of how environmental factors can affect embryonic development(Boudouresque & Verlaque, 2020). Additionally, *P. lividus* has been used to study the mechanisms of response to stressors, such as exposure to pollutants and changes in water temperature or acidification. *P. lividus* is also an important model organism for studying the genetics and molecular biology of echinoderms (Roccheri & Matranga, 2010). Its genome has been sequenced, and researchers have used this information to identify genes involved in a variety of biological processes, including development, immunity, response to

environmental stressors, biotechnology (Gambardella et al., 2021; Laport et al., 2018). This organism was selected as part of this thesis because of its role in modern sciences. In particular, in this work it was used as a tool to implement new research methods and protocols, as well as to increase our knowledge on this species to make it an even more valuable model organism.

At first, *P. lividus* was proposed to test a realistic mesocosm in combination with molecular analyses as an innovative approach to better understand the impacts of complex combinations of stressors and accumulation of organic contaminants. This approach aimed at addressing the limitations of standard ecotoxicology tests (Hellou, 2011) and improve the understanding of how pollutants impact coastal ecosystems and communities. The study also suggested that understanding the molecular processes involved in sensing and dealing with contaminants in this specific model organism could be useful for creating predictive diagnostic tools to assess threats to the marine environment.

Therefore, *P. lividus* larvae are an important resource for scientific research because they are easy to maintain in the laboratory and they have a well-defined developmental program that makes them an excellent model for studying the molecular and cellular mechanisms of development. They are used in aquaculture research as potential food source for other marine species of interest and they are also considered a promising candidate for the cultivation of sea urchins as a source of food for human consumption (Castilla-Gavilán et al., 2018; Zupo et al., 2018). For all these reasons, we aimed to investigate, for the first time through a molecular approach, the impact of maternal influences and culture conditions on the development and growth of *P. lividus* offspring. In fact, the larval stage of this animal is a bottleneck in the species' life cycle. This means that the survival rate during this stage is much lower compared to other stages, making it

a critical point (Zupo et al., 2018). This can be due to a variety of factors such as more sensitivity to environmental conditions, predation, and competition for resources [75-76]. Therefore, understanding factors that can affect the survival of sea urchin larvae is crucial for better evaluate results during an experiment, to better understanding the population dynamics of this species and more in general the health of the marine ecosystems in which they live. The findings from this investigation can help to increase our knowledge on this key species and, in addition, can help to develop protocols for the mass production of *P. lividus* for both research and aquaculture purposes.

Thus, previous studies have shown that *P. lividus* can be an appropriate model organism for studying algal metabolites potential(Ruocco et al., 2018) [77]. To study these active compounds from algal metabolites, researchers mainly use biological assays to test the activity on target model organisms. Bioassay-guided fractionation are generally used to isolate these compounds, characterize and study them for potential applications (Andras et al., 2012; Walton et al., 2014). One of the most common applications is in drug development [80]. For example, pigments like have been found to have anti-inflammatory, anti-cancer, and antioxidant properties (Kuczynska et al., 2015; Lourenço-Lopes et al., 2020; Pistelli et al., 2021). Furthermore, polysaccharides from diatoms have been found to have immunomodulatory properties, and lipids have been found to have anti-inflammatory and anti-cancer properties as well (Abdul et al., 2016; Somanader et al., 2022). In biotechnology, polysaccharides from diatoms have been found to be useful in the production of biofuels, and lipids from diatoms have been found to be useful in the production of biodiesel and bio-lubricants (Davis et al., 2012; Yi et al., 2017). Furthermore, diatom-derived pigments have been found to be useful in the food, cosmetic and pharmaceutical industries (Lebeau & Robert, 2003). Overall, the active compounds from diatoms have a great potential and could be used in various industries. Studies

continue to explore the potential of these compounds and to identify new ones with potential applications.

Considering this, an innovative method was tested for administering algal extracts, and eventually to carry out bioassay investigations, using *P. lividus* as a model organism. Indeed, traditional approach involves observing the behaviour of target animals forced to graze on them, but this method may reveal limitations, particularly in terms of how the compounds must be administered through suitable feeds and supplements and how to preserve their characteristics (Glaviano et al., 2021; María et al., 2014). For this reason, we proposed the use of encapsulation techniques as an innovative method to preserve their active compounds and administer them to an aquatic model organism. In details this study specifically focused on two types of benthic diatoms, *Nanofrustulum shiloi* and *Striatella unipunctata*, and investigated their effects when included in alginate matrices, which are a biocompatible and non-toxic delivery system, and fed to the sea urchins. The results obtained confirm that this inclusion in alginate beads may be a useful technique for isolating diatom-derived bioactive compounds.

In conclusion, given the importance of compounds derived from algae, as already mentioned, and given the success of the introduction of the molecular approach in the studies listed above, we decided to use this same method as a new approach aimed to study the mechanisms involved in the peculiar correlation between an early sexual shift in the shrimp *Hippolyte inermis* and the ingestion of *Cocconeis* spp. diatom. In summary, this shrimp undergoes a peculiar process of sex reversal promoted by the food ingested, in which it has been proposed that a specific algal compound from the diatom *Cocconeis* spp. triggers the development of the female phenotype (Zupo, 1994, 2001). However, traditional bioassays to identify this compound are complex and highly time-consuming. Therefore, this new approach has been proposed as an alternative to optimize the protocol

for an earlier identification of the molecular structure of this active compound. Nevertheless, *H. inermis* is not a model organism commonly used in other research fields and there are still few molecular tools available to study this species. For these reasons several steps were necessary in order to optimise a suitable protocol.

Section 3 reports a deeper approach into the implementation of automatic culture techniques for aquatic model organisms. Several animal and plant organisms are widely used in biological research to understand the functions of life forms and in aquaculture as live foods or as targets of production (Murthy & Ram, 2015). The use of small programmable devices can significantly reduce production costs and the need for personnel and fixed setups, by consistently and cost-effectively repeating standard operations with higher precision. The use of aquatic models in scientific research and aquaculture presents both opportunities and challenges. Studies from diet, culturing density, management of facility spaces, up to animal psychiatry with the aim of optimizing protocols and procedures, make them simpler, cheaper and more efficient as a fundamental step for the success of any model species (Calado et al., 2003, 2005). Although adult specimens collected in the field are usually prone to captivity problems, the management of conditioned organisms implies a wide spectrum of issues such as definition of long-term complete diets, set-up of high-density re-circulating systems, reduction of water–volume/organism ratio. Therefore, it is important to develop flexible, programmable and modular culture systems that facilitate the automatic production of demanding species, both for scientific and aquaculture purposes (Mutalipassi, di Natale, et al., 2018b). In the system outlined in chapter 7, an existing and patented system was employed, focused on its optimization to make it applicable to innovative cultures. In chapter 8, however, a new system as devised with the help of a technologically advanced

central control unit. As previously argued, a multidisciplinary approach and collaboration from multiple fields of study can revolutionize the way to face a research demand and can also lead to the development of new and innovative techniques. In line with this goal, we collaborated with a team of engineers to make it possible. In details, this thesis is structured as follows:

Summary of the thesis

Section 1

This first section contains the review chapters 1 and 2.

Chapter 1 comprehends the published review "Management and Sustainable Exploitation of Marine Environments through Smart Monitoring and Automation" (Glaviano et al., 2022), which explores the recent literature on the use of smart monitoring and automation in the management and sustainable exploitation of marine environments. The paper highlights the current state of the art in terms of the technology and methods used, as well as the challenges and opportunities associated with this approach.

In details, in this section I reviewed how monitoring of aquatic ecosystems has traditionally been done through intensive campaigns of direct measurements using probes and other boat instruments, as well as indirect methods such as aero-photogrammetry and satellite detection. These methods have been used for many years and have made significant, but limited, advancements. However, recent advancements in smart devices and networking technology, such as the Internet of Things (IoT), offer new opportunities for more accurate and precise measurements over larger areas. This is particularly important in light of the need to respond promptly to the frequent catastrophic impacts caused by drilling stations and intense transportation of dangerous materials over ocean routes. The coastal ecosystems are constantly changing due to increasing human activities, climate change, tourism, industrial impacts and conservation practices. Smart buoy networks (SBNs), autonomous underwater vehicles (AUVs), and multi-sensor microsystems (MSMs) can learn specific patterns of ecological conditions and with the use of electronic "noses", they enable the setting of innovative low-cost monitoring stations that can react in real-time to the signals of marine environments by autonomously

adapting their monitoring programs, and eventually sending alarm messages to prompt human intervention. These tools, according to multimodal scenarios, are dramatically changing both the coastal monitoring operations and investigations over large oceanic areas by yielding huge amounts of information, and partially computing them in order to provide intelligent responses. However, the major effects of these tools on the management of marine environments are yet to be realized, and they are likely to become evident in the next decade. This review also examines the most striking innovations applied by various research groups around the world from an ecological perspective and analyses their advantages and limitations to depict scenarios of monitoring activities that will be possible in the next decade.

Chapter 2 contains the book chapter “Automatic Culture of Crustaceans as Models for Science” (Glaviano & Mutalipassi, 2022). It offers an overview about traditional methods of cultivation that require large space and trained operators. To overcome this, modern research and aquaculture are increasingly turning towards integrating new technologies to improve efficiency, reduce costs and decrease the need for personnel. This is done by automating tasks such as feeding, live food production, and monitoring and controlling the culture's conditions. This can enhance the growth rate of the crustaceans and improve their overall health. Additionally, constant monitoring can also help prevent potential losses due to random problems. Several proposals have been designed and several prototypes and systems are currently in use. The main approaches are automatic feeding systems, automatic live food production and administration, and automatic monitoring and control of the culture. In this section, examples are focused on crustaceans in detail, because they are considered to be very important in various fields such as scientific research, aquaculture, and the ornamental market. They are often used

as aquatic models in scientific research to study the functions of living organisms and ecosystems. In aquaculture, they are considered as a valuable food source and in the ornamental market, they have a high value for their rarity.

Overall, this chapter aimed at providing a comprehensive overview of the current state of the art in terms of new technologies for marine research and automated culture techniques for aquatic model organisms, highlighting the most recent developments, challenges, and opportunities in these fields.

Section 2

This section contains the Chapters from 3 to 6, as reported below.

Chapter 3 focuses on the study "Morphologic and genic effects of organic pollution on the reproductive physiology of *Paracentrotus lividus* Lmk: a mesocosm experiment" (Glaviano et al., submitted). Conventional ecotoxicity tests enable the identification of one or few more substances which can have a major harmful effect on organisms. The benefits of such methods are clear because they may provide important information on the impacts of a single pollutant on the physiology of a model species, but they do not consider the impact of a mix of natural pollutants, as they are normally co-present in the environment. Consequently, an implementation of methods to fully understand the environmental impacts of complex combinations of contaminants is required. This may be accomplished by concentrating the seawater and testing the physiological responses of individuals and communities employing a more realistic mesocosm. Such a kind of investigations could enable significant progresses in understanding how contaminants

actually impact coastal ecosystems and communities. To this purpose, in this study, we employed the common sea urchin *Paracentrotus lividus* as a model organism. This sea urchin represents an economically relevant species for the seafood market and a resource for scientific research. In addition, it plays a crucial role in the ecology of Mediterranean coastal ecosystems because it is one of the main grazers in algal and seagrass ecosystems. Therefore, increasing pollution events impacting shallow coastal ecosystems might influence its reproductive potential and the ecology of economically relevant communities. As an innovative approach to face the limitations of standard ecotoxicology tests, we proposed a realistic mesocosm, in combination with the introduction of molecular analyses, to better understand the impacts of complex combinations of stressors and accumulation of organic contaminants in this well-known model organism and more in general in the marine environment. In addition, the understanding of the effects of substances discharged from human activities into the season marine species can be extremely important for forecasting and managing the possible environmental damage associated with their rise and spread. Understanding the molecular processes involved in sensing and dealing with classical or new contaminants in such a useful model, might be highly useful for creating predictive diagnostic tools to assess the threats to marine environment.

Chapter 4 focuses on the published paper "Gene expression detects the factors influencing the reproductive success and the survival rates of *Paracentrotus lividus* offspring" (Federico et al., 2022). In this investigation, for the first time, molecular approaches were applied on cultured sea urchins to understand the impact of maternal influences on the development and growth of their offspring, as well as how culture conditions affect these outcomes. The growing demand for *P. lividus* roe, a popular food

delicacy, is putting pressure on its wild populations. To mitigate this, aquaculture facilities can help reduce the impact of human activities on these stocks. To do this, research is needed to improve the efficiency of aquaculture practices for *P. lividus.* The results showed that the outcomes of in vitro fertilization of this animal are influenced by maternal influences, but these effects are largely determined by culture conditions. Additionally, 23 genes related to stress response and skeletogenesis were found to be differently expressed in sea urchins cultured under different conditions, and these effects were largely modified in offspring from different groups of females. These findings will be important for developing protocols for the mass production of *P. lividus* for both research and industrial purposes.

Chapter 5 focuses on the published study "Two benthic diatoms, *Nanofrustulum shiloi* and *Striatella unipunctata*, encapsulated in alginate beads, influence the reproductive efficiency of *Paracentrotus lividus* by modulating the gene expression" (Glaviano et al., 2021).

Understanding the physiological effects of algal metabolites is crucial for the identification of bioactive compounds. Invertebrates can be fed live diatoms or dried and pelletized diatoms that are added to compound feeds. However, these methods may have limitations, as some compounds may be released in the water before they are ingested. In this study, it has been proposed, as an innovative method, encapsulation techniques. They can be an effective step in bioassay-guided fractionation, as it helps to preserve active compounds. In details we investigated the effects of including two benthic diatoms, *Nanofrustulum shiloi* and *Striatella unipunctata*, in alginate matrices (a biocompatible and non-toxic delivery system) were tested when fed to sea urchins *Paracentrotus lividus*. The results showed that *N. shiloi*, which is known to have toxic effects on sea urchin

larvae, fully retained its activity after being included in alginate beads. Additionally, both diatoms affected the embryogenesis of *P. lividus* by altering the expression of several genes involved in stress response, development, skeletogenesis, and detoxification processes. The study suggests that the inclusion in alginate beads may be a useful technique for isolating diatom-derived bioactive compounds.

Chapter 6 focuses on the study "Emerging molecular approaches as an alternative to traditional bioassays" (Glaviano et al., manuscript to be submitted).

The study proposes a new molecular protocol as an innovative approach to investigate *H. inermis* shrimp. This shrimp is of special interest due to its unique process of food-determined sex reversal, in which an algal compound can induce the early development of the female phenotype. This is a result of co-evolution with a specific diatom, *Cocconeis* spp. Traditional bioassays for identifying the molecular structure of this algal compound are a long and complex process, which leads to various challenges such as finding a sufficient number of ovigerous females, given the anthropogenic threat to *Posidonia* meadows where these animals live. Additionally, culturing a large number of sensitive larvae and post-larvae poses a risk to their health and stress levels. This protocol may also lead to insufficient numbers of animals available for evaluating the distribution of sexes in treatment replicates. Furthermore, animals may be too stressed at the end of the culture period, potentially altering their biological response to treatment. Thus, the establishment of a new molecular approach, as an alternative and support to traditional bioassays, is crucial to optimize investigations and identify the molecular structure of this apoptogenic compound. The final phase of the study was to determine the effectiveness of this molecular approach by validating previously obtained transcriptome results.

1.3.1 **Section 3** contains Chapters 7 and 8.

Chapter 7 focuses on the study "Automated culture techniques applied to a continuous rearing of an ascidian *Botryllus schlosseri*" (Glaviano et al., manuscript in preparation). Specimen models are extensively used in biological and chemical sciences to test specific hypotheses. Ascidians are well-developed models, because they share common ancestors with vertebrates. Among them, the colonial ascidian *Botryllus schlosseri* is classically adopted within a range of biological disciplines, due to its unique life cycle, including cyclical events of programmed cell death and also due to an aptitude to regenerate the whole-body. In addition, it exhibits interesting cellular processes of self/non-self-recognition (histocompatibility). The improvement of culture protocols and rearing systems is important to correctly exploit model-organisms for scientific purposes, providing a continuous supply of individuals for experiments. The development of flexible and modular systems that may help the automatic production of delicate species is also important to reduce the rearing costs. Here, we provide evidence that specifically designed automatic systems are efficient, as compared to manual rearing systems, for maintaining healthy colonies in the laboratory for medium-term or long-term experimentations. In fact, a remarkable advantage of the system herein proposed is represented by the reduced daily cares required, that also moderate the involvement of experienced operators.

Chapter 9 focus on "A smart automated culture system for aquatic model organisms tested on the isopod *Idotea baltica basteri*", using a CR1000X data logger. The system is designed to control two tanks through the use of various sensors to monitor the tanks and effectors to control and react to changes in desired parameters.

The automation of culture systems for aquatic model organisms has the potential to transform the way scientific research is conducted. The ability to control and monitor the environment where these organisms are grown can improve the quality and the consistency of the data collected, as well as reduce the need for expensive daily care or the involvement of experienced operators. The use of a CR1000X data logger is a powerful tool in automating these systems, as it allows for the collection, analysis, and storage of data, as well as the control of various sensors and effectors.

Here, we investigated the potential set up of an automatic culture system for aquatic model organism, using a CR1000X data logger. The system was designed to control two tanks through the use of various sensors to monitor the tanks and effectors to control and react to changes in desired parameters. Our aim was to make this system fully automatic, with the ability to analyse data and adjust settings to optimize the culture and the set-up. The data logger was programmed to perform data analyses, such as calculating averages, determining maximum and minimum values, and more. This could allow for the identification of patterns and trends in the data, which can be used to optimize the conditions for the aquatic culture. Additionally, the system aimed to eliminate the need for significant daily care or the involvement of experienced operators, and to make it possible to monitor and control the system remotely. The data logger was configured to send data via a communication protocol such as Ethernet, which allowed for remote monitoring and control of the system.

This chapter aimed at describing the details the system and its logic along with the results obtained applying it to the culture of a model species for the marine research. In order to investigate its potential use for scientific researches, we tested the system with the culture of the isopod *Idotea baltica basteri*. However, this is intended to be a proof of concept, because it can be applied to other aquatic model organisms.

Section 3

Period abroad

During my Ph.D., I spent three months abroad in Portugal collaborating with Professor Rui Rosa, who is based at Laboratório Marítimo da Guia, in Cascais and a Professor at the University of Lisbon. The goal of Professor Rosa research is to comprehend the effects of climate change on marine life, encompassing everything from cells to ecosystems, through a multi-disciplinary and comprehensive approach. His team is actually investigating the collective impact of climate change stressors such as ocean warming, acidification, and hypoxia on marine invertebrates and vertebrates of ecological significance.

In line with the objective of my Ph.D. project, I took advantage of this opportunity to engage with a new field of biological research and work with a new model organism (*Sepia officinalis*). My background about model organism culture allowed me to interface and optimize the experimental system for simulating hypoxic conditions to expose the model organisms for experimental purposes through programming an Arduino with C. The results shown in the chapter are preliminary and will be reviewed with additional analysis planned in our collaboration.

In detail, this study "Lower hypoxia threshold can affect behaviour in early life stages of cuttlefish" aimed to explore the innate chemical recognition abilities of newly hatched cuttlefish in both normal and severe hypoxia conditions. This was accomplished by exposing the cuttlefish to odors from both predator (*Scyliorhinus canicula*) and non-predator (conspecific *S. officinalis*) sources. The research aimed to shed light on the effect of hypoxia on the chemical recognition abilities of cuttlefish and its potential implications for their survival and behaviour in their natural habitats. The results of this study would

provide insights into the impact of hypoxia on the sensory abilities of marine organisms and how it affects their interactions with their environment.

Section 4 contains only the conclusions and future directions taking into account the findings presented in previous chapters.

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Section 1

Chapter 1

Journal of *Marine Science and Engineering*

MDPI

Review

Management and Sustainable Exploitation of Marine Environments through Smart Monitoring and Automation

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Abstract: Monitoring of aquatic ecosystems has been historically accomplished by intensive campaigns of direct measurements (by probes and other boat instruments) and indirect extensive methods such as aero-photogrammetry and satellite detection. These measurements characterized the research in the last century, with significant but limited improvements within those technological boundaries. The newest advances in the field of smart devices and increased networking capabilities provided by emerging tools, such as the Internet of Things (IoT), offer increasing opportunities to provide accurate and precise measurements over larger areas. These perspectives also correspond to an increasing need to promptly respond to frequent catastrophic impacts produced by drilling stations and intense transportation activities of dangerous materials over ocean routes. The shape of coastal ecosystems continuously varies due to increasing anthropic activities and climatic changes, aside from touristic activities, industrial impacts, and conservation practices. Smart buoy networks (SBNs), autonomous underwater vehicles (AUVs), and multi-sensor microsystems (MSMs) such as smart cable water (SCW) are able to learn specific patterns of ecological conditions, along with electronic "noses", permitting them to set innovative low-cost monitoring stations reacting in real time to the signals of marine environments by autonomously adapting their monitoring programs and eventually sending alarm messages to prompt human intervention. These opportunities, according to multimodal scenarios, are dramatically changing both the coastal monitoring operations and the investigations over large oceanic areas by yielding huge amounts of information and partially computing them in order to provide intelligent responses. However, the major effects of these tools on the management of marine environments are still to be realized, and they are likely to become evident in the next decade. In this review, we examined from an ecological perspective the most striking innovations applied by various research groups around the world and analyzed their advantages and limits to depict scenarios of monitoring activities made possible for the next decade.

Keywords: IoT; buoy; aquaculture; coastal; connectivity; transmission; real time; network

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1. Current Policies for Environmental Monitoring and Conservation

The monitoring of marine environments has attracted increasing attention due to the growing concerns about climate change, along with intensified transportation activities, possibly producing direct, indirect, and stochastic impacts. In fact, a key challenge in

contemporary ecology and conservation management is the accuracy of tracking of the spatial distribution of human impacts, including oil spills and chemical pollution, along with the evaluation of environmental quality and fishery activities [1]. Automation is an important part of the new generation of information technology, and it represents the ultimate achievement in the development of ocean monitoring programs. Various emerging technologies developed in the last decade include smart devices for the collection of information and their sharing over networks, as well as emerging technologies such as the Internet of Things (IoT), often foreseen as the future solution to an intelligent monitoring assembly [2].

The systems currently in use generally consist of observatories connected to a network system lying on the seafloor or connected to the surface by, for example, a buoy. In the first case, an example of a stable observatory is the Dense Ocean Floor Network System for Earthquakes and Tsunamis (DONET) by the Japan Agency for Marine-Earth Science and Technology (JAMSTEC). DONET is a submarine-cabled real-time seafloor observatory network intended for large-scale research and earthquake and tsunami monitoring. The program, which began in 2006, consists of several phases involving an increase in the number of observatories.

This system concept consists of a high-reliability backbone cable, which provides the power line and the communications channel, connecting several nodes with different measurement instruments [3].

Buoy systems are widely applied as well to monitor ocean environments, and meteorological and oceanographic instrumentation platforms able to share meteorological and environmental data in real time are critical to promptly respond to critical events. The development of newer buoys is able to improve early detection and real-time reporting of events in the open oceans, which is fundamental for the forecasting and reporting of tsunamis. For example, forecasting and reporting of tsunamis were made possible by the development of newer buoys able to improve early detection and real-time reporting of events in the open oceans [4,5]. Similarly, the realization of systems able to detect the presence of pollutants in the marine environment (including hydrocarbons, often requiring prompt reactions due to ship collisions and other disasters) has become extremely complex, involving various technologies and integrated know-how [6] further discussed below. Stations for deep-ocean assessment and reporting of tsunamis were developed ad hoc by NOAA (https://www.ndbc.noaa.gov/dart/dart.shtml; accessed on 30 November 2021) to acquire critical data for real-time forecasts in key regions [7]. The network is presently composed of 39 stations (Figure 1). This station system was named DART®, and it consists of bottom pressure recorders (BPRs) anchored to the seafloor coupled with a companion moored surface buoy for real-time communications [4]. An acoustic link transmits data from the BPR on the seafloor to the surface buoy. However, the main constraint for ocean monitoring systems is represented by communications, because it is almost impossible to deliver the measured data to remote monitoring sites without the aid of satellite communications [8]. To extend the communication coverage of a buoy network, a wireless mesh network (WMN) can be adopted (i.e., a communication network containing multiple radio nodes consisting of mesh routers and clients organized into a mesh topology). Since mesh routers can forward a message deriving from other nodes (even outside the transmission coverage of their destination), a multi-hop relay network (MHRN) may be arranged. An MHRN can extend the coverage of wireless communications, and it provides line-of-sight (LOS) links between couples of nodes. Mesh networks provide many advantages, including reliability, robustness, self-organization, and self-configuration [9].

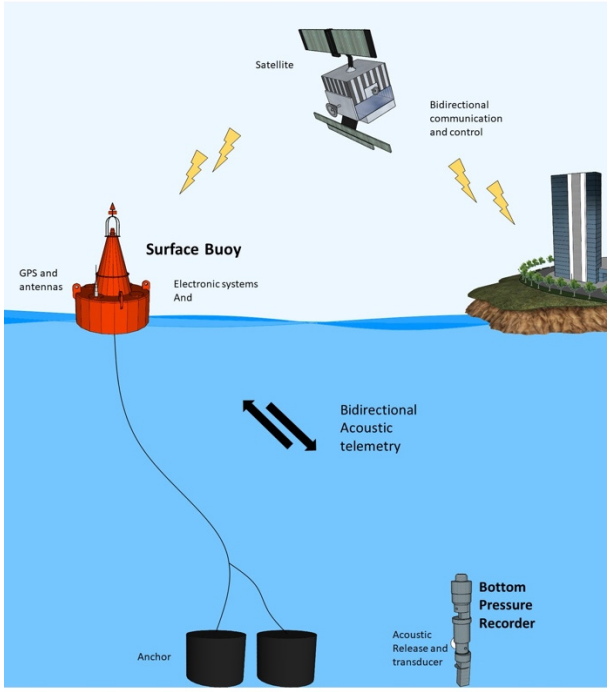

Figure 1. DART System set by NOAA (from www.noaa.gov, modified; accessed on 31 July 2021), containing 39 special stations transmitting GNSS data using bidirectional communications.

Thus far, it is evident that marine monitoring of natural environments is a tremendously wide field of study, taking advantage of various disciplines and comprising several aspects including the biology of species, the ecology of aquatic environments, the technology of new devices, and the chemistry of water as revealed by probes, with the inclusion of newer smart tools for detection and transmission. A complete analysis of all these aspects cannot be achieved and discussed within a single literature synthesis. For this reason, here we analyze the current literature to present several (but not necessarily all) recently developed methodologies and technologies to improve marine monitoring methods in order to highlight new trends and modern perspectives on the study of coastal and offshore environments, which are changing fast due the introduction of important innovations. In addition, we introduce some newly developed tools and experimental data collected at our laboratory in order to broaden the analysis of coastal tools with the introduction of smart sensors and autonomous monitoring buoys, facilitating video monitoring and immediate answers to critical events.

2. Sensing and e-Noses

The technological limits of probes and transmission devices must be taken into account when planning innovative monitoring stations and vessels. Some critical issues impose specific requirements for probes and monitoring stations, including simplicity, autonomy [10], adaptability, scalability, and robustness [11]. Some features should be assured due to the harsh characteristics of the marine environments [12]. Among these, we need to address the following specifically [13]:

- Self-standing devices: equipment should be designed against possible acts of vandalism, which are more frequent than commonly expected;
- Hardware robustness: all equipment needs strong resistance due to currents, waves, tides, typhoons, and other physical impacts producing frequent aggressions to weak structures;
- Salinity: sensor and actuator nodes need to have very high levels of robustness against corrosion and be adapted to a high electrical resistance to the medium;

- Stability of communications: specific techniques must be adapted to bad weather conditions (that can affect the stability of radio signals) and to the oscillation of the antennas due to waves and storms, which can cause unstable communications [14];
- Costs: energy storage and collection (eventually using energy accumulators) must be considered due to long communication distances and the need for probe functioning, data storage and transmission, and ultimately motion structures;
- Distance between receiving stations and buoy or mooring devices: sensor coverage needs to be carefully calculated because of the large areas often covered by a monitoring network [15];
- Stationary position: in the case of both fixed buoys and autonomous vehicles, the position of the sensor nodes should be assured, and its location should be assessed with high reliability because of the continuous movement in the fluid environment;
- The optical signal response is too low when compared with other targets and that one may have under certain circumstances of vegetation, soils, and also strong geometric effects (e.g., sun-view angle effects from optical data).

For these reasons, various monitoring systems have been developed in different areas, also according to the specific variables under analysis. Among them, the most powerful approaches employed to obtain sensitive data and rapidly compute them include synthetic aperture radar (SAR) [16], computer-aided imaging, and network analysis [17]. All these approaches account for some critical issues, including low detection capability (i.e., when wind speeds that are too low or too high influence the functioning of the SAR) or worse functioning during given times (e.g., at night, when sunlight is not available). In addition, both in oceanic environments and in coastal areas, hyperspectral and thermal imaging [18] and hydrodynamic mathematical modeling of stationary phenomena [19] may represent a possible solution.

Among the most modern and powerful systems, however, we must consider the chemical sensors for electronic nose-like systems [20–22]. Recently, a smart system based on electronic noses able to monitor the presence of pollutants (particularly hydrocarbons) on the sea surface was proposed [20]. The system was suggested to be employed, together with traditional methods, for a complete and exhaustive analysis of the marine pollution caused by hydrocarbons. It is composed of an array of sensors, a flow chamber, and electronics, and it was initially tested at the laboratory bench and then in the sea, demonstrating its efficiency and reliability in the detection of hydrocarbon pollutants present on the surface. It allows for an early intervention strategy from designated entities, as well as from the autonomous underwater vehicles (AUVs) themselves, when equipped for these circumstances. In addition, an e-nose-like technology may be integrated into an AUV in order to perform a dynamic check of the pollution status over a given area, and this possibility is increasingly stimulating various research groups because various noses are presently under study for implementation into smart vehicles able to independently monitor large coastal and oceanic areas [23–25]. This extension to the basic functions of AUVs was also performed by earlier prototypes [6], and it could embody an invaluable innovative contribution to the prevention strategies presently adopted throughout the world in this field, possibly establishing the basis for future multimodal marine monitoring implementations.

A number of different approaches have been employed to provide real-time acquisition of environmental data, especially to provide immediate reaction to incidents involving petroleum tanks or oil spills in coastal or oceanic areas, where continuous monitoring may be limited by economic or technical constraints [16]. Spills or leaks, as well as accidents [26,27], can induce dramatic consequences on the marine environments, and their immediate localization (followed by restoration activities) is critical to reduce long-term impacts over the marine biota. In these cases, various monitoring approaches have been widely applied in the past, such as hyperspectral and thermal imaging [18] and hydrodynamic mathematical modelling [19]. However, these large-scale approaches exhibit some limitations when the pollution sources are of a small size and the waves of pollution have

not yet been distributed over larger areas. In addition, weather conditions and light availability may drastically reduce their detection capabilities. To this end, newer intelligent technologies primed the development of AUVs (described in the next paragraph), independently sailing over large areas and able to ride out customized or pre-loaded explorations according to the needs of scientists and administrators [6]. This innovative approach is based on signals produced by electrochemical sensors reacting to the presence of possible pollutants [22], the signal of which is immediately sent to reference stations where the signal may be interpreted and eventually converted into an alert message, prompting the intervention of specialized personnel to assure marine environment preservation.

In parallel to atmospheric issues, as mentioned above, hydrocarbon pollution is one of the most serious concerns for the health of marine ecosystems, and the strategies for its timely monitoring have grown in complexity and number in the last decade. To this end, an AUV equipped with an *e*-nose-like system was proposed [20], employing sensors set both at the laboratory bench and at sea. The results confirmed the feasibility of the approach and the good reliability of the data acquired, confirming the possible employment of this system within an integrated marine monitoring tool.

The high costs of offshore mooring systems and traditional oceanographic cruises have suggested the use of innovative technologies, often based on intelligent devices and small monitoring platforms automatically collecting a wide range of environmental and meteorological data [28]. These approaches reached lower costs thanks to the new opportunities offered by emergent tools, representing cost-effective solutions to the need of modularity, flexibility, and real-time observing systems. Their affordability is guaranteed by the efforts dedicated to the design, development, and realization of new oceanographic devices, leading to rapid advances in the fields of probes and intelligent vehicles. In addition, innovative molecular technologies tremendously improved biodiversity studies, particularly in the case of microbes, rare species, "soft species" (or extremely small species), and cryptic species (to be studied combining molecular and morphological information [29,30], while new sensors and in situ technologies are being applied to the identification of life forms in remote deep-sea habitats [31,32].

In general terms, *e*-nose technologies are based on arrays of sensors connected to specific unit boards able to analyze the sensor's signals, compare their results, and compute an answer according to pollution thresholds set by the user. For some applications, photoionization detectors were employed, whose driving force relies on vacuum ultra-violet radiation capable of ionizing the volatile organic compounds (VOCs) contained in the air over the seawater [6]. In this case, the sensors do not analyze the chemical or physical properties of the seawater. They detect the VOCs present in the air immediately over the water surface, just like a "nose" exploring large areas along the coastline searching for the "smell" of petrol [20]. For these applications, a concentration of 100 ppm of each hydrocarbon among the ones most frequently present in polluted seas (e.g., gasoline, kerosene, diesel fuel, and crude oil) is considered sufficient [26,27]. The smart modules employed for these purposes are normally trained to evaluate the responses of various probes after the determination of the most relevant features among all the data collected by e-noses by means of principal component analysis (PCA). Using this system, the detected stimuli may be classified according to different levels of warning, depending on the intensity of the concentration of pollutants.

3. Autonomous Vehicles and Monitoring Platforms

Unmanned vehicles (UMVs) represent a significant innovation, improving the quality, affordability, and costs of environmental monitoring (Table 1). They are also used in the military field for the inspection of areas and targets of strategic interest [33], and they are divided into three kinds: AUVs, autonomous surface vehicles (ASVs), and remotely operated underwater vehicles (ROUVs). These vehicles can be also deployed in the air (unmanned aerial systems (UASs)), at the sea's surface (ASVs, also known as unmanned surface vessels (USVs)), or in the water column (AUVs). UMVs have various applications,

such as gathering oceanographic and meteorological data [34–39] and monitoring sea ice [40] and wildlife [41–44]. Most ROUVs are equipped with at least a video camera and lights. The main difference between these types is that an operator controls the ROUV, while AUVs and ASVs operate autonomously. Thus, some innovative vehicles are capable of sensing the environment and navigating on their own. UMVs include semi-submersibles and unmanned surface crafts.

Table 1. Features of unmanned vehicles (UMVs) classified according to the types (unmanned aerial system (UAS), autonomous surface vehicle or unmanned surface vessel (ASV/USV), autonomous underwater vessel (AUV), remotely operated underwater vessel (ROUV), and gliders). The main features are indicated in terms of environment explored, control, navigation system, and propulsion type.

	Operates			Controlled by		Navigation System		Propulsion	
UMV	**In Air**	**Water Surface**	**Under Water**	**Operator**	**Independent**	**GPS Navigation**	***e*-Compass**	**Propellers**	**Variable Buoyancy**
UAS	X			X	X	X		X	
ASV/USV		X			X	X		X	
AUV			X		X		X	X	
ROUV			X	X			X	X	
Glider	X	X	X		X	X	X		X

The advantage, with respect to aerial photogrammetry and other large-scale monitoring approaches, is that the measures are quite direct, punctual, and characterized by precision and accuracy, even if large territories may be explored for longer times by smart AUVs. Their employment in association with other classical monitoring systems can increase accuracy and efficiency, because the movements of autonomous vehicles can be semi-randomly influenced by alarms sent by satellites or other monitoring sources, modifying the programmed maps of cruises. Such systems may also find wide application in critical coastal zones, such as in marine protected areas (MPAs), because they are left free to iteratively explore transects and continuously transfer to reference centers (on land) signals of "all normal" conditions or, alternatively, warning messages prompting immediate inspection by coastal guards or other marine authorities [45–47]. Several MPAs have been set in Europe in the last decade after the evaluation of marine sites of ecological interest [48], where ship transits are totally or partially forbidden, and consequently, oil spills should be avoided. Since continuous and punctual environmental monitoring in these areas is critical, automation of smart monitoring activities may represent an obvious solution.

AUVs are widely used for monitoring survey and data collection. They can be equipped with various types of sensors, such as sonar, video cameras, and the means for measuring conductivity, temperature, pressure, and salinity, among other factors. AUVs collect information through sensors. Parameters such as the water temperature and speed are simply measured and easily interpreted. Other types of data are more complex to collect and analyze because they require further interpretation to convert the records into meaningful information. Therefore, the selection of sensors is important for successful detection. Equally important is the diagnosis of the problem, which requires the ability to analyze and interpret the data collected by eliminating sensor noise and therefore making the data reliable [49–52]. They have the advantage of huge spatial coverage, but they are limited by a small resolution [53]. The risk is that the collected data might not be representative from a temporal point of view. As part of the research, they can be involved in data collection for bathymetric and magnetic fields and conformation of the seabed [54]. They are also used for the evaluation of water parameters in specific locations, such as in the areas surrounding hydrothermal processes or coral reefs [55]. Currently, they find application in various fields ranging from scientific research to industrial purposes. In industrial applications, AUVs are used for the monitoring and maintenance management of oil, gas ducts, and electrical lines [56]. Evidently, AUVs and ASVs represent the most

recent advances in the field of smart tools compared with ROUVs, which were introduced several decades ago and have been improved in terms of efficiency and cost in the last few years. Additional equipment is commonly added to expand the vehicle's capabilities. These may include sonars, magnetometers, a still camera, a manipulator or cutting arm, water samplers, and instruments that measure the water clarity, water temperature, water density, sound velocity, light penetration, and temperature [57].

ASVs and AUVs suitable for marine monitoring can vary from relatively small vehicles lifted by one or two persons and deployed from a small inflatable boat to large diesel-powered surface vessels [58]. In particular, smaller vessels are able to operate with a high level of autonomy and are also capable of staying at sea for several months. In contrast, larger surface vehicles often tend to be more tightly controlled. Surface vehicles have the advantage of being able to continuously receive GPS position data while navigating, and their locations can be accurately recorded at all times. Subsurface vehicles do not receive GPS data while they are immersed and therefore must generally rely on depth measurements and dead reckoning using electronic compasses [59,60]. Moreover, ASVs can operate safely in hazardous locations and at night and can cover much larger areas, mitigating the risk of crew fatigue. In some cases, they can independently operate off large ships [61].

ASVs started to be developed at an academic level in 1993, when the MIT presented its first vessel, called ARTEMIS [62,63]. The newer ASV, called the Shallow Water Autonomous Multipurpose Platform (SWAMP), is a full-electric catamaran built with the purpose of being a modular multi-functional vehicle, having several applications for a range of missions, such as geomorphological analysis, water sampling, and physical and chemical data collection in harsh environments [64]. This vehicle has four thrusters, azimuth pump-jet thrusters that are flush with the hull, small-draft soft foam, an unsinkable hull structure with high modularity, and a flexible hardware and software architecture [64]. Generally, USVs are associated with unmanned surface vehicles (USVs) [65]. Usually, USVs are equipped with a central processing unit and different memories for saving and providing a preliminary management of the acquired data (e.g., compression and classification). In addition, batteries and photovoltaic panels are equipped to increase the electrical autonomy as much as possible, which generally turns out to be one of the major limiting factors [60]. ENDURUNS is an example of a system that integrates both an AUV and an USV system. The USV is equipped to support the power requests of both systems with photovoltaic panels and rechargeable battery packs. The peculiarity of this AUV is the ability to move using two different modes. The first, thruster mode, allows it to move in a precise and controlled way to perform transects parallel to the seabed and collect data with great accuracy. The second mode is called glider mode and allows it to cover larger areas for a longer time, as consumption is significantly reduced [66–68]. The USV autonomously follows the AUV, providing information for accurate geo-localization of the acquired data. Data transfers between the AUV and the USV are realized through acoustics communication or through a wireless connection [53]. It is also important to establish threshold values at the beginning of the mission for correct data processing. The last phase is represented by adaptation, in which the mission plan can be redesigned by changing the detection scheme and the trajectory of the vehicle [69]. The AUTOSUB Long Range 1500, which is being designed, built, and operated by the National Oceanography Center, is a highly capable AUV with the potential of providing measurements that would have been previously impossible to collect, therefore allowing key advances in marine ecology studies. This vehicle will be built to be able to reach a depth of 1500 m [70,71].

Finally, an underwater "glider" (Table 1) is a specific type of AUV which employs variable-buoyancy propulsion instead of traditional propellers or thrusters. It houses sensors capable of making multidisciplinary oceanographic observations with long-term deployments (months) and has the ability to cover large distances (hundreds to thousands of kilometers) because it has significantly greater endurance compared with traditional AUVs [72]. The typical up-and-down, sawtooth-like profile followed by a glider can

provide data on temporal and spatial scales unattainable by powered AUVs and which are much more costly to sample using traditional shipboard techniques. Four commercially available electric underwater gliders represent the main opportunities in this field: the Slocum electric [73], the Seaglider [34], the Coastal glider [74], and the Sea Explorer [75]. In addition, other gliders are under development, including Spray [76]. Coastal gliders are designed to be applied in the littoral zone (they are self-ballasting from essentially fresh to full ocean water) with a faster maximum speed (2 knots, according to Imlach and Mahr [74]). The Deep Glider, on the other hand, is designed to operate at depths of 6000 m [77]. These vehicles mostly extract energy from wave motion and convert it directly into forward motion. The vehicles also use solar or wind power to charge batteries used to power the navigation systems and the sensor payload.

4. Experimental Data

As mentioned above, the main advantage of coupling e-noses with smart autonomous vehicles relies on the possible customization of analytical procedures, as well as on the rapidity in intervention policies suddenly made possible after an accident or any type of pollution event. Attempts to quantify the ecological effects of special coastal areas, such as MPAs and MPA networks, are usually hampered by a lack of well-designed monitoring studies [78,79]. The management plans for an MPA network aim at protecting and conserving biodiversity and other natural values within protected areas. However, coastal monitoring in an MPA is not limited to the detection of oil pollution and the mapping of VOCs, because various ecological descriptors may be crucial to follow the chemical and physical state of key environments along the coastal waters in a timely manner [80,81], such as in seagrass meadows and recruitment areas. To this end, we designed and realized an innovative system for marine environmental monitoring whose main features are represented by the employment of an innovative probe carried aboard a smart ASV (Figure 2). Although the realization of the monitoring system is still in progress, it may be worth it to present the data obtained to date as a preliminary description of these innovative tools based on the newest technologies appearing on the market. In particular, we designed the prototype of a simple and inexpensive floating ASV able to independently move within an MPA located around the Isand of Ischia and send real-time data to a land-based station located at the local laboratory of Stazione Zoologica Anton Dohrn. The floating ASV was equipped with three electric propellers mounted under a floating plastic base, containing a glass bell that protected the main components. One of the main innovations was represented by the presence of pioneering probe technology.

The probe was a multi-metal detector produced by SensiChips [82], named "smart cable water" (SCW), based on the impedance generated by the presence of various pollutants. Such probes must be trained prior to be applied for ecological purposes, because their reactions to patterns of various substances are singular and not-linear. In this light, they represent a complex though interesting means to afford biomonitoring of coastal ecosystems. SCW is a multi-sensor microsystem (MSM) produced to monitor the presence of toxic chemicals (TICs), pollutants, hydrocarbons, and organics in water [83,84]. At the core of SCW there is SENSIPLUS, a microsensor platform which can interrogate on-chip and off-chip sensors with its versatile electrical impedance spectrometer (EIS) and potentiostat. Analyses performed with EIS allow for exploiting the RedOx dynamics of catalytic noble metals to aid the fine discrimination of chemicals along with the measurement of the conductivity and permittivity spectra. The on-chip potentiostat is used for a number of voltammetric or amperometric measurements and real-time discrimination of pollutants.

By cycling the electrodes with overvoltage, the device prevents or mitigates the formation of biofouling. Consequently, SCW may be considered a reliable multiparametric water analysis microsystem. Thanks to its analytical instruments and availability of catalytic interdigitated electrodes, SCW (Table 2) also represents an experimental microsystem for discriminative measurements (Figure 3).

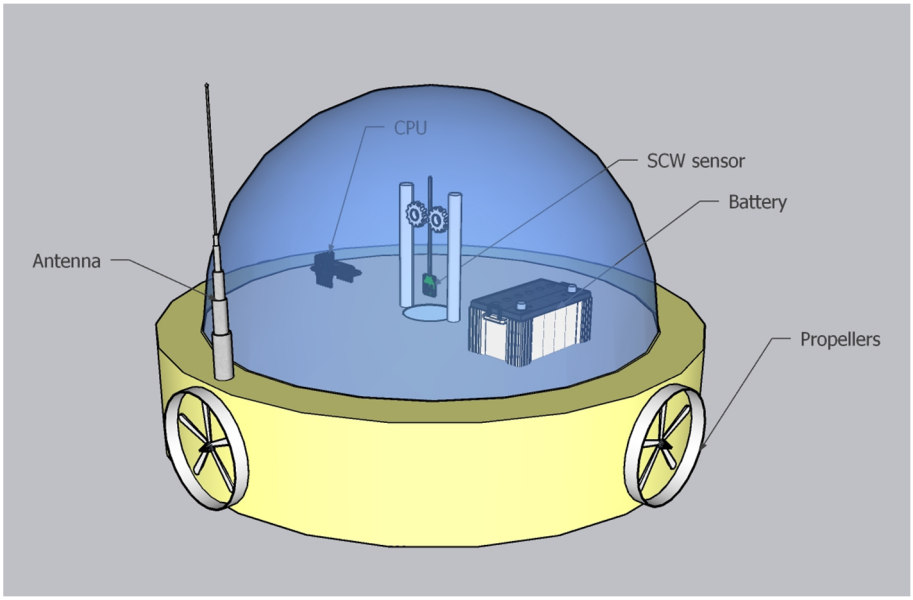

Figure 2. An experimental ASV equipped with an innovative probe under development in our laboratory. An SCW is located on board an ASV, and the CPU directs the movements of the instrument over a network of fixed points to transmit data sets and, eventually, alarms according to thresholds set by local administrators. The probe is automatically extracted from the marine water at given time intervals and mopped to avoid corrosion of the metal plates.

Table 2. Technical specifications of SCW used for our smart monitoring test.

ELECTRICAL	
Supply voltage	1.5–3.6 V
Max current	0.4 mA continuous when reading on-chip sensors with EIS
Size	12 × 15 mm, 3 mm thickness
Interface	I^2C or SENSIBUS, single data wire multidrop sensor array cable interface, 1.5–3.6 V
Unique identifier	OTP 48 bits unique device identifier, 16 bits user-defined
ELECTRICAL IMPEDANCE SPECTROSCOPY	
Frequency	From 3.1 mHz to 1.2 MHz
Vpp output sinewave	From 156 mV to 2.8 Vpp
Coherent demodulation	1st, 2nd, or 3rd harmonic
Output	Reciprocal of real or imagery component
Wide measurement range	From ohms to 100 MΩ
TEMPERATURE	
Range	−40–125 °C
Accuracy	±0.1 °C
Thermodynamics	Calorimetry, enthalpy, and exothermic or endothermic
ELECTROCHEMICAL METHODS	
pH	From 3 to 14, potential of platinum vs. clads-platinum
ORP	Total oxidation and reduction potentials
RedOx	Reduction or oxidation activity (free chlorine, hardness)
Voltammetry	Specific reduction or oxidation potentials
Anodic stripping voltammetry	Measures heavy metals
Electro-catalysis	Noble metal IDEs measure current specifically
IMPEDANCE METHODS	
Conductivity spectroscopy	Resistivity, salinity, EC, TDS, and absorption dynamics
Dielectric spectroscopy	Turbidity, SS, biomass total and active, and hydrocarbon detection

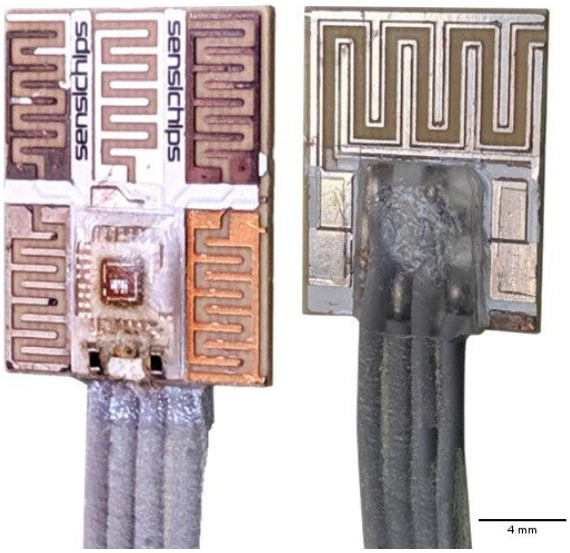

Figure 3. Front (left) and rear (right) sides of SCW adopted to produce a smart ASV for coastal monitoring (see Figure 2).

As mentioned above, an SCW needs to be trained to recognize pollutants and other substances of ecological interest. For this purpose, various amounts of key compounds (such as nitrogen and phosphorus compounds) were tested and used to calibrate the probe. Our results indicate that low amounts of important pollutants were detected by the instrument, but a full set of permutated measurements is needed to train the instrument to recognize compounds in any pattern of reciprocal concentrations.

Another constraint is represented by the oxidizing power of the seawater, because continuous immersion in water rich in NaCl produces fast deterioration of some of the metal plates, drastically reducing the performance, as demonstrated by our tests. For this reason, the SCW was mounted over the ASV by means of an immersion device able to move the SCW up and down at various time intervals, protecting it with frequent washes in distilled water followed by mopping and drying of its surface. However, this SCW-equipped ASV was demonstrated to be quite promising for coastal monitoring, because its performance may improve through auto-training and also because of the easy installation over small smart vehicles wirelessly connected to the control stations on land.

5. Autonomous Monitoring Networks

The increase in the exploitation of marine resources enforces the necessity to develop new methods of environmental monitoring which, with the integration of new technologies, make the reaching of new frontiers possible in the field of biological features, namely for environmental, physical, and chemical parameters and sampling surveys [85]. In fact, in recent years, several projects had the goal of identifying new tools for the optimization of monitoring and sampling techniques for the improved assessment of an environmental status, which is the basis of several international management policies [86,87]. The conception of new models of structures for data collection is necessary to cope with the different types of marine environments in which the survey is carried out to increase the operational range either in time or space [64]. While multiparametric cabled bases are a well-proven solution for the remote and continuous monitoring of marine environments [84], the implementation of more autonomous solutions is an important future prospect to ideally allow data collection at any depth and distance from the coast. In this light, network complementation with surface or aerial (radio frequency transmission) and underwater (acoustic) video monitoring may represent the smartest solution.

Video monitoring, in fact, can also be realized by taking advantage of a fixed-point cabled camera installed over a platform [88] or a mobile underwater television (UWTV) consisting of a towed camera sled. The sled is positioned on the seabed and dragged along

a transept. Care must be taken to try to keep the vessel speed stable, as it is affected by the surface conditions [89]. The advantages of the UWTV solution lay in the fact that if used properly, it allows for obtaining a relatively constant measurement while being more accurate and less invasive than trawling surveys. For example, the Scottish government and Joint Nature Conservation Committee [90] considered the use of UWTVs an excellent solution to identifying any new areas potentially eligible to become MPAs [91].

Upon the set-up of various autonomous monitoring vehicles (AUVs), a network composed of AUVs moving around a single buoy may produce timely maps of the marine areas under control (Figure 4). The network should contain a master buoy equipped with a wireless link receiving data from the AUVs and, eventually, satellite communication to an inshore station in order to raise warning signals to the station as well as to check the real-time evolution of pollution events.

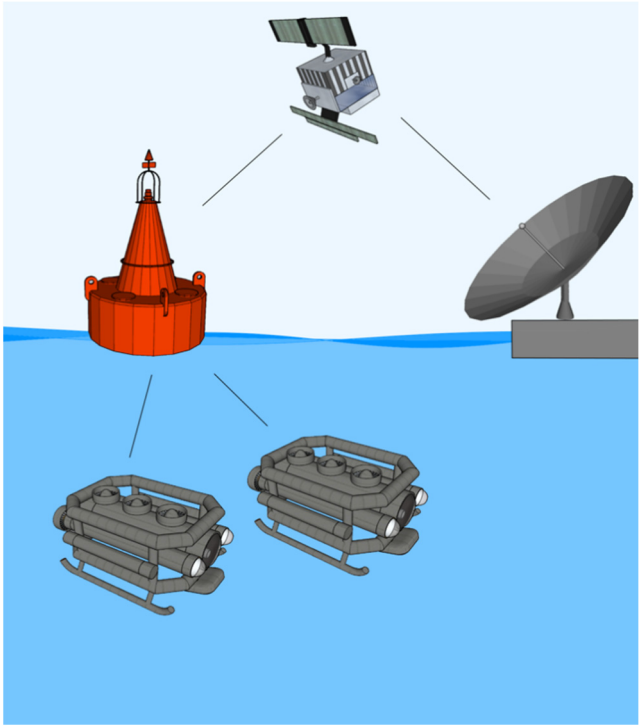

Figure 4. Real-time marine monitoring multimodal scenario (from [20], modified). A master buoy (red) receives pollution data from the AUVs (two gray vessels) and sends data to the land through satellite communications.

6. Marine Permanent Infrastructures

Currently, the largest existing networks of underwater observing stations are represented by permanent infrastructures specifically intended for multidisciplinary monitoring and research in the fields of geology, oceanography, and ecology. The advantage of permanent infrastructural networks is that they can be connected directly to the coast or through a succession of nodes [85,86]. Connection by a cable transmission line directly provides power and real-time data transmission to and from the marine observatory. However, networks of this magnitude are very expensive.

The operation costs for this kind of infrastructure are really high, considering the involvement of suitable ships and specialized equipment. Moreover, given the complexity and multidisciplinary nature of the projects, the use of specialized personnel is required in various areas, such as engineers, marine scientists, and data analysts [85,92]. Although the possibility of having a connection with the shore confers numerous advantages by finding an easy solution to the problems of energy supply and data transmission, at the same time, these prove to be a limit if the site of interest is not close enough to the coast [93]. Permanent

structures tend to also be limited due to their restricted spatial coverage and unpredictable bias in monitoring results that can be influenced by the infrastructure's presence [68]. To overcome these limitations, most of these infrastructures are integrated with mobile nodes that allow observations to be extended over a much larger area, taking into account different geographical gradients and different depths. A network designed by different nodes, including mobile ones, allows for collecting data in a more extensive and continuous way, making it possible to follow animal movement across different spatial gradients [94] and energy flux interchanges [95]. The data collected are transmitted through a cyber infrastructure, making it possible for anyone with an Internet connection to download the data in real time. Raw data are archived and read by a system code that separates them into data streams based on the content. According to the requirements, multiple levels of data products are processed with different algorithms to make them easier to consult at different levels of complexity. Each platform hosts several integrated scientific instruments, and they can contain multiple "nodes" to which the integrated instruments are attached, as well as a means for transmitting the data to the shore. Some examples of cabled observatories that integrate remote control systems and interactive sensors are the following.

The Ocean Networks of Canada (ONC) [96] is a research facility hosted and owned by the University of Victoria. This network operates with several ocean observatories in the deep ocean and coastal waters of Canada from the west and east coasts and the Arctic. It continuously collects data in real time, which are made available for scientific research, governments, and industry. Through the use of cabled observatories and remote-control systems, the ONC enables the development of several projects [97].

NEPTUNE is among the largest observatories. This observatory has several nodes, with various cabled instrument platforms and mobile crawlers that can cover around 15 km of linear distance with a depth oscillation of about 500 m [85]. This observatory is equipped with various instruments that can be used in different applications, such as a seismograph to monitor earthquake activity, bottom pressure recorders for real-time tsunami monitoring systems around the world, and specialized hydrophones to track marine mammals' activities [98,99] and investigate how they are influenced by human activities. Specialized sensors, cameras, and remotely controlled sampling devices make NEPTUNE's site easily adaptable for monitoring [98] commercially relevant fishery resources (such as the sablefish *Anoplopoma fimbria*) with life cycles that involve small-scale and large-scale geographic movements with both vertical and horizontal changes [100,101].

The American Ocean Observatory Initiative (OOI) funded by the National Science Foundation was designed as a long-term project to collect ocean data. The Ocean Observatories Initiative is made up of five major research components with several associated arrays located in the northern and southern Atlantic and Pacific according to the demand of the scientific community. Each array is composed of fixed and mobile platforms [102]. A platform can be stable, fixed, or mobile. Mobile components can move up and down in the water column or be a glider, which is able to move in three dimensions. Each platform hosts several integrated scientific instruments and can contain multiple "nodes" to which the integrated instruments are attached, as well as a means for transmitting the data to the shore. The OOI instrumentation is involved in the support of several research projects, including climate variability, ocean food webs, biogeochemical cycles, and coastal ocean dynamics and ecosystems.

The European Multidisciplinary Seafloor and water column Observatory (EMSO) [103] consists of a system of regional observatories located at key sites around Europe. Each platform is equipped with multiple sensors sited along the water column and on the seafloor. They constantly measure different parameters. Data are collected and available to different users, from scientists and industries to institutions and policy makers [104]. The EMSO infrastructure range runs at the European scale from the coastal area to the deep sea and open ocean, operating with both stand-alone observing systems and nodes connected to shore stations through fiber optic cable [105]. The data in both cases are transmitted in real time either through the cables or through acoustic networks featured by satellite-linked

buoys [106]. Data are collected from the surface of the ocean to the seafloor. In addition to generic sensors, specific modules with different instrument combinations are deployed to be able to respond to specific objectives [107]. Many physical and biological applications require observation of the physical and ecological parameters (such as concentrations of oxygen and chlorophyll) at high-resolution time series data over long periods. Other systems for marine ecological research require photo and video imaging, acoustic recording, and in situ collections [65–85].

KM3NeT is a research infrastructure located in the Mediterranean Sea which houses the next-generation neutrino telescopes. Still nearing completion, this structure aims to have a detector volume of several cubic kilometers of clear seawater [108]. The main purpose of this project is to allow an innovative framework for studying neutrinos from distant astrophysical sources. Nonetheless, given the arrays of thousands of sensor modules, this research infrastructure will also house instrumentation for other scientific investigations for long-term and online monitoring that may find application in such fields as marine biology, oceanography, and geophysics [108].

The Joint European Research Infrastructure of Coastal Observatories (JERICO) is a network of coastal observatories providing a European Research Infrastructure (RI) dedicated to the observation and monitoring of marine coastal seas to provide high-quality environmental data as tools for scientific researchers and societal and policy needs [109,110]. It comprises JERICO-S3. In parallel, JERICO-RI is an integrated pan-European multidisciplinary and multi-platform research infrastructure dedicated to the assessment of changes in the coastal marine system. JERICO-S3 officially started in 2020, entitled Marine coastal observatories, facilities, expertise, and data for Europe. Its aim is to be involved in the cooperation of coastal observatories in Europe by the implementation and improvement of the coastal structures of a European Ocean Observing System and to cooperate with other European initiatives. There are currently 10 structures between the different partner nations. These facilities provide wired observatories, AUVs, fixed and multi-platform structures, and calibration laboratories to allow the carrying out of different projects [111]. An example of some of these projects currently underway is the study focused on Algerian Basin (AB) circulation through the monitoring line ABACUS [112] through the AB between Palma de Mallorca and the southern part of the basin [113]. These projects involve partners both public (e.g., university and research institutes) and private (e.g., private non-profit research institutes) from European and non-European countries.

7. IoT Hardware Modules

The European Research Cluster of the Internet of Things defined the IoT as a technological revolution, consisting of a dynamic global network infrastructure with self-configuring capabilities. It is based on standard and interoperable communication protocols. In this system, physical and virtual "things" have identities, physical attributes, and virtual personalities, and they use intelligent interfaces natively integrated into an information network [114]. The IoT is characterized by the integration of various devices equipped with sensing, identification, processing, communication, actuation, and networking capabilities [115] (Figure 5).

The term Internet of Things was initially created in 1999 by Kevin Ashton, an expert in digital innovation [117]. IoTes (i.e., the "objects" taking part of the network) can be variously defined. Firstly, IoTes can be defined as intelligent objects, or "things having identities and virtual personalities" operating in smart spaces and using intelligent interfaces to connect and communicate within social, environmental, and user contexts [118]. IoTes are also considered an extension of the Internet with objects, devices, sensors, and items not ordinarily considered computers [119]. In addition, the IoT is understood as a global network infrastructure linking physical and virtual objects (IoTes) through the exploitation of data capture and communication capabilities [120]. Finally, the IoT can be regarded as a way to promote information interaction by linking people, things, and objects autonomously and intelligently without any temporal or spatial constraints [121,122].

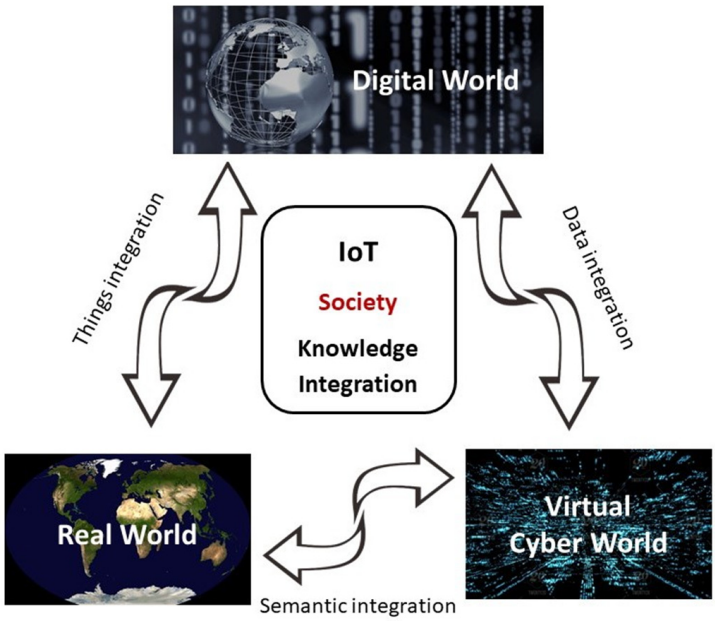

Figure 5. Knowledge integration for the logical relationships among the digital and cyber world as connected with the real world ([116], modified).

Even if the IoT and IoTes are still evolving, their effects are beginning to be seen and are making great strides, offering universal solution media for an interconnected scenario [123]. This is mainly due to the fact that the IoT guarantees high-speed and accurate data with secure processing and an improved client or user experience [124,125]. Its development depends on dynamic technical innovation in a number of important fields, from wireless sensors to nanotechnology [126]. In fact, the IoT can be applied to various fields of our daily life, such as eHealth (a relatively recent health care practice supported by electronic processes and communication) [127], security, entertainment, smart cities, defense, and many other fields [128]. The IoT can be used to manage soil moisture, irrigation and drainage systems, and crops in smart farming systems. Finally, the IoT is useful to monitor the conditions of marine environments, allowing scientists to monitoring such physical parameters as the water temperature, dissolved oxygen, salinity, pH, and turbidity [129]. Smart health sensors are used to collect human physiology information as well and use gateways and clouds to analyze and store the information and wirelessly send the analyzed data to caregivers for further analysis and review [130]. The IoT can also be operated in smart cities for (1) improving infrastructure, public transportation [125], and electrical conductivity thanks to smart grids that combine the information and communications technologies into an electricity network [131] and (2) helping predict natural disasters with the combination of sensors and their autonomous coordination and simulation [132]. However, the IoT is not limited to public uses. It can also be privately adopted for smart home and security systems, such as by natively connecting several household devices to the Internet [133].

Domingo [134] proposed the architecture of an IoT network in three layers: (1) perception, (2) network, and (3) application. The main function of the perception layer is to identify specific objects and gather information. It is formed mainly by sensors, actuators, monitoring stations (such as cell phones, tablet PCs, smart phones, and PDAs), nano-nodes, RFID tags, and readers and writers. Depending on the type of sensor, the information to be referred can be the location, temperature, orientation, motion, vibration, acceleration, humidity, or chemical changes, among other details. The collected information is then passed to the network layer for its secure transmission to the information processing system. This network layer consists of converging, privately owned, wired, or wireless networks where the transmission technology can be chosen (e.g., 3G, UMTS, Wi-fi, Bluetooth, infrared, or ZigBee) depending upon the features of sensor devices. Its main function is to transfer the

information obtained from the perception layer to the middleware layer. It receives the information from the network layer, stores it in the database, and autonomously makes some decisions based on the results and the agreed protocols. The application layer provides global management based on the object's information processed in the middleware layer. Finally, the business layer is responsible for the overall management of the IoT system, including applications and services. In particular, this layer eventually builds management models, graphs, and flowcharts, and it proposes the future actions and operative strategies based on the data received from the application layer [132].

Evidently, the IoT represents a future challenge in many technological applications, minimizing efforts, offering the use of efficient resources, and guaranteeing accurate quality data and a high speed of reaction. The reliability and validity of the data, performance, security, and privacy are additional advantages. However, various issues will need to be addressed in the future, such as privacy issues (hackers can break into the system and steal the data) and unemployment, because some activities performed by human operators will be replaced by machines [125,133].

8. The IoT Applied to Marine Environmental Monitoring

IoT-based technologies, as well as wireless sensor networks (WSNs), a subset of IoT, can be applied to the monitoring and protection of marine environments [13]. In particular, monitoring activities employing IoT technology can be used for ocean sensing and monitoring of water quality [134], coral reef protection, offshore and deep-sea fish farms, and wave and current watching [13].

The development of an adaptive, scalable WSN must foresee such critical properties as autonomy, scalability, adaptability, durability, and simplicity [135]. On the other hand, the design and deployment of a lasting and scalable WSN for marine environment monitoring should consider all of the following peculiar challenges mentioned in the second paragraph. Other issues can concern the devices and sensor nodes, which can be highly reliable because of the difficult deployment and maintenance. In addition, their coverage needs to be carefully evaluated, because their application over large areas far from direct control and with expensive, delicate equipment should be protected against possible acts of vandalism [13,135].

Overall, an online marine monitoring system needs (1) sensors adequate to measure seawater features, (2) a controller or processor unit to compute the data from sensors, and (3) communication equipment to send data from the processing unit to the cloud via ground stations. Sending large amounts of data (as large images or videos) to the cloud requires the combination of the IoT and cloud computing, because satellite communications may be expensive, in terms of both money and energy consumed [136,137] (Figure 6), and sensing stations should be relatively small and light.

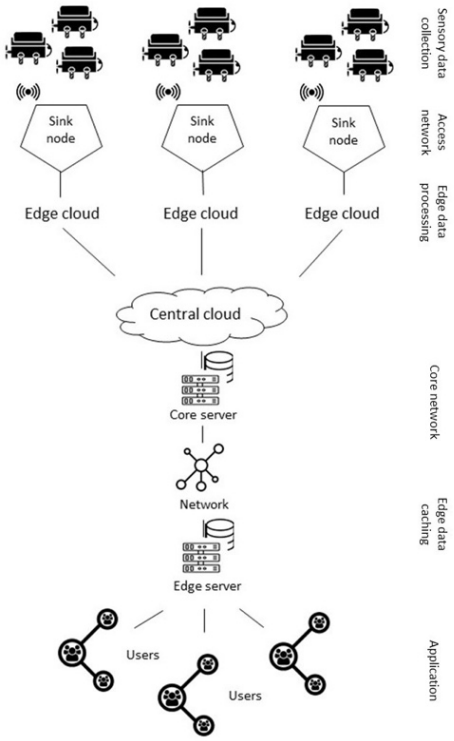

Figure 6. Physical architecture of smart monitoring systems with data collection networks.

9. Monitoring Applied to Aquaculture and Fishery Productions

Various technological applications of artificial intelligence (AI) were set for improving the sustainability of monitoring in coastal waters and oceans, as well as in aquaculture and smart fishery plants, and they are widespread nowadays [138]. In particular, the attention of scientists in AI-inspired fisheries focused mostly on monitoring the automation of fishery resources (mainly detection, identification, and classification). However, it is still unclear how fishers perceive AI needs and how governments exhibit a tangible strategy on the regulation of AI concerning smart fishery systems to promote the value and potential of the techniques of AI-inspired fisheries. AI has great influence on catch monitoring across fishery systems at sea [139], and several AI applications improved fishing activities, helping the economic evaluation of commercial fleets [140]. In addition, a fishery may be helped by electronic monitoring of the catch and bycatch [141], as well as the detection and forecasting of fishing grounds [142], eventually applying mathematical models to simulate fishing vessel behavior [143]. This also helps to reduce fishery wastes [144] by optimizing the sorting operations. Finally, automation of the monitoring of illegal fishery methods [145] is also possible for reducing the negative impacts of fisheries on coastal areas. The AI technologies of fish farming mainly focus on the means for optimizing the efficient use of resources in ecosystem management [146]. In several instances, fishery and ecological monitoring have been strictly interconnected. In fact, sustainable fisheries are related to environmental monitoring [147]. Various authors stressed the scope of smart fisheries because of the "epidemic of plastics entering the sea". This warrants urgent action if humanity is to stave off a collapse in fish stocks. Additionally, oil spills [148] and global changes [149], as mentioned above, are topics of great concern [150], prompting not only issues of environmental conservation but also large impacts on fisheries [151], requiring accurate and modern monitoring activities. The employment of smart systems able to autonomously tune their activities according to local perturbations and able to be trained for the detection of various compounds (both in the water and over the water, evolving into e-noses) is boosting improvements in various fields of environmental monitoring.

A special case of monitoring marine environments is the one applied to aquaculture activities [152,153], primarily because these activities may impact various coastal areas when practiced in cages, pens, floating tanks, and raceways deployed in open waters [154]. There is great concern about the potential environmental effects of marine finfish cages on the water quality [155] and a large interest in developing an ecologically responsible industry [156,157]. Several reviews [158] have broadly addressed this topic [159–161].

In addition, aquaculture ponds may be considered very special marine environments, and they need continuous monitoring and real-time reactions to negative changes impacting the organisms contained therein [162]. In addition, in this case, artificial intelligence and IoT devices may be applied to improve production efficiency and reduce impacts and risks. In fact, the connection between good environmental conditions and seafood health in aquaculture has been documented [163–165]. Sea cages can be more than 45 m in diameter and 30 m deep, and they need frequent inspections. Although a single cage can contain high value production [166,167], the level of surveillance of the product and of the closer environments is often low [168]. As for tank aquaculture, various remote sensor systems were proposed to have a conveyed gathering of sensor hubs organized together and be able to exchange the crude information up to a base station through an IoT network [169,170]. Using Arduino-like hardware and a few simple probes, automatic farming systems were developed based on IoT platforms (Figure 7).

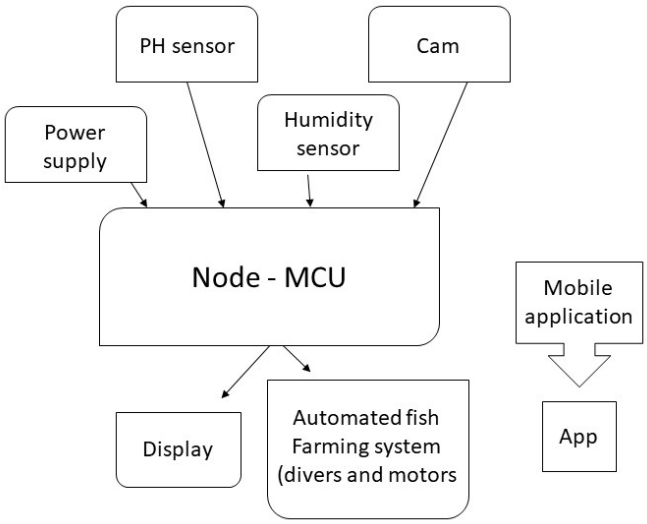

Figure 7. Block diagram of the system proposed in [169] for IoT control of aquaculture productions (modified).

All IoT systems for aquaculture are to be considered smart systems based on intelligent sensors, intelligent processing, and intelligent control. Their functions consist of data collection, real-time image acquisition, wireless transmission, intelligent processing, warning messages, and auxiliary decision making [171–173]. Any aquaculture IoT monitoring system fundamentally consists of water quality monitoring stations, including meteorological stations, water quality control stations, and on-site and remote monitoring centers. These structures are supported by a central cloud-processing platform [174]. The water monitoring stations are provided with monitoring sensors and take advantage of wireless data collection terminals. Local data are collected in ponds and transmitted to monitoring centers. In particular, such water quality parameters as dissolved oxygen, pH, and ammonia concentration are key elements to allow prompt answers to production issues. Generally, weather stations are also used to acquire in real time such meteorological data as wind speed, wind direction, and air humidity. The system analyzes the relationships between the water quality parameters and weather changes to predict water quality trends and ensure the optimal water quality in the culture tanks. The tank controllers include independent control terminals, an electrical control box, aerators, and other equipment,

with the control terminal receiving wireless instruction from the control equipment. On-site and remote monitoring centers based on wireless sensor networks and the Global System for Mobile Communications (GSM) central servers with central cloud processing platforms are included, favoring an intelligent control algorithm for water quality to achieve data acquisition, smart data processing, alarms, and their mailing to human managers [175]. Central cloud processing platforms provide the basis for decision making for farmers by providing a variety of models and algorithms of quality monitoring, feeding, and pond management [176]. These strategies reduce the risks of product losses, reduce the pollution of local environments by increasing the efficiency of culture procedures, and also reduce the need for using drugs, with obvious advantages for local environments.

10. Conclusions

Environmental monitoring solutions must be adapted to each individual situation, because communication systems and the rapidity of responses differently influence the monitoring activities in various environments. Evidently, pollution is concentrated off the coastal areas [177], where anthropized urban settlements are mainly located and maritime traffic is intense [178]. In the case of the Mediterranean, for example, which is almost completely surrounded by lands, ecosystems may be extremely fragile and vulnerable because their waters are slowly renewable, thus making them sensitive to all kinds of pollutants, especially when derived from commercial traffic, industrial pollution, or touristic activities [179]. In parallel, these areas are characterized by valuable and fragile environments such as seagrass meadows and coralligenous areas [180,181], and they deserve a higher degree of monitoring and conservation practices [182]. This task is partially accomplished by the institution of MPAs and sanctuaries, but again, they require a higher level of monitoring and immediate reaction to stresses produced by anthropic activities in order to conserve key reproductive areas and fragile environments [183,184]. In this case, communications are not the most important problem, since the presence of coasts closer to the monitoring areas guarantee a fast transfer of information to the computing centers [176]. In contrast, oil pollution has become a matter of serious environmental concern in all oceans [26], with petroleum hydrocarbons (gasoline, kerosene, fuel oil, etc.) penetrating shallow and deeper environments through spills or leaks, as well as after frequent accidents [27]. Here, the rapid delivery of signals becomes critical because coastal stations are quite far away, and satellite communications become indispensable.

An ocean-sensing and monitoring network is a monitoring system that has basically been applied since the last century because oceanographic and hydrographic research vessels were previously adopted for this purpose. A water quality monitoring system usually monitors water conditions and quality, including water temperature, pH, turbidity, conductivity, and dissolved oxygen (DO) in bays, lakes, rivers, and other water bodies. A coral reef monitoring system typically monitors coral reef habitats and the surrounding environments. A marine fish farm monitoring system checks water conditions and quality, including the temperature and pH. It measures the levels of waste and uneaten feed in a fish farm, as well as fish conditions and activities including the presence of dead fish. A wave and current monitoring system measures waves and currents for safe and secure waterway navigation [13]. The most common tools traditionally used to monitor marine environments are satellite imagery, underwater devices with various sensors, and buoys [184]. These devices transmit data by means of satellite communications or close-range base stations, which present several limitations and elevated infrastructure costs. Unmanned aerial vehicles (UAVs), as described above, are an alternative for remote environmental monitoring which provides new types of data and ease of use. These techniques are mainly used in video capture-related applications in its various light spectra and do not provide the same data as sensing buoys, nor can they be used for extended periods of time [184]. However, it is important to stress that monitoring the marine environment is quite challenging, because it requires waterproof robust technology to endure the high levels of humidity and salinity, wave collisions, and extreme weather conditions [135].

In this light, the development of newer "noses", coupled with the powerful features of various kinds of UMVs as classified above, may represent a tremendous innovation toward the collection of data in an efficient way, with minimum costs and fast delivery of strategic information. In this review, we have described, from an historical perspective, the main strategies of monitoring coastal and ocean areas, showing that several smart solutions are presently available, although most of them still need complete engineering to reach full applicability, perfect automation, and their best performance.

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Chapter 2

Automatic Culture of Crustaceans as Models for Science

Francesca Glaviano[1,2,#] and *Mirko Mutalipassi*[3,#]

Introduction: culture of Crustaceans for ornamental market, research, and aquaculture purposes

Crustaceans are emerging model organisms in various fields ranging from marine biology to ecotoxicology and physiology. They are part of a wide variety of aquatic models used to investigate the function of live organisms and ecosystems. Crustaceans have some key characteristics that give them many advantages as models: they are small in size, cheap to culture and maintain if compared to vertebrates, for example mice and rats [*1*]. Small research centres need facilities able to culture various aquatic organisms that can answer researcher's needs. Non-automatized procedures, although capable of good efficiency, need plenty of space and trained operators [*2*].

Modern research and aquaculture are increasingly projected towards the integration of new technologies, through the use of automated and intelligent control systems. These implementations can lead to a more efficient control of the culture's conditions, such as water quality, stocking density and feeding rate allowing a constant and automatic regulation of the conditions of cultivated organisms (Figure 1).

The chance to make automatic measurements of values such as temperature, oxygen, pH, ORP, etc., it allows to optimize their efficiency by reducing labour and utility costs.

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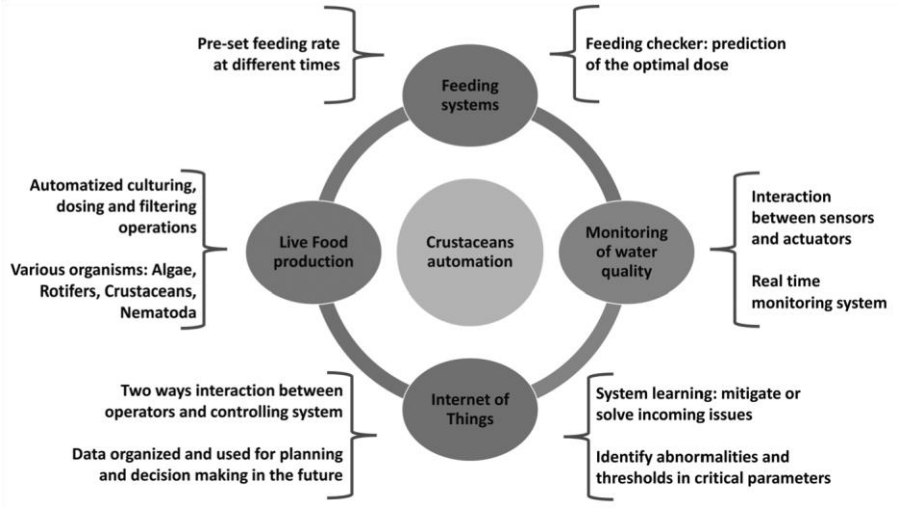

Figure 1. Summary of the potential application related to crustacean's automation.

Automatic devices can not only improve a large-scale system as for aquaculture but they can also largely simplify the culture of small laboratory organisms and it offers interesting applications for the purposes of scientific or biotechnological research [*1*]. If automated culture systems of established aquatic model organisms, such as *Danio rerio*, are widely present on the market, few researchers and industries focused on the development of small, flexible, programmable, and modular culture systems able to culture various aquatic species, such as crustaceans. The culture of Crustaceans, not only for aquaculture but for ornamental and research purposes too, can be considered a multimillionaire market [*3*]. In fact, species belonging to *Palaemon*, *Lysmata*, *Stenopus*, *Periclimenes*, *Hymenocera*, *Clibanarius* e *Mithraculus* genera were reared and studied in the frame of the optimization and, in some cases, automation of culture techniques [*2*]. Among this interesting species, many studies focused on *Lysmata* ones due to their interesting physiology, i.e., peculiar sex reversal and hermaphroditism, but also the high selling price in the ornamental market [*3*]. Rearing systems, such as *planktokreisel*, has been extensively used to achieve the reproduction of species with demanding larval phases, such as *Jasus edwardsii*, and it is one of the first devices on the long road to automation of farming systems. The idea of semi-automatic devices, able to remove degrading organic matter or change water to achieve optimal chemical parameters or assure an optimal water flux, has led scientist to accomplish the reproduction and rearing of various marine species of crustaceans, assuring low mortality. Variation of the original productive plant designed in 70', 80' and 90' were made to optimize recirculation, water flow and to simplify maintenance and operation activities [*2*]. Such modification assured a different movement of water inside the cone-truncated cylinders as well as a reduction in potential damage to larval feeding and swimming appendices. Although these rearing apparatuses were not able to culture all the considered species, as in the case of *Mithraculus forceps* larvae, they can in general assure not only a high output in term of biomass and number of individuals, but also

the healthy state of reared organisms, making them useful for scientific purposes, where physiological responses of model organisms can be altered by stress.

Small culture systems, able to administer food, to check and maintain chemical and physical water parameters, may dramatically reduce production costs and the need for personnel. In addition, the ability of such devices to be monitored continuously may reduce potential losses related to power failure or caused by breakage of water pumps or other instruments [*4*]. Mutalipassi developed a compact culture system that can be modulated on the necessity of various cultured aquatic organisms. Various software, in Ladder language, can be uploaded into the central control unit (PLC) to meet the needs of a range of species. In addition, this system can be used to culture different life stages, not only adult individuals but also larvae of delicate and demanding species. This automatic culture system, set to work without the intervention of trained biologists and intensive daily maintenance, has been devised to meet the needs of cultured species and reduce research and laboratory resources while optimizing spaces and the manpower. The efficiency of automatic culture system depends, as demonstrated in many automatic or semi-automatic culture systems [*2*], on various chemical, biological and mechanical parameters can influence the efficiency of automatic culture systems, as demonstrated in many automatic or semi-automatic culture systems. The efficiency, in terms of health as well as production and costs, can be improved including in the system a robust mechanical and chemical filtration, frequent water changes, a correct feeding, the use of ozone and a careful setting of optimal densities of cultured individuals. Culture process was also improved by a fine tuning of the operational software, providing efficient water changes.

In addition to decapods, various copepod species have been used as model organisms in various research fields. For example, *Tigriopus fulvus* has been used as a model for ecotoxicological studies. Other species, such as *Temora stylifera* and *Centropages typicus*, were used as live feed for a variety of aquaculture species, as substitution of rotifers and other species. Copepods production had to face many issues linked both to feeding unicity and needs and to management difficulties due to the small size of nauplii and larval phases [*3*].

How automation and digitalization support aquaculture

Aquaculture researchers produced a great amount of knowledge that contributed to the industrialization of this productive field. Aquaculture operations require sophisticated tools and specially designed facilities, which “have evolved through intensive research and a great deal of innovation”. As seen in other fields, advancements in technology have supported the development of aquaculture despite many products of technology not developed specifically for application in farming systems have found applications in this area. The improvements in aquaculture technologies have provided a supporting basis to the evolution of aquaculture systems where artificial intelligence, automation and integrated management are key points of the productive system.

The use of computer monitoring and automation in aquaculture has grown during the last years. Benefits aims at: reduction of energy and water waste; greater process efficiency; reduction of labor costs; optimization of animal health conditions.

Robotics has made substantial advances in a wide range of applicative fields including aquaculture [*5*]. In this field, robotics acquired an important role in many applications, ranging from feeding to monitoring water quality, to the cleaning of fouling on the cages and the monitoring, through ROVs, of cage integrities, finishing with security surveillance in open sea. Automation projects play a fundamental role in improving economic profitability. For example, optimization and appropriate management of feeding improves the growth rate of the marine animal and the realtime monitoring of physicochemical parameters of water provides information used to carry out corrective actions for optimal conditions of cultivation [*5*]. The main factors to consider in the design of an intelligent control software are ease of use and intuitiveness, otherwise the non-expert user in the informatic sector will not be able to interface with it; flexibility and adaptability, so that the chosen software is interchangeable with other products. Aquaculture is becoming increasingly important for global food production. On one side oceans are dramatically overfished, limiting the future development of fishing activities; on the other side, only 2–3% of the global food production comes from the oceans despite the oceans cover more than 70% of the earth surface. In addition, aquaculture for non-food purposes is a growing industry that can encompass great revenues with its direct and indirect association with human activities. In fact, beyond human consumption, marine organisms provide various services that can be used, such as biotechnology [*6*]. Non-food aquaculture can provide live feeds, food production, natural restocking and can provide bioactive molecules for pharmaceutical, cosmeceutical and nutraceutical industries, among others. To ensure sustainable and efficient aquaculture production, modern technology should meet aquaculture production needs with the aims to improve energy efficiency, plant availability, product quality, and overall productivity. In an integrated shrimp aquaculture, biological entities (the cultured organisms but also cocultured species or biological filters) and non-biological components, such as water parameters or instruments, should be monitored together [*5*]. All the sampled data is channelled to a central command system, which responds by sending signals (motor pathways) to regulators (for example, aerators, water flow control devices) which in turn act according to information fed into the system in the form of algorithms. The continuous monitoring of all the aspects of shrimp aquaculture, from environmental conditions to physiological parameters and metabolic outputs, allow commercial aquaculture facilities to optimize their efficiency by reducing labour and utility costs, and decreasing the environmental impacts.

Automatic feeding system for aquaculture

Shrimps are popular seafood and among all the cultured species, *Litopenaeus vannamei* is the preferred one due to its culture characteristics and consumer acceptance. The shrimp farming, not differently from the rest of the aquaculture field, relies on intensification, improved feed management and reductions in labour costs to expand and produce wealth. Into the frame of the limitation of production costs, the amount of used food for kilogram of produced shrimp, the source of nutrients and the biological wastes produced are important variables that should be taken into consideration. The cost and quality of shrimp food is generally adequate, and the development of the new techniques is focused on the reduction of the waste, of the

personnel work-time and on the environmental improvements in aquaculture farms [*7*]. In aquaculture, personnel have to face many problems in relation with the feeding routines. These involve the depth of the pond or the shape and culture nets, the differences among solid, liquid feed and, in case, medicines, the risk of working on operational boats in open sea, sometimes miles away from the coast. Automatic feeding systems, expressly conceived for aquaculture, were designed to face these issues in both indoor (plastic or concrete tanks) and outdoor (ponds, cages, river estuaries) aquaculture. First experimental automatic feeders were food dispensers, able to pump a volume of water containing live food or dry food, triggered by an electronic timer. The following step was to develop a system that could be activated, providing food, through photoelectric and motion sensors that detect certain animal behaviour. At the same time, commercial feeding robots were developed to feed different foods, in various tanks, differentially. Usually, these projects are based on a robot that move on a rail and that is managed by a PLC or via Wi-Fi by a computer (Storvik feeding robot) and Arvo-Tec robot feeder system (Arvo-Tec 2010). In some other cases, automation of the aquaculture processes led to the development of an automatic boat, designed to operate in ponds, by untrained personnel [*8*]. The aim is to sell on the market a system that can be used by farmers, with low or no instruction, but involved in aquaculture activities. The boat can be easily controlled by remote, due to a RF transmitter and receiver that can operate up to a range of 3–5 kilometres. In addition, the inbuilt video transmitter and receiver enable the farmer to have a "first person" point of view of the boat movements. The boat is built with an electric engine, powered by LiPo battery (or by solar plate). This technic solution has two advantages: on one side, it allows to reduce noise that can disturb cultured organisms, on the other side it helps to contain building and maintenance costs. It is provided with two different food-pumping mechanisms, the first mechanism was designed for dosing solid food, the second for dosing liquid food and medicines. In addition, the system can be easily modified, for example changing the RF wireless communication with Wi-Fi module. With Wi-Fi, the operators can manage all the operations from anywhere in the world.

This cheap, low-tech system cannot be easily adapted to large, highly productive farms. Digitalized feed monitoring and distributor is the answer in terms of automation as well as digitalization of the feeding process. The development of integrated feeders is one-step through the optimization of the aquaculture performances, especially if referred to shrimp aquaculture. In fact, generally, benthic shrimps are grazers that externally masticate their feed. Cultured species are usually benthic and they have a limited capacity to store food inside their digestive tract which result in the need to eat reduced amount of food throughout the day. For this reason, as the number of feedings is increased, shrimp performance in aquaculture is improved [*9*]. This also means that food that has not been eaten immediately is exposed to leaching and, as consequence, to a decrease in nutritional value. In fact, many of the essential nutrients are water-soluble and evidences demonstrate significant reductions in growth for shrimp offered feeds leached for more than one hour [*9*]. Consequently, industry is moving towards the development of automated feeders that would allow to dose multiply small quickly consumed meals throughout the day minimizing the effect of leaching and addressing the shrimp physiological needs to have access to nourish over a prolonged period [*10*]. Integrated feeding

systems have been developed coupled with automatic aerator and monitoring system. Automatic feeder, working with pre-set feeding rate at different times of the day and night, comprises a control panel and a real-time alarm system able to send SMS messages to operators in case of problems thanks to the embedded microcontroller. The activities of the feeder are subjected to feedback given by temperature, salinity and oxygen probe connected to the monitoring system that, at the same time, can power the aerator in case of needs. Offering multiple meals can be very labour intensive and economically impracticable and the only way is to use automatic and, if possible, digitized devices. Several investigations demonstrated an enhancement of growth performance in shrimp cultured with multiple feeding throughout the day [*9*]. The enhancement is not only due to an increasing in the availability of feeds but also to a reduced degradation and degeneration of the food that is in contact with water for a reduced amount of time.

In addition, in the frame of the new concept of aquaculture farm where digitalization and automation should be fully integrated in the management of the production process, the automated feeders are developing to be more "accurate" not only in the set dose, but also in the prediction of the "optimized dose" that should be administered, and some methods were developed to detect left over feed to stop feeding. Initially, estimated food waste was measured by suspending a sheet below the sea cage during the feeding period, retrieving it after feeding, and counting the left over feed pellets and then by using an underwater camera and image analysis tool to detect and count leftover pellets. To automatically detect the optimal dose of food that should be administered, acoustic technologies have been applied in this field in fish aquaculture and then, in Tasmania, Australia, in a shrimp aquaculture plant [*9*]. This sensor-based feed control system uses sonic technology to obtain indirect measure of feeding intensity, thanks to complex filtering algorithms that can analyse and recognise the unique sound of shrimp feeding and identify its intensity. This acoustic system, coupled with temperature and dissolved oxygen probes, allows a complete management of the feeding procedure as well as real time observation of water parameters, such as dissolved oxygen, that can give indirect indications on how the water parameters change over time as consequence of the food dosage. This acoustic system can be applied in many aquaculture farms, with various productive methods ranging from extensive to super intensive conditions, giving the possibility to provide real time adjustment of feed input based on the real needs of the cultured shrimps. Experiment performed in pond culture of Pacific whit shrimp *Litopenaeus vannamei*, comparing acoustic feeding system with standard feeding strategies demonstrated the advantages of integrated, automatic, and digitized system in terms of growth performance, production, water quality and economic returns [*9*]. In addition, on-demand acoustic feedback feeding system has been developed and has proved to be a reliable tool in shrimp farming. This acoustic on-demand system responds to the signature clicking noise produced by shrimp feeding and produced a higher production and value of *L. vannamei* produced in semi-intensive pond culture [*10*].

Similar systems, used in fish aquaculture but that can be easily converted for shrimp farm purposes, are commercially available. These systems are based on Doppler pellet sensor, CAS pellet sensor and camera sensor (Akvasmart, Norway). Visual systems were developed to obtain the status of growth and health of the shrimp

culture, data that can be integrated with other water parameters. Underwater image visibility technology, developed for aquaculture in ocean environments, were used in association with image defogging technology to work in culture ponds too. The visual system was improved and enhanced with detection technology that can give feeds to automatic feeding devices about the remaining feed at the bottom of the pond [*11*]. The increase of the number of daily feedings in conjunction with a system able to administrate the right amount of food, allow an increase in feed inputs with the consequence of an increased intake and growth as well as an increase value of each production, without the negative consequence related to an overfeeding (increase in productive costs, pollution etc.).

Automatic live food production and administration

Aquaculture needs live feeds for its productions. Live food production is a very intensive and costly manner. Automatized and/or integrated live food production systems have been developed by many authors to simplify, standardize, and kept constant production of the most common or interesting species. Dehasque et al. (1997) developed two culture systems designed to culture *Artemia salina* and Rotifers, respectively [*12*]. In the first system, the one dedicated to *A. salina*, repeated decapsulation procedure, automatic rinsing, concentration, hatching, enrichment, and feeding are completely automatized and take place in one recipient, reducing of approximately four times the manpower needed if compared with the manual method. Rotifers automated culture system was designated with a reversed filter system, in which, during the harvest, the rotifers first pass through a bigger filter (about 300 μm) with the function of debris removal and provided an aeration collar to avoid clogging of the filter screen. Rotifers are then concentrated outside the central cylindrical filter (about 60 μm). After harvest, the settling of the rotifers occurs in the same recipient. Papandroulakis developed at the Aquaculture Department of the University of Crete, an automatized system able to culture phytoplankton, rotifers, and crustaceans, such as *Artemia salina*, with the aim to produce feeds for aquaculture [*13*]. Another intensive system to culture crustaceans, but also shellfishes and fishes, as live food, was developed by the University of Trømso in collaboration with the Aquaculture Department Polarmiljosenteret of the Norwegian Polar Institute as part of the ALFA project (Development of an automated innovative system for continuous live feed production in aquaculture hatchery units), founded by EU (https://cordis.europa.eu/project/id/512789/reporting). The system is coupled with photobioreactors and rotifers culture system (CROPS), to cover all the aspects of the larval development. A novel optical algal density monitoring system based on colour image analysis techniques was also developed for the continuous assessment of algal density and the control of quality and growth rate of the culture. The project was integrated by system led to the footprint reduction, such as solar panels as electricity source, as well as with devices to control carbon dioxide concentration, pH, nutrient contents, and other parameters. Two full-scale complete systems were built and tested in Greece and Norway and adaptations were made to optimise output according to local conditions.

Alternative live foods have been developed to replace traditionally used living organisms such as *Artemia* nauplii and rotifers. Nematodes appear to be a promising food source for commercial penaeid larval culture and various studies have been

performed to find the most promising species. Among various nematode species, *Panagrellus redivivus* demonstrated to be a highly promising species on both mass production and nutritional value point of view. Schlechtriem et al. (2004) investigated various techniques to mass culture nematodes with an enhancement of their nutritional values [*14*]. These techniques, based on oat media, enable shrimp hatchery operators to rely on an inexpensive, standardized, semi-automatized and permanently available live food for first-feeding fish larvae.

Copepods have been demonstrated to be interesting organisms for scientific (as model organisms) and aquaculture (as live food, especially for larvae) purposes. Various systems have been developed for intensive copepod production, taking into consideration that different species need different environments, i.e., harpacticoid copepods need larger surfaces instead of deeper tanks needed by calanoids [*15*]. Whatever the species considered, tanks for copepod culture are usually of a volume of 100–1000 litres, with gentle aeration and they are renewed every 3 weeks, and re-circulating systems are equipped with specific filtration systems [*3*]. Payne and Rippingale developed a semiautomatic system designed for the brackish water copepod *Gladioferens imparipes* [*16*]. This semiautomatic system is designed to separate the various larval stages thanks to a complex system of decreasing mesh nets. A first net, with a pore diameter of 150 μm, was used in the cylinder dedicated to the culture of males and ovigerous females. A second net, with a pore diameter of 53 μm was used to collect nauplii and it works, in addition, as an overflow system. The system was automatized by a PLC produced by Toshiba that allows the routine activities of valves, pumps, lights etc. Buttino and colleagues developed in 2012 at the Stazione Zoologica Anton Dohrn of Naples a complex system of intensive aquaculture designed for the copepods *Temora stylifera* and *Centropages typicus* [*17*]. This system used and improved the ideas of Payne and Rippingale allowing the concentration of larval stages using positive phototaxis and the selective division of different larval stages using nets with various pore diameters. The system was designed with an efficient filtration system made by a protein skimmer, UV sterilizer lamp and a bio-mechanical filter and it was managed by a PLC.

Finally, automatic culture and administration of marine micro-algae should be taken into consideration. In fact, marine microalgae are increasingly used as both feed for marine organism, constituting both a source of energy as well as the essential vitamins and PUFAs (as in the case of Penaeid larviculture), and as source of high value fine chemicals, therapeutics, and health foods. Large scale microalgae culture systems provide the possibility of delivering a continuous supply of high-quality microalgae although the newest culture systems appear quite expensive and with high operational costs. Control over the growing culture is necessary to maximize the production and an automated control system should take important decisions with respect to fertilising, harvesting, lighting and temperature to prevent the reduction of the efficiency and economical losses. Several control systems have been developed for this purpose, especially for the in-situ growth monitoring of the unicellular cultures. Most promising technologies are flow injection analysis, based on turbidimetric measurements, technique based on the monitoring of oxygen production, based on the measurement of the pressure inside a closed reactor and the use of optical density as a turbidimetric measure of biomass through spectrophotometry [*18*]. Sananurak designed a small scale (260 litres), highly

sophisticated, although expensive, integrated continuous production system for *Tetraselmis suecica* and the zooplanktonic rotifer *Brachionus plicatilis* [*28*]. Considering the huge needs of microalgae in Penaeidae aquaculture plant, the increase in efficiency scaling up the production system and the need for an automatic control of culture conditions, Erbland and colleagues designed a large scale photobioreactor (170 litres of volume) built using cone-bottom, polyethylene tank, equipped with fluorescent lamps, monitoring and control system that measured temperature, pH and optical density of the microalgal culture [*20*].

Automatic monitoring and control of shrimp aquaculture

Real time monitoring of environmental parameters are very important for both shrimp aquaculture and paddy farming. Constant control of the water quality to keep the concentration of the water environment parameters in the ideal range can enhance the growth rate of cultured organisms, affect dietary utilization, and reduce the probability of diseases. Gathering information of water physic-chemical parameters is the key activity to perform the appropriate technical intervention to prevent harm to aquaculture production and is crucial to keep up sufficient conditions and avoid unfortunate circumstances that cause the failure of aquaculture [*21*]. Automatic monitoring and control systems can be used to face some serious issues like wastage of water. An integrated aquaculture can be controlled using aquaponics plants, which requires consistent water quality checking procedures that depend on intense information securing, communication, and handling. The connection between the hydroponic and aquaculture sections, that are the two main components of aquaponics, relies on ideal water quality conditions and on the monitoring of them.

Real time monitoring take advantage of various probes and sensors, which are the source of data for the automatic system, and actuators. Sensors and actuators are usually connected to microcontrollers built using various boards, for example Arduino or Raspberry [*22*]. The latter is preferred by some authors due to the intrinsic Wi-Fi module. Microcontroller monitors the output of sensors and it logs all the data using a data logging system. Actuators are activated or stopped, or in some cases their activity can be modulated, on the base of the software loaded on the microcontrollers and according with sensors output and pre-set thresholds. Several authors focused their attention on few kinds of sensors, such as turbidity, dissolved oxygen, and pH, although modern aquaculture relies on the real time monitoring of a large variety of physical and chemical parameters, such as Ammonia, Carbonates, Nitrate, etc.

The environmental monitoring of the water parameters, in both aquaculture plant or ponds, can be improved including Internet of Things (IoT) ZigBee-based wireless sensor network based on low power microcontrollers that are capable of collecting, analysing and presenting the whole data using an easy-to-access Graphical User Interface. Preetham proposed an IOT based aquaculture monitoring and control system, able to continuously observe the water quality factor and to take preventive steps early to harm for water animals [*21*]. The proposed architecture is composed by power module, sensor module, controller module and output module. In addition, a large variety of innovative sensors, that use new concepts and techniques, are replacing the traditional methods of water quality measurement, based mainly on UV measurement, Mass Spectrometry and amperometric sensors. Optical sensors,

Microelectronic Mechanical System and Biosensors are used to measure different water quality parameters. These sensors that use emerging techniques can be merged in a single system as demonstrated by some authors [*23*]. They are, if compared with the previous generation of probes and sensors, more selective, sensitive, cheap and user friendly. The combined use of ZigBee wireless network and new probes allow aquaculture plants to apply wireless and highly efficient systems for real time monitoring to aquatic animal production. To make the communication network more efficient, sensors and microcontrollers should be connected using a MESH topology, where all nodes cooperate to distribute data amongst each other, with routers linked to end-devices and a coordinator node. These technologies have been applied successfully on aquaculture shrimp. Internet of Things in many cases was applied in rural context, such as tiger shrimp aquaculture in south east of Asia thanks to the low required power, the redundancy of nodes and the user-friendly graphical user interface. The automatic monitoring is strictly linked to the real-time alarms provided by the control system. On one side, automatic monitoring and control systems for aquaculture should monitor data coming from probes in continuous to identify abnormalities and identify thresholds in critical parameters, acting consequently on the actuators to mitigate or solve incoming issues. On the other side the automatic monitoring and control system, using the Internet of Things, should interact with the operator(s), that can be based all over the world, communicating issues and action taken to solve them. The interaction between operator(s) and controlling system via open-source app, such as instant messenger service, allows a two-ways communication, where operators are not only passive spectators of the controller routines, but they can issue orders based on the analysis of data available online [*22*]. For these reasons, monitoring and control system in aquaculture plant should meet four fundamental requirements:

- The operating routing should be systematic, performing the programmed activities at regular intervals with minimum or no deviation.
- Information should be always available and easy to access also for low trained personnel.
- Basing on the data collected by the system and available online, personnel should be able to interact with the system, giving new commands and reacting promptly to the encountered issues, via open-source software(s).
- Data should be organised and used for planning and decision making in the future, with the prospect of software implementation of new commands and routines to face common issues.

Considering the attention that has been given in recent years in the implementation of automation and intelligent systems applied to aquaculture and industrial systems, several models have been designed and tested [*24*]. There are automated control systems used in aquaculture whose main purpose is to acquire and record data; programmable logic controller (PLC) systems [*4*] and systems that integrate artificial intelligence for a more complex control, advanced interpretation and problem solving.

Understanding the basic architecture of these systems, making it intuitive and integrable, can allow not only to use new and completely innovative systems, but also to update and renew systems already in use without excessive economic expenditure.

As a guideline, a typical process control software application is composed of various modules in which the different tasks are sorted. It includes: data acquisition, communication between the different hardware components, information acquisition and management, creation of an easily accessible database, graphic interface for interaction with the user who can control the system through specific functions. The acquisition of information, through probes and smart devices, uses analogue and digital inputs and outputs, each with its own reference protocols [*5*]. The manmachine interface is made up of blocks (that is, a set of functions that encoded the instructions that perform specific activities) in turn connected in such a way that they can cooperate in creating an effector monitoring and/or control circuit. The process control software must be able to make available both data spreadsheets and graphical diagrams that are simpler and more immediate to interpret, which at the same time allow simple consultation even to the interaction of processes [*21*].

Through these automated control systems, it is possible to access both real-time data and historical databases and any alarm signals. Furthermore, if, in addition to automation, one can integrate an artificial intelligence, it may be able to process information collected, immediately and based on historical data, to develop useful statistics and future forecasts. To maximize the efficiency of these systems, a remote controller software is required. It allows you to interact with the system remotely, through dedicated control nodes. In this way, in addition to receiving alerts and messages in real time changes can be made to database blocks.

The internet of things revolution

Industrialization processes are associated with technology improvements. No other period has been as rich in scientific breakthroughs and technological innovations as the 20th century. Science and technology have been protagonists of these changes: the technology of the twentieth century has improved the lives of billions of people, while science has changed the very conception of man has of himself and of his role in the universe. At the same time and in sync with the latest technological developments that have revolutionized the last few decades, radically changing our lives, a new means of communication has also developed: the Internet, which in just over twenty years has grown exponentially, passing from a few thousands of connections, estimates of the International Telecommunications Union, which counted at the end of the 1980s and a few billion people connected all over the world through the use of different terminals such as computers, smartphones and tablets. The evolution of the Internet has experienced two distinct phases that have revolutionized the lifestyles, habits, and behaviours of everyone, from citizens to institutions to companies: the World Wide Web phase in the 1990s and the Mobile Internet phase in the 2000s. Today, the Internet is entering its third phase of development: The Internet of Things or "Internet of Things" which, as Kevin Ashton, co-founder of the Auto-ID Centre at the Massachusetts Institute of Technologies (MIT) argues, "the Internet of Things have the potential to change the world, just like the internet did. Maybe even more".

The potential of this new evolution lies in the fact that with the Internet of Things the devices connected to the network, which are therefore able to exchange information, will grow exponentially since they will be able to connect to the network not only computers and mobile phones, but also everyday objects from means of transport to household appliances. This will generate a mole of information many orders of magnitude higher than the one the Internet has generated which, if used correctly, will allow the creation of new products and services for users.

The Internet of Things (IoT) is the extension of the internet in the sense known today, from people to objects. It is the construction of a network that places objects and their interaction in the centre [*25*]. More properly, it is the passage of a connection network for end-user-devices to an interconnection network made of physical objects able to communicate and cooperate (Figure 2) with each other through the internet connection, becoming smart devices (intelligent devices). To apply the IoT concepts it is necessary that various conditions are met. The most important is to make sure that all the devices used, which need to make communications on the network, have the ability to access the Internet, directly or indirectly. Moreover, they must have a unique digital identity to be recognized and distinguished. In the presence of simpler

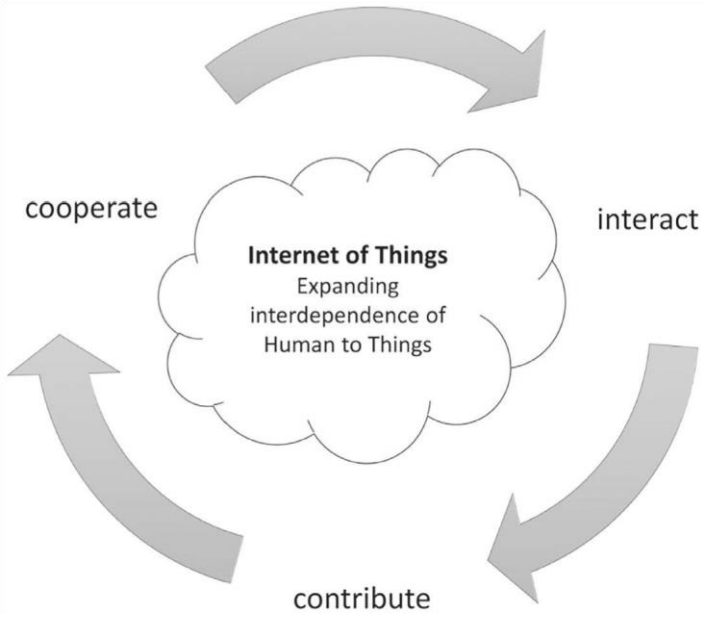

Figure 2. Interdependence of Internet of Things.

devices (such as simple sensors) which do not natively have network cards, it will be possible to connect them to a device that has gateway functions, or that is responsible for forwarding to the central server all the information received.

The integration of sensors in the network gives the possibility to obtain and elaborate data. When we refer to the elaboration of the collected data, a fundamental part concerns the management of Big Data. Big Data is a field that deals with the ways to analyse and then extract information from a large amount of data that would otherwise be too large or complex to be managed. Furthermore, although a survey

with many cases offers greater statistical power, at the same time a very extensive data collection in terms of volume, speed and variety is very difficult to consult and can potentially lead to a higher rate of false interpretations.

The life sciences have a long history of dealing with large quantities of data, and recent advances in experimental capabilities have vastly increased the amount of data that needs to be stored and analysed. Biology is notoriously fragmented in its methods, goals, instruments, and conceptual frameworks. Often, different groups—even within the same subfield—disagree over preferred terminology, research organisms, and experimental methods and protocols [*26*]. Using interoperable databases and file formats to integrate data from different sources so that they can be used and re-used across a variety of research contexts. Databases need to be accessed through a common 'query' system, and this raises the question of which terminologies should be used to classify the data and integrate them with other data, and what are the implications of such choices. We should acknowledge that no data are 'raw' in the sense of being independent from human interpretation, assessing which data are reliable and which are not. But it does not take into account that data are often extensively processed artefacts resulting from highly planned interactions with the world; nor does it do justice to the observation that biologists have different views of what counts as reliable data, or what counts as data in the first place [*27*].

In details, the data are collected through the sensors, stored generally in a cloud where they are analysed, and the information obtained is available for the users according to the designer's purposes. The sensory network that the IoT allows to implement is able to produce data that, compared to the current ones, are more reliable and derive from statistically more relevant samplings [*28*]. In fact, many of the barriers due to the various passages that the information must go through from the moment of its acquisition to use/interpretation are removed. This is understood as the possibility of constant monitoring of all events and processes of a given environment/ business. In general, the IoT allows devices to be monitored and controlled remotely through special network infrastructures, creating a direct interaction between the real world and computer-based systems. The application of these concepts to the world of industry makes it possible to improve the efficiency and accuracy of the processes, as well as obtaining economic benefits, thanks also to the fact that the cases in which human intervention is required are reduced. The Internet of Things is in fact revolutionizing the market and numerous opportunities for growth and development are being created. The use of a system implemented by IoT applied to the culture of marine organisms such as crustaceans, both as model organisms for scientific research purposes and for production for commercial purposes, could be extremely useful and revolutionary. The idea is to have an intelligent system with special sensors for measuring the fundamental parameters for breeding and special effectors to intervene autonomously in such a way that all measured values always remain within the optimal limits (Figure 3).

Therefore, the primary purpose would focus on optimizing the production processes, making them on the one hand more efficient in terms of quantity and quality of the product and at the same time lowering production costs (intended both from an economic and temporal point of view since it would reduce the use of human resources). Considering a crustacean culture, with the use of IoT it could be possible to optimize the use of water and primary resources, in consequence of an adequate

real-time analysis of the values in the tanks and an intelligent management of food additions and supplements. The changes of water, if necessary, will be regulated by the intelligent system itself which will consider every variable, even the weather forecast (which, in some cases, can affect the quality of the incoming water where it is possible to directly draw on sources of external natural water), consulting both local sensors and open-data environmental/meteorological services.

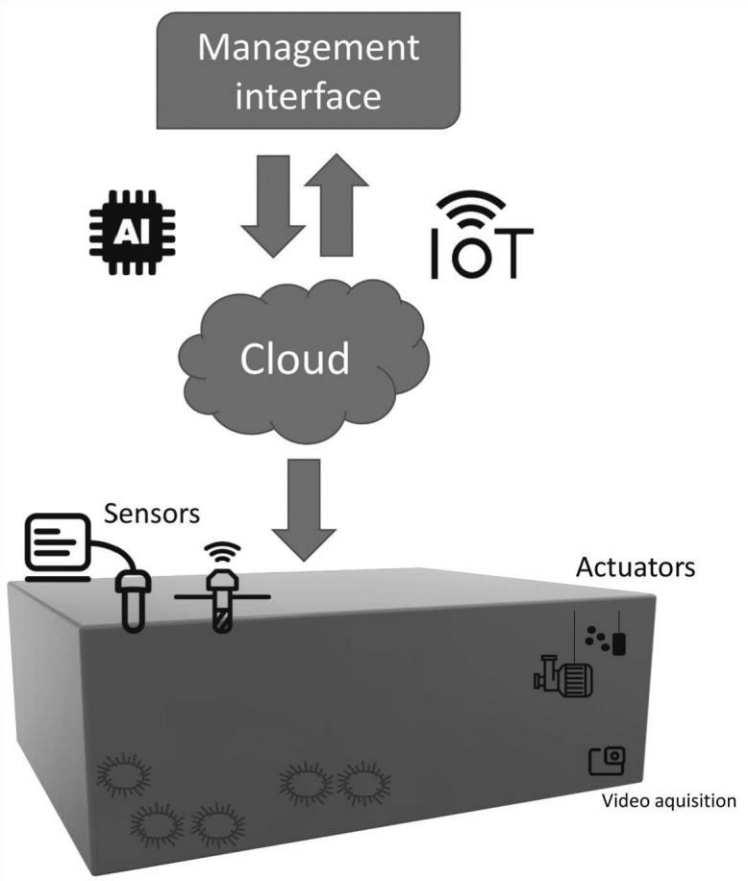

Figure 3. "Interactive operators" capable of reading environmental signals, carry out assessments and respond with programmed actions.

These sensors must be connected to the internet and the measurements made will be sent to an online platform, which will have the purpose of collecting the data. This platform will give the possibility to clearly view the data collected, for example by generating intuitive and easily understandable graphs. The system itself will also be able to analyse these data and understand where it will be necessary to intervene (however, leaving manual intervention possible) to correct them, for example through specific actuators for each value. Furthermore, the platform will be able to autonomously interrogate open-access platforms or private databases to consult data collected in previous comparable farms, to be able to reason on the actual need to intervene based on current conditions. In details, the data are collected through the

sensors, stored generally in a cloud where they are analysed, and the information obtained is available for the users according to the designer's purposes. The sensory network that the IoT allows to implement is able to produce data that, compared to the current ones, are more reliable and derive from statistically more relevant samplings [*28*]. In fact, many of the barriers due to the various passages that the information must go through from the moment of its acquisition to use/interpretation are removed. This is understood as the possibility of constant monitoring of all events and processes of a given environment/business.

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Section 2

Chapter 3

Morphologic and genic effects of organic pollution on the reproductive physiology of *Paracentrotus lividus* Lmk: a mesocosm experiment

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Keywords: dissolved organic compounds; recirculated aquaculture systems; gene expression; larvae; sea urchin

Abstract

A considerable amount of coastal contamination is caused by organic wastes, even if our attention is commonly focused on chemical pollutants and contaminants. Most organic pollutants are represented by highly dilute soluble compounds and particles deriving from dead organisms. This complex combination, consisting of suspended particles and dissolved macromolecules, has a significant impact on coastal planktonic and benthic organisms, also playing an active role in the global cycles of carbon. In addition, production practices are nowadays shifting towards recirculated aquaculture systems (RAS) and the genic responses of target organisms to the organic pollution are still scarcely addressed by scientific investigations. The reservoir of organic matter dissolved in the seawater is by far the least understood if compared to that on land, cause only a few compounds have been identified and their impacts on animals and plants are poorly understood. The tendency of these compounds to concentrate at interfaces facilitates the absorption of dissolved organic compound (DOC) onto suspended particles. Some DOC components are chemically combined with dissolved metals and form complexes, affecting the chemical properties of the seawater and the life of the coastal biota.

In this research, we compared the reproductive performances of the common sea urchin *Paracentrotus lividus* cultured in open-cycle tanks to those cultured in a recirculating aquaculture system (RAS), where organic pollution progressively increased during the experiment, even not reaching deadly concentrations. Sea urchins were cultured for seven months under these two conditions and their gametes were collected. Embryos resulting by *in vitro* fertilization were analyzed by *Real Time qPCR* to identify possible effects of pollution-induced stress. The fertility of sea urchins was evaluated, as well as the gonadosomatic indices and the histological features of gonads. Our results indicate that

organic pollution, event at sub-lethal concentrations, may hardly impact the reproductive potential of this key species and that chronic effects of stress are revealed by the analyses of survival rates and gene expression.

Introduction

Global concern about the possible negative impacts of pollutants on ecosystems and humans has extensively increased in the last decades. Thousands of pollutants, most of which are organic, have entered the environment because of such human activities as industrialization, agriculture and coastal urbanization (Daughton, 2005; Dachs and Méjanelle, 2010). Indeed, coastal areas, including transitional waters, are subject to considerable human influences on a global scale, because a major proportion of the world's population historically resides in regions near the water bodies, inducing increased anthropogenic stresses to coastal ecosystems (Muir and Howard, 2006; Steffen et al., 2007). Even if the effects of organic contaminants on aquatic organisms are well-known, the short-term consequences of several compounds released into the marine environment received insufficient attention (Asher et al., 2007; Grzybowski et al., 2009). Organic pollutants, in coastal areas and elsewhere, have not been thoroughly investigated, partially due to analytical limitations and the limited cooperation among different scientific fields, such as environmental and analytical chemistry, marine biology and oceanography (Asher et al., 2007; Valiela, 2016). An undetermined number of chemicals have potentially been released into the environment, but they have still not been acknowledged in the scientific literature (Gigliotti et al., 2005; Jurado et al., 2005; Asher et al., 2007). In addition, the classical methods of investigation only permitted the identification of a small number of organic contaminants, using costly and time-consuming procedures (Valiela, 2016). Nowadays, due

to huge advancements in analytical instruments, hundreds of emerging contaminants were identified up to trace levels in the last decades, tracking their actual accumulation. Several compounds deriving from a variety of applications, including drugs and cosmetics, agricultural herbicides, combustibles, detergents, aquaculture effluents and others, are constantly introduced into the marine environment (Gibbs, 1993; Muir and Howard, 2006).

In particular, it has been stated that aquaculture practices locally affect the aquatic surroundings (Aubin et al., 2006). The inorganic nutrient accumulation (mainly nitrogen and phosphorus) leads to eutrophication, and an increase in organic wastes in the ecosystems produces various negative effects both to the aquatic biota and to the same organisms cultured in RAS (recirculating aquaculture systems) tanks (Cole et al., 2009). Taken together, these issues and the chemical pollution can lead to oxygen depletion, alteration of the water quality, decline of aquatic communities, algal blooms, mass mortality and habitat loss (Boesch and Paul, 2001). Organic pollutants can access the coastlines through a variety of pathways, and they enter the biogeochemical cycles by sinking, and through bioaccumulation processes, after their first introduction (Gigliotti et al., 2005). Their environmental distribution strongly relies on the physical-chemical characteristics of the compounds, except in the proximity of source points. Once these compounds reach the water, they flow until they are either decomposed, absorbed on sediments, or become sinking particles (Fleeger et al., 2003; Steffen et al., 2007)

The majority of ecotoxicology research have focused on evaluating the effects of single contaminants or simple combinations of pollutants (Muir and Howard, 2006). To mirror these realities, ecotoxicological investigations and approaches must undergo significant innovations (Jurado et al., 2005; Dueri et al., 2008), because several evidences indicate the

direct and indirect effects of organic pollutants on aquatic habitats, despite only 20% of previous studies focuses on oceans (Fleeger et al., 2003). In addition, aquaculture practices in RAS have been largely improved and the present trends of research are aimed at implementing new culture techniques in recirculating systems (Ahmed and Turchini, 2021), because of the urgent need to reduce the water consumption and the impacts of polluted wastes on marine coastal communities (Midilli et al., 2012). To this end, several species historically cultured in open-cycle tanks, or even in cages, are progressively transferred to RAS, in order to reduce the impacts, and in view of green aquaculture technologies (Gunning et al., 2016). This shift, however, also imposes to have clear mind about the actual effects of pollutants normally increasing in RAS, when the density of cultured organisms is high and the limits of the life support systems (LSS) are met (Zohar et al., 2005). To this end, a good knowledge of the effects of organic pollutants on the physiology of cultured organisms, along with their genic responses, becomes vital.

In this study, we employed the common sea urchin *Paracentrotus lividus* as a model organism. This sea urchin represents an economically relevant species for the seafood market and a resource for scientific research. In addition, it plays a crucial role in the ecology of Mediterranean coastal ecosystems because it is one of the main grazers in algal and seagrass ecosystems. Therefore, increasing pollution events impacting shallow coastal ecosystems might influence its reproductive potential and the ecology of economically relevant communities. In addition, *P. lividus* is extensively adopted in embryological and developmental biology studies, and it is a perfect model organism for ecotoxicological and physiological surveys, thanks to its easy management in the laboratory and the transparency of embryos, which permits to follow the early stages of development.

Embryotoxicity tests on sea urchin embryos can be rapidly completed (currently, at 24, 48 and 72 h; (Matranga et al., 2010) on a huge number of individuals at the same time (Bonaventura et al., 2005), and the effects on embryonic differentiation can be observed both at the morphological level and the molecular level (Roccheri et al., 2004; Pinsino et al., 2011). Moreover, while the planktonic larval stage represents a useful indicator for short-term events, the settled individuals of *P. lividus* can be indicators for long-term phenomena. Here, we aimed at investigating the effects of waters contaminated with organic pollution on the sea urchin *P. lividus* at morphological and molecular levels, by adopting a mesocosm experiment. In particular, adult sea urchins were cultured for seven months in open-cycle tanks and compared to individuals cultured in a recirculating aquaculture system (RAS), where organic pollution progressively increased during the experiment, in the absence of water changes. The reproductive performances of the sea urchins in these two conditions were analysed, as well as the gonadosomatic indices and the gonadic state, investigated by means of histological techniques. The expression levels of seventy-nine genes involved in the stress response, as well as in development/differentiation processes (such as those involved in the skeletogenesis) were evaluated by Real Time qPCR, to identify the functional pathways affected by organic compounds.

Materials and methods

Experimental set-up

Adult sea urchins, *Paracentrotus lividus*, were collected by scuba divers in the Gulf of Napoli (Italy) and carried to the laboratory in thermostatic bags, to avoid stressing increases of temperature. They were gradually acclimatized in open-cycle tanks for two weeks prior to start the experiment, that was carried out for 7 months in four adjacent circular tanks. The

diameter of tanks was 90 cm on average and the height was 66 cm (until the surface water level). Each tank was filled with 405 litres of filtered saltwater, previously pumped from a pipeline located off the harbour of the Procida Island (about 60 m off the seashore). Two tanks were set as a Recirculating Aquaculture System (RAS), where organic pollution progressively increased during the experiment, never reaching deadly concentrations. Each RAS tank was equipped with an external mechanical filter (Whale, SICCE, Italy) and a skimmer (Seachem Aquavitro, Italy). In addition, the RAS tanks were equipped by a set of five submersible smart pumps XStream SDC (SICCE, Italy) mounted on the inner walls of the tanks through magnetic supports coated with protective gum, to dissipate vibrations. The pumps were managed by the smartphone app *Contrall* (Apple store and Google Play store), in order to become smart devices controlled through Wi-Fi connection. The app *Contrall* provided real-time feedback on the status of the pumps and an alarm system which was activated in case of anomalies. The pumps in the upper part of the tanks were positioned in counter-clockwise and upward direction, and three pumps in the lower side were positioned in clockwise and downwards direction. These settings allowed to create two different and contrary currents that mixed and oxygenated the water. The pumps were also connected to an ORP probe, constantly measuring the value in each tank. The ORP controller permitted to set an ORP lower limit and, when the probe read values under a threshold, the pumps were activated mixing and aerating the water until the ORP values reached values above the set threshold.

The two control tanks, in their turn, were managed in open cycle conditions, to guarantee a continuous exchange of seawater. They continuously received clean seawater pumped from a pipeline set off the harbour of the Procida Island (Bay of Naples), filtered into a large sump connected to a protein skimmer, and finally directed into a distribution pipeline reaching the

tanks. The output water was re-directed to the sump, where an overflow permitted to wash it out in the harbour of Procida. Twelve complete water changes per day (every 2 h) were assured by the water pumped into the open cycle system. An aeration device was also set inside each experimental and each control tank, to maintain dissolved oxygen (DO) at healthy levels for sea urchins and guarantee water circulation in the tanks.

After the complete setup, thirty sea urchins (*P. lividus*) were added to each tank (both test tanks and control tanks), i.e., 20 females and 10 males (female/male ratio of 2:1). The sex of sea urchins was previously determined under the optical microscope, based on the dimorphism in terms of shape and size of five dermal plaques visible around the anus (Brundu et al., 2022) During the experiment, the sea urchins reared in RAS tanks and those in the control tanks were fed twice a week *ad libitum* on a highly proteic pellet (Greenvet, Italy). The main water parameters, namely temperature, dissolved oxygen, redox potential, salinity and pH were checked manually three times a week. Nitrites, nitrates, phosphates and ammonia concentrations were checked using a colorimetric test (by adopting standard analytical kits for the photometer AL450, Aqualytic, Germany). The above-mentioned data measured in RAS tanks were compared to those measured in open cycle tanks.

Biotic and abiotic variables

Physical and chemical variables of the seawater were measured every three days in each tank. Water samples were collected in 50 ml beakers from the tanks to be analysed by the filter photometer AL450 (Aqualytic, Germany). Chemical analyses served to monitor the concentration (ppm) of nitrites, nitrates, ammonia and phosphates. Physical variables were also monitored. Temperature was daily recorded at noon by an alcohol thermometer; salinity was measured by means of a refractometer (TTBH Pte Ltd, Singapore); dissolved O_2 was

measured by means of an Oxygen portable meter (ProfiLine oxi 3310, WTW, Germany); the pH was measured by a multiparametric probe (XS Instruments®, PC 7 Vio, Italy). In addition, behaviour, spawning, mortality and the health status of sea urchins were daily checked in the tanks and recorded in a spreadsheet.

In vitro fertilization for morphological and molecular analyses

Five sea urchins of each gender were injected with 1 ml of 0.5 M KCl through the peristomal membrane, to stimulate the contraction of gonads and to obtain the gametes. The subjects were then vigorously shaken, and females were placed with their mouths up, over 50 ml beakers, until the gametes were released into filtered (0.22 μm Millipore) seawater, to facilitate the collection of oocytes, which were rinsed three times with clean seawater to remove possible organic residuals. Sperms were collected dry from the gonophores to avoid premature activation that takes place when the sperms remain in direct contact with seawater. The eggs obtained were pooled in Petri dishes (diameter 14 cm) filled with filtered seawater. Embryos were incubated in a thermostatic chamber at 18°C for 48h until reaching of the *pluteus* stage; subsequently larvae were fixed in glutaraldehyde (4%) and observed under an optical microscope to evaluate the percentage of malformations, according to (Mcedward, 1984; Pagano et al., 1986). The significance of differences was determined by means of t-tests.

After 48 hours post-fertilization, about 5,000 fertilised eggs were collected from each of five females. The samples were centrifuged at 4 °C for 15 minutes at 3,500 rpm. The embryos were then conserved in RNAlater (Qiagen, Hildesheim, Germany), frozen in liquid nitrogen and then stored at -80°C until use.

Total RNA was extracted using *Aurum Total RNA Mini Kit* (BioRad, Hercules, California, USA). Using a NanoDrop spectrophotometer (ND1000 UVVIS Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). The quantity of total RNA extracted was determined by the absorbance at 260 nm and the purity by the 260/280 and 260/230 nm ratios. To obtain cDNA, 1000 ng of total RNA was retrotranscribed for each sample using an *iScript cDNA Synthesis kit* (BioRad, Milan, Italy). Additionally,

adults were weighed, sacrificed and dissected; their gonads were extracted and weighed (fresh weight) for the evaluation of the gonadic indices (GI%). The evaluations of the GI% were performed on all specimens in the test tanks as compared with all the specimens still present in the control tanks at the end of the experiment.

Histological analyses

The gonads of one male and four females for each treatment were collected, fixed in Glutaraldehyde solution (4%), dehydrated in ascendant ethanol, clarified in methyl benzoate and included in paraffin according to Zupo et al., 2018. Sections of 5 µm were obtained with the microtome (Leica) and stained with haematoxylin to observe the presence of morphological alterations. Histopathological indices were calculated according to (Costa et al., 2013).

Variations of gene expression

The variations in the expression of 27 genes involved in the stress response, 43 genes involved in development/differentiation processes, 8 genes involved into skeletogenesis and 9 in detoxification processes (see Table S1 in the Supplementary Materials for their biological functions) were evaluated by *Real Time qPCR*. Undiluted cDNA was used as a

template in a reaction containing a final concentration of 0.3 mM for each primer and 1× FastStart SYBR Green master mix (total volume of 10 µL) (Applied Biosystems, Monza, Italy). PCR amplifications were performed *CFX96 Touch Real-Time PCR Detection System* (Bio-Rad Laboratories, Inc.), using the following thermal profile: 95° C for 10 min, one cycle for cDNA denaturation; 95° C for 15 s and 60° C for 1 min, 40 cycles for amplification; 72° C for 5 min, one cycle for final elongation; one cycle for melting curve analysis (from 60° C to 95° C) to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability, all real-time qPCR reactions were carried out in triplicate.

For all Real-Time qPCR assays, the results of each cDNA sample were standardised with the mRNA level of the housekeeping genes *18S rRNA* and Cytochrome c oxidase used as reference genes, whose expression levels are rather stable throughout the development. Fluorescence was measured using *Bio-Rad CFX Maestro software* (Bio-Rad Laboratories, Inc.). The values of C(t) obtained and the efficiency values for each pair of oligonucleotides are analysed and normalised against the internal control by REST programme (*Relative Expression Software Tool*) based on the Pfaffl method, and the expression values of the gene of interest relative to the control were reported (Pfaffl, 2001; Pfaffl et al., 2002). Relative expression ratios greater than ± 1.5 were considered significant.

Data collection and statistical analyses

A Student *t*-test was applied to determine the significance of differences between two sets of samples whose normality of variance was previously tested by means of a Shapiro-Wilk test. In trials where a larger number of groups were compared, one-way ANOVA was adopted, based on the type of data to be analysed, to determine the significance of differences between

experimental groups. In the same experiment, similarity matrices were obtained for all considered parameters, in order to check the relationships among variables, for all the considered datasets. Correlation matrix analyses were used to display the correlation coefficients among the seawater parameters and mortality events in the tanks. For the evaluation of the GI%, sea urchins were weighed, sacrificed and dissected; their gonads were extracted and weighed (fresh weight) and the index was calculated according to the formula proposed by Fabbrocini and D'Adamo (2010) and Keshavarz et al. (2017):

1) GI = gonadal wet weight (g)/sea urchin wet weight (g) × 100

Histopathological indices were calculated using the formula proposed by (Costa et al., 2013):

$$I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j}$$

where, Ih is the histopathological index for the individual h; wj is the weight of the jth histopathological alteration; ajh is the score attributed to the hth individual for the jth alteration and Mj is the maximum attributable value for the jth alteration (in the case all alteration are present at the maximum diffusion). The Ih was determined following the concepts of the differential biological significance of each analysed alteration (weight) and its diffusion (score). The weights ranged from 1 (minimum severity) to 3 (maximum severity), while the score varied from 0 (not present) to 6 (diffuse). As histopathological alterations, we considered: the presence of lipofuscin (w=1); cells in atresia (w=2); cells in necrosis (w=3). The diffusion was calculated using the presence/absence of each alteration in 6 random pictures taken at magnification of 40x for each specimen. A PERMANOVA test was performed to determine the significance of differences in the Ih index among all

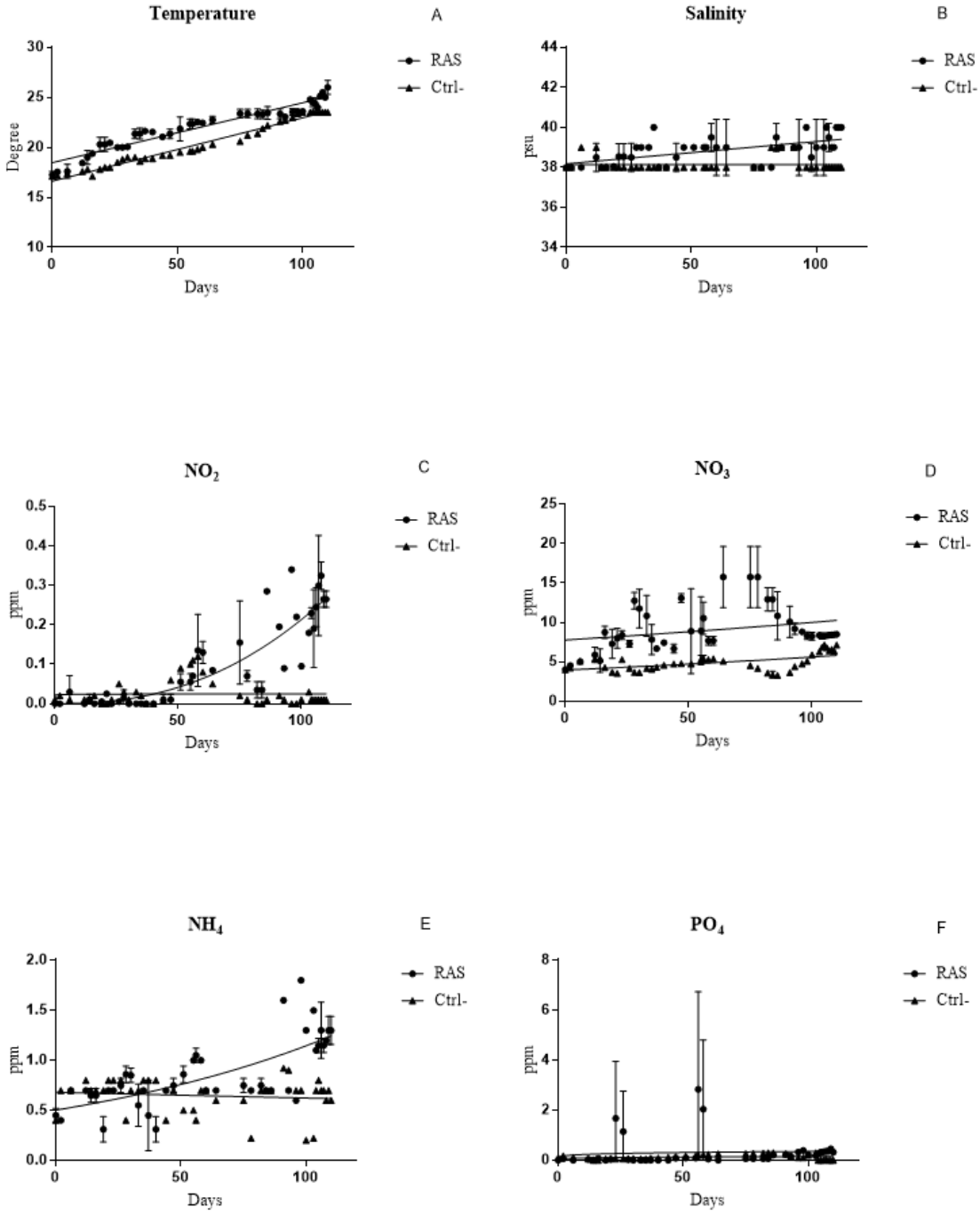
treatments. All graphs and statistical analyses were processed using GraphPad Prism 8.0 (GraphPad Software, San Diego, California USA, www.graphpad.com).

Results

Seawater chemistry

The concentration of NH_4 in the water of both RAS tanks (test tanks), ranged between 0 and 1.8 ppm (a maximum at 1.8 ppm was recorded at the end of the experiment). Taking into account the average between the mean weekly concentrations of NH_4, (Figure 1E) it showed similar time trends in both RAS tanks. In the control tanks (open cycle) the concentration of NH_4 was characterized by a gradual increase from the 60^{th} day until a stable concentration of 0.9 ppm in the last days of the experiment (Figure 1E). The trends of ammonium concentration significantly differed in experimental tanks with respect to the control tanks in ($p < 0.05$; Table S2). The concentration of NO_2 in the water of RAS tanks, ranged between 0 and 0.39 while in control tanks (Figure 1C) it ranged between 0 and 0.12. The Student *t*-test showed a significant difference between RAS tanks and the control tanks, ($p < 0.05$; Table S3). The concentrations of NO_3 in the water of RAS tanks exhibited very irregular trends and ranged between 4.1 and 18.5. The weekly average of NO_3 concentrations indicated trends from the two RAS tanks with a significant deviation in almost every time, as compared to the control tanks (Figure 1D). The nitrate concentration in control tanks ranged between 3.1 and 7.3, thus it remained consistently lower than in the RAS tanks (Figure 1D) ($p < 0.05$; Table S4). The concentration of PO_4 ranged between 0 and 5.6 in the RAS tank 1 and between 0 and 0.45 in RAS tank 2 (Figure 1F). The mean concentration of phosphates in control tanks ranged between 0 and 0.3, with no significant differences between the RAS and the control tanks ($p > 0.05$; Table S5). The temperature in the RAS tanks varied along with external

temperature, continuously increasing from March to August (Figure 1A). In the control tanks the temperature trends were characterized by a gradual increase, but the maximum was 23.5°C, reached in the last period. The Student *t*-test indicated significance of the difference ($p < 0.05$; Table S6). The pH ranged between 7.8 and 8.2 in RAS tanks (Figure 1H) while in the control tanks the pH significantly differed ($p<0.05$; Table S7) and it was consistently above 8. The concentration of dissolved O_2 was between 3.5 and 7.4 ppm in RAS tanks while in the control tanks it was significantly different ($p<0.05$; Table S8) and ranged between 6.1 and 7.4 ppm (Figure 1G). The salinity of water ranged between 38 PSU and 40 PSU in RAS tanks while in control tanks it significantly differed ($p < 0.05$; Table S9) and remained stably around 38 PSU, with a few increases at 39 PSU (Figure 1B).


Temperature
A
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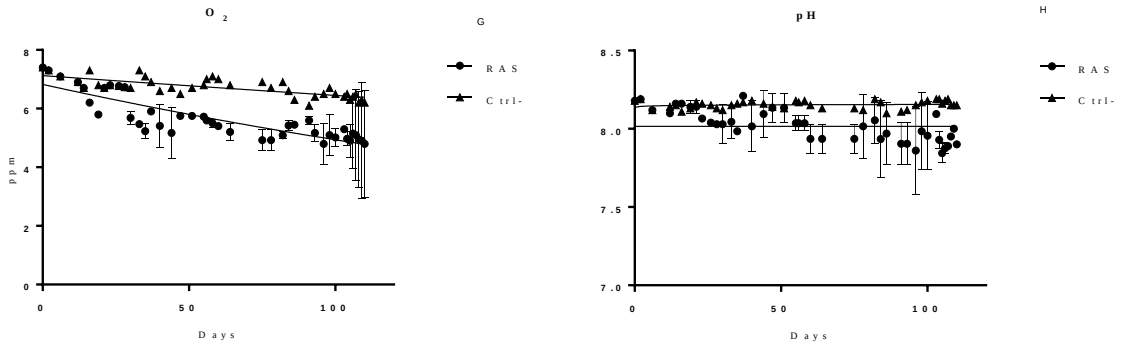


Figure 1: Chemical and physical water parameters trend in RAS tanks and control (ctrl) tanks.

Survival rates and water conditions

Significant mortality differences were detected between control tanks, where no mortality was detected, and RAS tanks ($p < 0.05$; Table S10; Figure 2). The water descriptors in RAS tank 1 and 2, analysed by means of correlation matrices, indicated a significant relationship between sea urchin mortality and the presence of records of water descriptors out their optimal ranges. In particular, a significant difference between RAS systems and the control tanks was detected in all physical and chemical descriptors of water in the last days of the experiment, when an increase of mortality was recorded, especially in the last days (Figure 2).

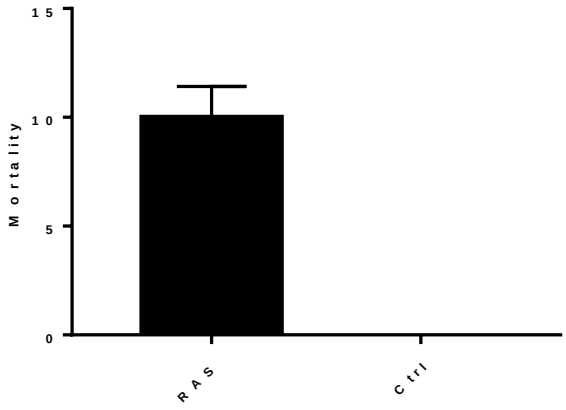


Figure 2: Mortality rates of animals reared in the RAS tanks and control tanks.

The correlation matrix (Figure 3A) for the RAS tank 1 showed that NH_4 is negatively correlated (-0.35) with NO_3, and positively correlated (0.41) with PO_4. NO_2 exhibited a moderate positive correlation (0.45) with PO_4. As well, NO_3 was positively correlated with temperature (0.41), and a negatively with the pH (-0.49). PO_4 was positively correlated (0.42) with temperature. The pH exhibited a positive correlation (0.57) with the dissolved O_2 and in fact the two parameters increased in parallel. The dissolved oxygen exhibited a negative correlation with the mortality (-0.51). A moderate negative correlation (-0.61) between temperature and pH was also observed, as well as a moderate positive correlation (0.42) with the salinity. However, temperature exhibited a moderate correlation (0.45) as well with sea urchin mortality in all the tanks and a negative correlation (-0.79) with the dissolved oxygen, indicating a strong decrease in the dissolved oxygen of the water when the temperature increased.

In the RAS tank 2, the correlation matrix (Figure 3B) showed for NH_4 a positive moderate correlation with temperature (0.33), while the correlation indices were 0.47 with salinity,

0.41 with pH and 0.42 with dissolved O_2. NO_2 exhibited a strong positive correlation (0.84) with PO_4 and temperature (0.75). As well, a moderate positive correlation between NO_2 and salinity (0.6) was indicated by this analysis. PO_4 exhibited a strong correlation with temperature (0.82), as well as a moderate correlation with mortality (0.29) and salinity (0.59). Temperature showed a moderate negative correlation with pH (-0.65), while its correlation indices were 0.45 vs. mortality, and 0.81 vs. salinity. The pH showed a moderate correlation with the dissolved O_2 (0.53). The dissolved oxygen exhibited a negative correlation with the mortality (-0,49). Increases of NO_2 concentration corresponded to increases of PO_4, and when also phosphates increased, mortality events were recorded. In contrast, the corelation matrices in control tanks did not indicate any relationship of the seawater descriptors with mortality events, because of a total absence of mortality.

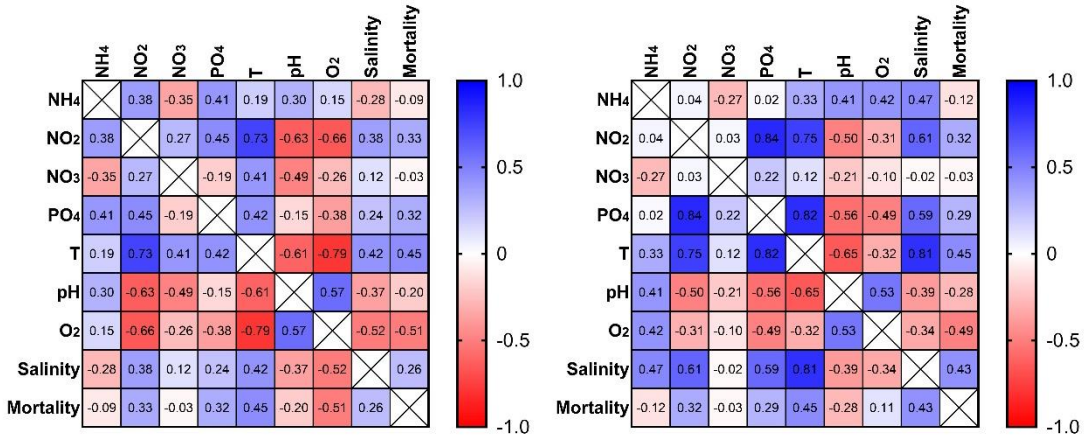


Figure 3: Correlation matrix for different measures from RAS tank 1 (on the left). Correlation matrix for different measures from RAS tank 2 (on the right).

Efficiency of *in-vitro* fertilization tests and gonadosomatic indices

Fertilization rates ranging from 98% to 100% were observed using gametes deriving from animals from both RAS and control tanks ($p > 0.05$). In contrast, different results of larval

development and malformations were observed between the two types of treatments. In fact, the tests in the RAS systems produced at 48 hpf: 20,93% (± 2,23) of embryos still at the blastula stage; 21,75% (± 2,76) of embryos still at the gastrula stage; 5,85% (± 4,31) still at prism stage (Table S11A); 26,4% (± 8,91) malformed plutei and only 31,85% (± 4,3) of normal plutei (Figure 4A; Table S11B). Inversely, the control tanks produced only 0,15% (± 0,07) of embryos still at the blastula stage; 27,4% (± 4,81; Table S11A) of malformed plutei and 72,45% (± 4,88) of normal plutei (Figure 4B).

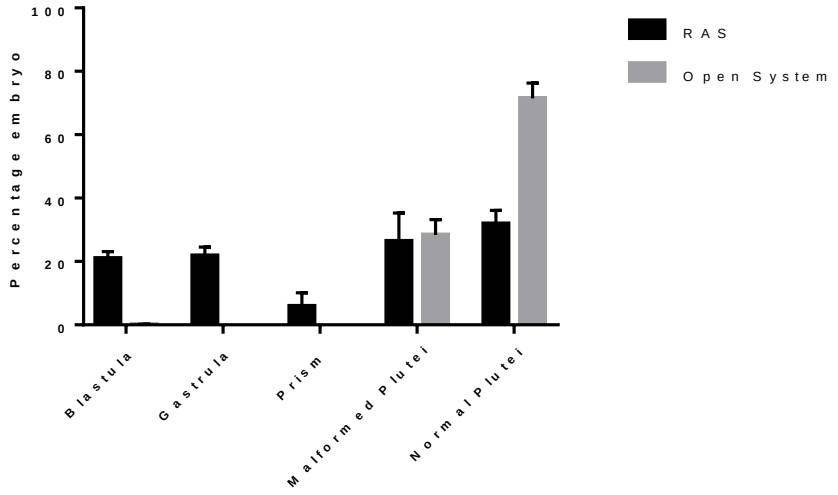


Figure 4: Percentage of malformed and delayed embryos at 48h post fertilization from sea urchins reared in RAS tanks.

The differences between the two treatments were significant ($p < 0.05$; Table S11B). Despite the significant differences in the production of healthy offspring, the adult sea urchins collected at the end of the experiment exhibited not significant differences in the gonadosomatic indices between the two different culturing systems (Figure 5, Table S12).

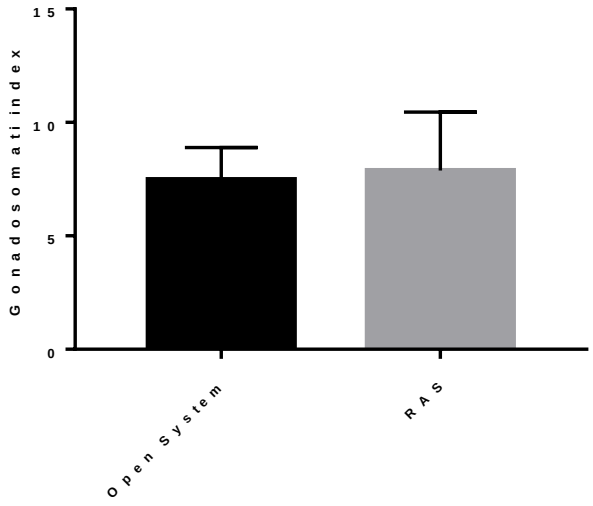


Figure 5 Gonadosomatic indices recorded at the end of the experimental period from sea urchins reared in RAS tanks and control tanks.

Histological analyses

The histological analyses indicated that all tested specimens were at the stage of sexual maturity, with no substantial differences among the housing methods (Figure 6). The only noticeable differences between the open system and the RAS consisted in the absence of sperms in the interstitial spaces of the testis belonging to specimens cultivated in RAS (Figure 6 A, B, C).

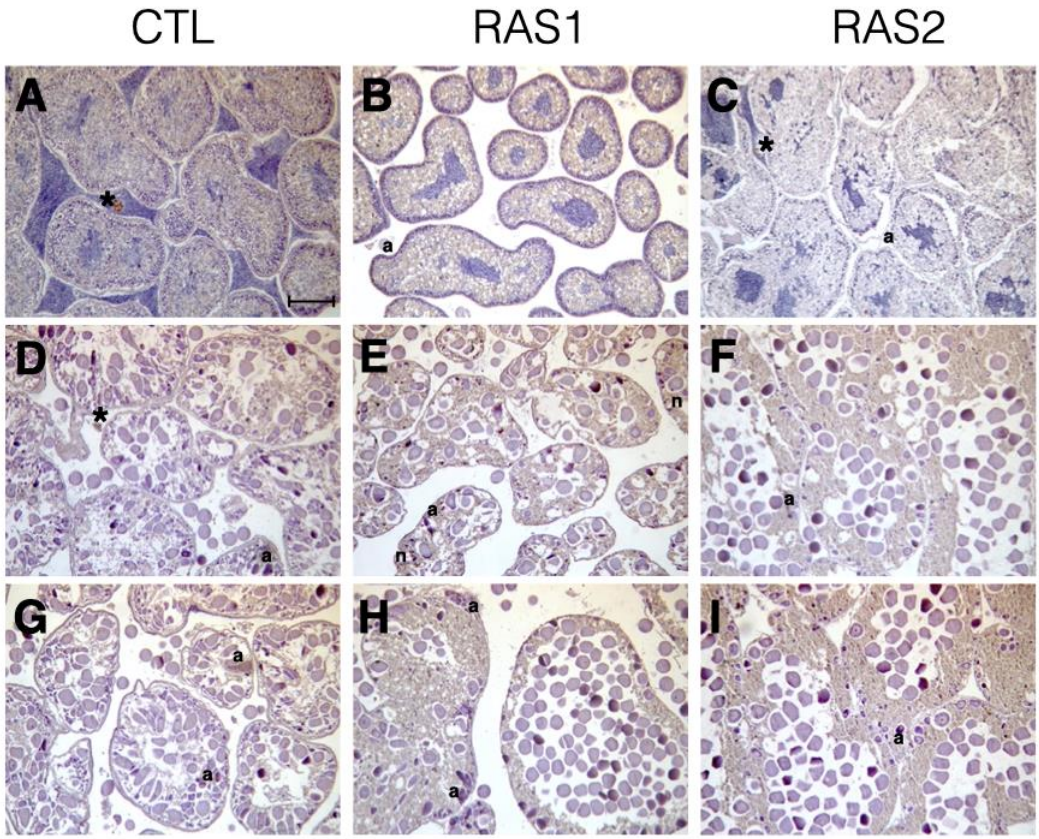


Figure 6. Overview of gonads sections from specimens raised with different water circuitry. A, B, C, testicles; D – I ovary sections; CTL: control system; (* lipofuscin aggregate, a atrasia, n necrosis) scale bar = 100µm

The ovaries exhibited a similar pattern in control and in RAS-reared specimens, but variable maturation levels were exhibited, with the presence of mature eggs in the interstitial gonad space. The histopathological index, as well, did not reveal significant differences among treatments (Figure 7)

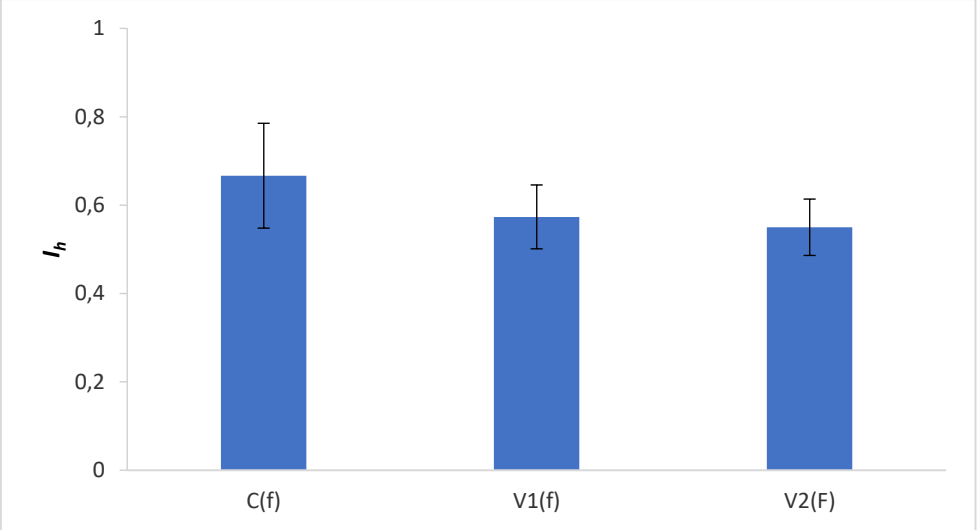


Figure 7. Ih: the graph shows the comparison of Ih among difference treatments, C = control tanks, V1 and V2 = RAS.

Stress genes

The variation of gene expression levels was followed by Real Time qPCR (Table S1, Figure 8) for the genes of interest, belonging to four functional classes. The results referred to twenty-seven genes analysed showed that the plutei deriving from the RAS tanks had a significant variation in the expression for most of the genes analysed (Figure 8; Table S1). In the case of plutei deriving from RAS tank 1, twelve genes increased their expression levels (Figure 8): *caspase 3/7*, *CASP8*, *ChE*, *GS*, *GST*, *hsp56*, *hsp60*, *hsp70*, *hsp75*, *hsp90*, *NF-kB*, *PARP* and *p53*. Six genes reduced their expression levels: *CYP-2UI*, *cytb*, *GRHPR*, *HIF1A*, *MTase* and *TNF*. Similarly, in the case of plutei deriving from the RAS tank 2, fourteen genes increased their expression levels (Figure 8): *ARF1*, *caspase 3/7*, *CASP8*, *ChE*, *GS*, *GST*, *hsp56*, *hsp60*, *hsp70*, *hsp75*, *hsp90*, *NF-kB*, *PARP* and and *p53*. Six genes decreased their expression levels: *CYP-2UI*, *cytb*, *GRHPR*, *HIF1A*, *MTase* and*TNF*.

Development and differentiation genes.

Concerning the forty-five genes involved in the development and differentiation processes, the results showed that the plutei deriving from the RAS tanks have a further significant variation in expression for most of the genes analysed (Figure 8; Table S14A). In the case of plutei deriving from RAS tank 1, six genes increased their expression levels: *CM-K*, *FOXA*, *FoxG*, *Foxo*, *Wnt5* and *Wnt6*. Instead, sixteen genes decreased their expression levels: *BRA*, *CREB*, *FZ-7*, *GFI1*, *GOOS*, *H3.3*, *HH*, *JAK*, *JNK*, *KIF19*, *nodal*, *OneCut*, *TAK1*, *tcf4*, *TCF7* and *VEGF*. Similarly, in the case of plutei deriving from RAS tank 2, eight genes decreased their expression levels (Table S14B): *CM-K*, *FZ-7*, *FOXA*, *FoxG*, *Foxo*, *Wnt5*, *Wnt6* and *Wnt8*. Conversely, sixteen genes decreased their expression levels: *BRA*, *CREB*, *FZ-7*, *GFI1*, *GOOS*, *H3.3*, *HH*, *JAK*, *JNK*, *KIF19*, *nodal*, *OneCut*, *TAK1*, *tcf4*, *TCF7* and *VEGF*.

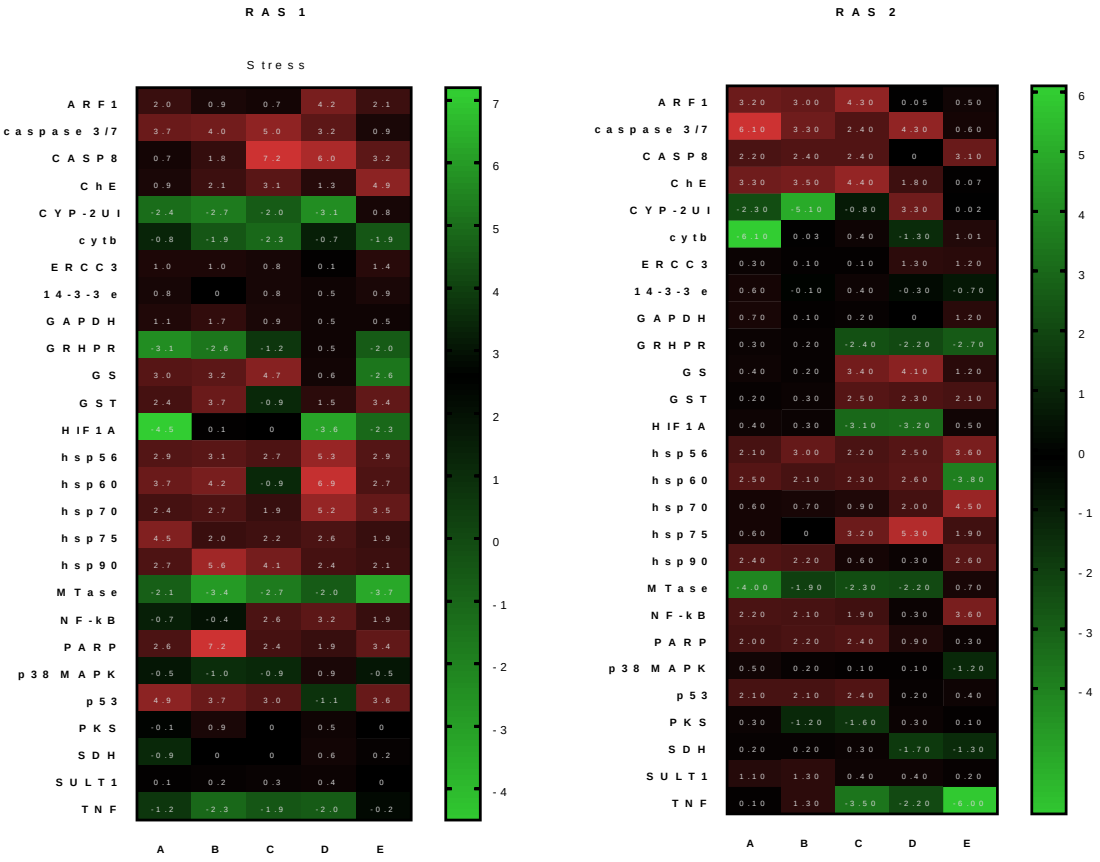
Skeletogenic genes

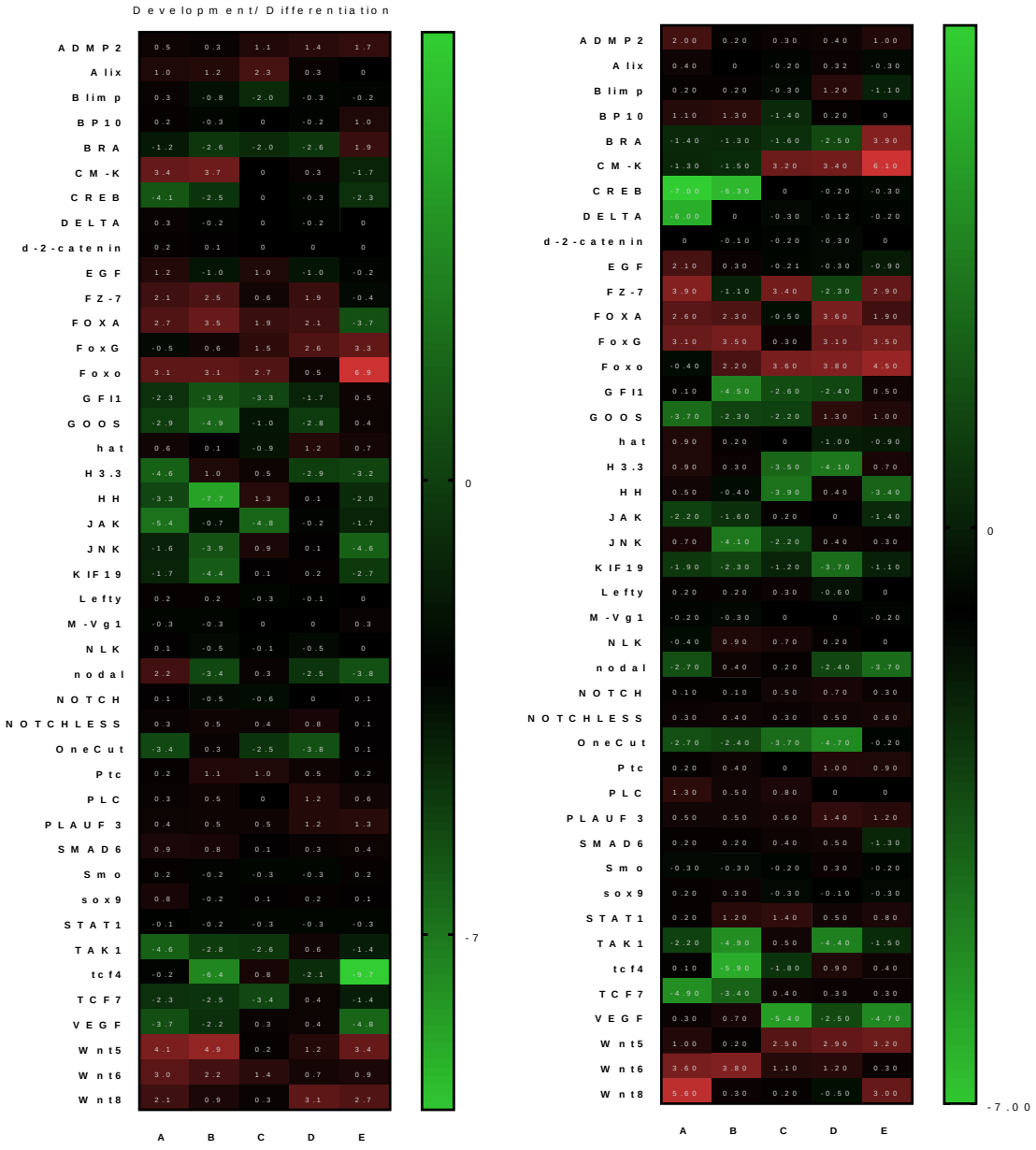
The results referred to eight genes involved in skeletogenesis showed that the plutei deriving from the RAS tanks had consistently a significant expression variation for most genes analysed, resulting down regulated. In the case of plutei deriving from the RAS tank 1 (Figure 8; Table S15A): *C-jun*, *Nec*, *p16*, *p19*, SM50 and *Uni* were down-regulated. Similarly, in the case of plutei deriving from RAS tank 2 (Table S15B): *BMP5-7*, *C-jun*, *Nec*, *p16*, *p19*, *SM30*, *SM50* and *Uni* were down-regulated.

Detoxification genes

The genes involved in detoxification processes showed that the plutei deriving from the RAS tanks had an expression variation for some genes analysed. In the case of plutei deriving from RAS tank 1 (Figure 8; Table S16A), *CA*, *MDR1* and *NADH* exhibited significant variation

of their expression. Similarly, in the case of plutei deriving from RAS tank 2 (Table S16B), *CAT*, *MDR1*, *MT* and *NADH* exhibited significant variation.




Development/ Differentiation
ADMP2 0.5 0.3 1.1 1.4 1.7
Alix 1.0 1.2 2.3 0.3 0
Blimp 0.3 -0.8 -2.0 -0.3 -0.2
BP10 0.2 -0.3 0 -0.2 1.0
BRA -1.2 -2.6 -2.0 -2.6 1.9
CM-K 3.4 3.7 0 0.3 -1.7
CREB -4.1 -2.5 0 -0.3 -2.3
DELTA 0.3 -0.2 0 -0.2 0
d-2-catenin 0.2 0.1 0 0 0
EGF 1.2 -1.0 1.0 -1.0 -0.2
FZ-7 2.1 2.5 0.6 1.9 -0.4
FOXA 2.7 3.5 1.9 2.1 -3.7
FoxG -0.5 0.6 1.5 2.6 3.3
Foxo 3.1 3.1 2.7 0.5 6.9
GFI1 -2.3 -3.9 -3.3 -1.7 0.5
GOOS -2.9 -4.9 -1.0 -2.8 0.4
hat 0.6 0.1 -0.9 1.2 0.7
H3.3 -4.6 1.0 0.5 -2.9 -3.2
HH -3.3 -7.7 1.3 0.1 -2.0
JAK -5.4 -0.7 -4.8 -0.2 -1.7
JNK -1.6 -3.9 0.9 0.1 -4.6
KIF19 -1.7 -4.4 0.1 0.2 -2.7
Lefty 0.2 0.2 -0.3 -0.1 0
M-Vg1 -0.3 -0.3 0 0 0.3
NLK 0.1 -0.5 -0.1 -0.5 0
nodal 2.2 -3.4 0.3 -2.5 -3.8
NOTCH 0.1 -0.5 -0.6 0 0.1
NOTCHLESS 0.3 0.5 0.4 0.8 0.1
OneCut -3.4 0.3 -2.5 -3.8 0.1
Ptc 0.2 1.1 1.0 0.5 0.2
PLC 0.3 0.5 0 1.2 0.6
PLAUF3 0.4 0.5 0.5 1.2 1.3
SMAD6 0.9 0.8 0.1 0.3 0.4
Smo 0.2 -0.2 -0.3 -0.3 0.2
sox9 0.8 -0.2 0.1 0.2 0.1
STAT1 -0.1 -0.2 -0.3 -0.3 -0.3
TAK1 -4.6 -2.8 -2.6 0.6 -1.4
tcf4 -0.2 -6.4 0.8 -2.1 -9.7
TCF7 -2.3 -2.5 -3.4 0.4 -1.4
VEGF -3.7 -2.2 0.3 0.4 -4.8
Wnt5 4.1 4.9 0.2 1.2 3.4
Wnt6 3.0 2.2 1.4 0.7 0.9
Wnt8 2.1 0.9 0.3 3.1 2.7
A B C D E
0
-7
ADMP2 2.00 0.20 0.30 0.40 1.00
Alix 0.40 0 -0.20 0.32 -0.30
Blimp 0.20 0.20 -0.30 1.20 -1.10
BP10 1.10 1.30 -1.40 0.20 0
BRA -1.40 -1.30 -1.60 -2.50 3.90
CM-K -1.30 -1.50 3.20 3.40 6.10
CREB -7.00 -6.30 0 -0.20 -0.30
DELTA -6.00 0 -0.30 -0.12 -0.20
d-2-catenin 0 -0.10 -0.20 -0.30 0
EGF 2.10 0.30 -0.21 -0.30 -0.90
FZ-7 3.90 -1.10 3.40 -2.30 2.90
FOXA 2.60 2.30 -0.50 3.60 1.90
FoxG 3.10 3.50 0.30 3.10 3.50
Foxo -0.40 2.20 3.60 3.80 4.50
GFI1 0.10 -4.50 -2.60 -2.40 0.50
GOOS -3.70 -2.30 -2.20 1.30 1.00
hat 0.90 0.20 0 -1.00 -0.90
H3.3 0.90 0.30 -3.50 -4.10 0.70
HH 0.50 -0.40 -3.90 0.40 -3.40
JAK -2.20 -1.60 0.20 0 -1.40
JNK 0.70 -4.10 -2.20 0.40 0.30
KIF19 -1.90 -2.30 -1.20 -3.70 -1.10
Lefty 0.20 0.20 0.30 -0.60 0
M-Vg1 -0.20 -0.30 0 0 -0.20
NLK -0.40 0.90 0.70 0.20 0
nodal -2.70 0.40 0.20 -2.40 -3.70
NOTCH 0.10 0.10 0.50 0.70 0.30
NOTCHLESS 0.30 0.40 0.30 0.50 0.60
OneCut -2.70 -2.40 -3.70 -4.70 -0.20
Ptc 0.20 0.40 0 1.00 0.90
PLC 1.30 0.50 0.80 0 0
PLAUF3 0.50 0.50 0.60 1.40 1.20
SMAD6 0.20 0.20 0.40 0.50 -1.30
Smo -0.30 -0.30 -0.20 0.30 -0.20
sox9 0.20 0.30 -0.30 -0.10 -0.30
STAT1 0.20 1.20 1.40 0.50 0.80
TAK1 -2.20 -4.90 0.50 -4.40 -1.50
tcf4 0.10 -5.90 -1.80 0.90 0.40
TCF7 -4.90 -3.40 0.40 0.30 0.30
VEGF 0.30 0.70 -5.40 -2.50 -4.70
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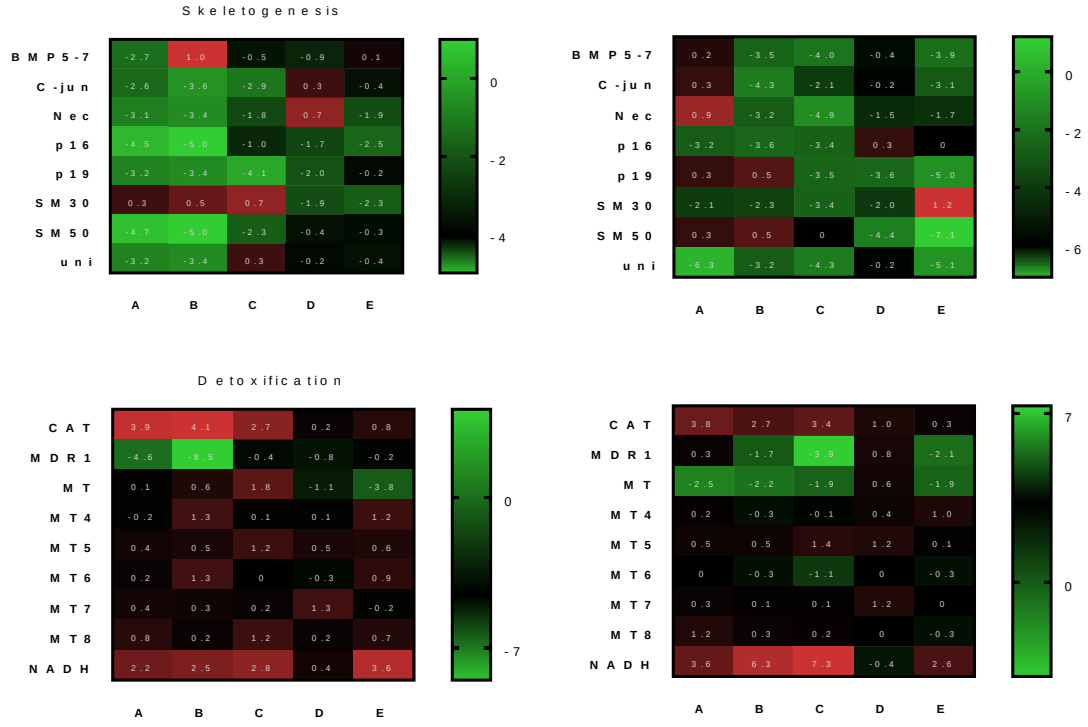

Figure 8: Heatmaps showing the expression profiles and hierarchical clustering of genes analysed by real-time qPCR in embryos deriving from *P. lividus* sea urchins reared in RAS tank 1 (on the left) and RAS tank 2 (on the right). A-B-C-D-E are the different replicates coming from different females. Colour code: red, up-regulated genes respect to the control; blue, down-regulated genes.

Discussion

Conventional ecotoxicity tests enable the identification of one or a few more substances, which can have a major harmful effect on organisms. The benefits of such methods are evident, because they may provide important information on the impacts of a single pollutant on the physiology of a model species, but they do not consider the impact of a mixture of natural pollutants, as they are normally co-present in the environment. Consequently, an

implementation of methods enabling fully understanding of the impacts of complex combinations of contaminants is required. This may be accomplished by concentrating the seawater and testing the physiological responses of individuals and communities employing a more realistic mesocosm. Such a kind of investigations could enable significant progresses in understanding how contaminants actually impact coastal ecosystems and communities (Dachs and Méjanelle, 2010).

In our experimental set-up, despite the presence of the skimmers, pumps and the external filters and their constant maintenance, the increase in nutrients and waste substances concentration, as well as the stochastic mortality events, induced gradual alteration of the water quality. As a result, the abiotic and biotic descriptors showed significant variations within a larger range in RAS tanks, as compared to those measured in the control tanks. Considering the high organic loads and the low dilution rates, nitrites, nitrates, phosphates, temperature and salinity might, as expected, accumulate more in RAS systems than in the controls reaching higher concentrations, while pH and DO were lower.

The most relevant difference in the systems was represented by a total absence of mortality in the control tanks, revealing good health conditions of the animals in the open system, while mortality events occurred, often contemporaneously, in the RAS tanks. The correlation matrix of data collected in RAS tanks indicated that, rather than a single descriptor or factor, the continuous exposure and the additive effects over time led to stress responses and, consequently, to death. However, their effects may still be considered “sub-lethal”, since most reared individuals reached the end of the experiment. The increase of temperature and dissolved oxygen have already been identified as critical factors in aquaculture in general (Qiang et al., 2019) and in echinoculture in particular (Siikavuopio et al., 2007). For this reason and according to the correlation matrixes, we can affirm that the increase in

temperature and the decrease of oxygen strongly affected the general quality of the water and the health state of the sea urchins, leading to mortality events in both RAS tanks. The volume of water in the RAS tanks was limited and, without any water changes, its quality was strongly influenced by high temperature, which in turn could amplify pollution effects. In fact, this evidence was well supported by the other water quality descriptors, which were strictly correlated with both variables. Accordingly, the temperature increases also induced an increase of salinity and PO_4 concentrations. The increase of the salinity was also moderately correlated with the decrease of DO in the RAS tank 1, while in the RAS tank 2 the salinity was moderately correlated with the increase of NH_4, NO_2 and PO_4. Considering its ecological adaptations, *P. lividus* has a considerable tolerance for salinity variations (Santos et al., 2022), but salinity increases can also lead to alteration in the microbial community influencing nitrification and denitrification processes (Xia et al., 2019). Dissolved oxygen is a crucial variable as well. It not only directly affects the health of sea urchins (Siikavuopio et al., 2007), but it is also fundamental for the decomposition of toxic substances (Zhang et al., 2020). Moreover, low levels of dissolved oxygen can limit nitrification and lead to the increase in CO_2 content, and consequently to the decrease of the pH value. In both RAS tanks, dissolved oxygen presented a negative correlation with mortality, indicating that the water quality was highly altered by a low concentration of dissolved oxygen. In fact, dissolved oxygen in aquaculture drops significantly when temperature and density organic matter increase (Zang et al., 2011). These variations were not recorded in the control tanks, where the water was continuously renewed.

Sea urchins are well known for being very sensitive to environmental fluctuations (Fernandez and Boudouresque, 1997), which can affect their reproductive cycle and lead to fertility decreases, or abnormal larval developmental (Byrne et al., 2011; Perez and Lehner, 2019).

Subsequently, in this study we reported how the progressive increase of organic pollution can affect the reproduction success of *P. lividus*.

Interestingly, the two different culture conditions did not affect sea urchin gonad growth. In fact, at the end of the experimental period, the gonadosomatic index exhibited no significant differences among the animals reared in the RAS system and in the control system. Sea urchin gonads are considered as structural storage tissue; the reserve take place both through gonad increase in size or lipids and carbohydrates accumulation (Klinger et al., 1988; Fernandez, 1997). Generally, nutrient storage and gonadic development in *P. lividus* (Boudouresque and Verlaque, 2001) and other echinoderms (Barker et al., 1998) were considered mostly linked to food quality and availability, instead of the water temperature. For this reason, during the experimental period, all the sea urchins were fed on the same diet *ad libitum*, to avoid any effect on the physiology of gonads. Nevertheless, gametogenesis can be affected by water temperature (Shpigel et al., 2004). Our histological analyses confirmed that all the adults tested (both males and females) achieved a sexual maturity stage, with no substantial differences. On the other hand, the results herein obtained from both the morphological observations on the larvae and the molecular analyses, indicate that an increased organic pollution may hardly impact the reproductive potential of this species. In fact, there is a significant difference between the percentage of malformed plutei at 48h, deriving from gametes produced by animals reared in the RAS system. Furthermore, our findings showed that more than 50% of embryos were delayed, being at 48 hpf at at the blastula or gastrula stage, some of them with evident apoptotic signals. These results evidenced a strong effect on *P. lividus* reproductive efficiency. These morphological observations are well supported by the evaluation of the expression of several genes related to different functional processes as stress response, development, differentiation,

skeletogenesis and detoxification. Almost all genes under analysis switched on, as compared to the controls. Most of the genes involved in development and skeletogenesis were down-regulated, justifying the morphological low success of embryonic development observed. In addition, it is evident that sea urchins exposed to these treatments attempted to detoxify, by increasing the expression of the specific genes involved in detoxification pathways. These findings indicated that the prolonged exposure of sea urchins to organic pollution, even if not inhibiting their gonadal maturation, was sufficient to affect common molecular pathways, altering some physiological mechanisms, which in turn can lead to morphological malformations in their offspring. Reproductive success is vital for the survival of any species. Failure of adult sea urchins to obtain embryos able to correctly develop, can might induce strong impacts on their natural stocks.

Considering the limitations of standard ecotoxicology tests, the realistic mesocosm tested in this study can be considered as an effective method which, in combination with molecular analyses, helps our understanding of the impacts of complex combinations of stressors and accumulation of organic contaminants in marine environments. In addition, the understanding of the effects of toxic substances discharged from human activities can be extremely important to forecast and manage possible environmental damages associated with their rise and spread. Understanding the molecular processes involved in sensing and dealing with classical or new contaminants might be useful to produce diagnostic tools to timely assess various threats to the marine environment.

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Chapter 4

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Article

Gene Expression Detects the Factors Influencing the Reproductive Success and the Survival Rates of *Paracentrotus lividus* Offspring

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Abstract: The increase in the demand for *Paracentrotus lividus* roe, a food delicacy, causes increased pressure on its wild stocks. In this scenario, aquaculture facilities will mitigate the effects of anthropogenic pressures on the wild stocks of *P. lividus*. Consequently, experimental studies should be conducted to enhance techniques to improve efficient aquaculture practices for these animals. Here, we for the first time performed molecular investigations on cultured sea urchins. We aimed at understanding if maternal influences may significantly impact the life of future offspring, and how the culture conditions may impact the development and growth of cultured specimens. Our findings demonstrate that the outcomes of in vitro fertilization of *P. lividus* are influenced by maternal influences, but these effects are largely determined by culture conditions. In fact, twenty-three genes involved in the response to stress and skeletogenesis, whose expressions were measured by *Real Time qPCR*, were differently expressed in sea urchins cultured in two experimental conditions, and the results were largely modified in offspring deriving from two groups of females. The findings herein reported will be critical to develop protocols for the larval culture of the most common sea urchin, both for research and industrial production purposes for mass production.

Keywords: aquaculture; carryover effects; skeletogenesis; stress response

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1. Introduction

1.1. Sea Urchins and Aquaculture

Sea urchins are a resource for the scientific research, besides their key role in the ecology of Mediterranean shallow ecosystems and their fishery importance. In fact, the Mediterranean *Paracentrotus lividus* has both an ecological and an economic importance, and an emerging demand for fresh animals characterizes the fish markets, because their gonads (also known as "roe") are considered a food delicacy worldwide and a substantial source of revenue. The roe consists not only of immature and mature gametes but also of germinal and connective tissues. Consequently, the number of gametes is not the only factor related to gonadal size, because the growth of nutritive phagocytes and accessory tissues also influence the size of gonads. This is important because it is possible to enhance the gonadal growth of farmed individuals by favoring the accumulation of nutrients independently from their actual reproductive power [1]. Aquaculture of echinoderms (sea urchins and sea cucumbers) is defined as "echinoculture", but this term often refers specifically to

sea urchin culture in the laboratory. Wild populations of *P. lividus* are presently facing changing environmental conditions and overexploitation of their stocks. This issue urgently needs action to ensure correct management of the resource. The implementation of eco-friendly and efficient aquaculture systems could help in overcoming the overexploitation of marine resources, including sea urchins. In addition, sea urchins play a pivotal role in scientific research [2] because they are widely used for ecotoxicological, physiological and embryological studies due to their easy handling in the laboratory and to the transparency of their embryos, ensuring that the first developmental stages can be easily monitored [3–5]. Sea urchin larvae are also employed as live feed for other cultured organisms, such as fishes and filter feeders (e.g., shellfish larvae). Consequently, they represent a valuable resource for the culture of other economically-relevant species [6].

1.2. Factors Influencing Larval Development

Several factors, such as diet composition, larval density and quality of the culture environment, may deeply impact the reproductive success of this species [7–10]. Variable feed abundance, feeding frequencies and species of live microalgae used for larval diets can severely influence larval growth, survival and metamorphic success [8,9,11,12] according to the cultured species of sea urchin. In addition, the nutritional quality of microalgae can vary depending on culture factors influencing their cell size, digestibility and biochemical composition [13]. For example, *Chaetoceros* spp. and *Tisochrysis lutea* contain relatively high amounts of long-chain PUFAs, but neither are rich in both EPA and DHA [14], and in their research were found to decreasein the absence of *Rhodomonas lens*. Castilla-Gavillán et al. [10] demonstrated that *P. lividus* larvae fed on *Rhodomonas* sp. contained higher total lipid content than those fed on other microalgae. Similar dynamics could have positive implications for the development of larval echinoderms. Previous studies [15] indicated that *Dunaliella* sp. may be sufficient to sustain the complete development of *P. lividus* up to metamorphosis, although small additions of other feeds (e.g., *R. lens*) may represent a useful improvement towards complete success of the larviculture. In contrast, some microalgae may have deleterious effects on the growth of post-larvae [16].

However, maternal and zygotic factors also influence and control the early development of sea urchins. Maternal factors include messenger RNA and proteins expressed during oogenesis. mRNA is also necessary before activation of the embryonic genome for early development, as they are involved in the regulation of metabolism, cell cycle and development [17]. When genome activation occurs in embryos, zygotic factors begin to be expressed. The first known developmental genes are expressed at the reaching of 16-cell stage; before this stage any developmental process is completely driven by maternal factors [18,19]. Besides their genetic contribution, mother sea urchins control the phenotypic development of their offspring in response to environmental conditions [20]. These maternal influences, which result in the combined effect of maternal phenotype and genotype [21], can have significant effects on the fitness and performance of the offspring [22–24]. In addition, they can influence the sea urchin population ecology [21,25]. However, factors experienced by sea urchin females during early development can also affect the phenotypes and fitness of their offspring.

In addition, the size of culture vessels and the density of larvae may represent key factors in determining rates of survival of larvae up to metamorphosis. An experiment conducted in small-scale 1.3-L culture vessels revealed an inverse relationship between larval density of a sea urchin (*Diadema antillarum*) and growth [26]. Previous authors obtained variable rates of survival by approaching different larval-rearing systems [27]. Different studies were characterized by variable feeding protocols, fertilization techniques and rearing devices [28]. In particular, previous research has indicated culture experiences within tanks of different volumes, besides the influence of additional rearing devices (e.g., aeration systems, palettes, procedures, etc.). Hence, we aimed to investigate if tanks of the same shape but different sizes might influence the survival of larvae. In fact, keeping constant the larval density and the concentration of microalgae feed, tanks of different

volumes are characterized by variable hydrodynamic conditions. For example, larger tanks need faster bubbling to ensure sufficient gas exchanges, when compared to smaller tanks, but aeration may interfere with the limited swimming capabilities of planktonic larvae, pushing them into some sectors on the bottom. However, an appropriate determination of the most favorable stocking density within a production-oriented recirculating aquaculture system (RAS) has not been performed. Consequently, the size of culture vessels, the quality of larval nutrition and the stocking density remain extremely relevant for the larviculture of sea urchins, especially when considering the feasibility of production for fisheries, restoration and scientific research. For these reasons, we tested the effect of the tank size in order to detect any influence on survival rates during the early development of these sea urchins.

We had two distinct aims in our manuscript: (i) we aimed at understanding if maternal influences may significantly impact the life of future offspring; (ii) we aimed at detecting how and if slight variations in the culture conditions may impact the development and growth of cultured specimens. In addition, we aimed at evaluating the best production sizes, in terms of tank volumes, to ensure correct growth and higher survival rates of *P. lividus* embryos. On the whole, we aimed at understanding how genic resources interact with environmental stressors to ensure maximum reproductive performance in a marine invertebrate. Furthermore, and for the first time, we tested the effect of these production units by performing molecular analyses (*Real-Time qPCR*) to check the expression levels of several genes involved in stress responses and skeletogenesis. In particular, we followed the variation of expression levels of twenty-three genes, which were first isolated in sea urchin embryos in response to different stressors [3,5,29–33]. In this study, gene expression levels are not responses related to a stimulus but must be intended as higher or lower levels of gene expression.

2. Results

2.1. Survival Rates

Even if the survival rates of larvae deriving from females A-B in the first three weeks of growth were not significantly different from those deriving from females C–D, when analyzed by means of paired *t*-tests (Table 1):

Table 1. Paired *t*-test (two-tailed *p* value) to compare the larval survivorships (A-B vs. C-D) in smaller and larger tanks. ** indicates $p < 0.01$.

Paired *t* Test	Smaller	Larger
***p* value**	0.0830	0.1169
***p* value summary**	n.s.	n.s.
t	1.980	1.758
df	8	8
Mean of differences (B-A)	−21.93	−8.778
SD of differences	33.22	14.98
SEM of differences	11.07	4.994
95% confidence interval	−47.46 to 3.610	−20.29 to 2.738
R squared (partial eta squared)	0.3289	0.2786
Correlation coefficient (r)	0.7608	0.8595
p value (one tailed)	0.0086	0.0015
p value summary	**	**
Significantly effective pairing	Yes	Yes

The performances of the two sets of larvae exhibited different patterns of development (Figure 1).

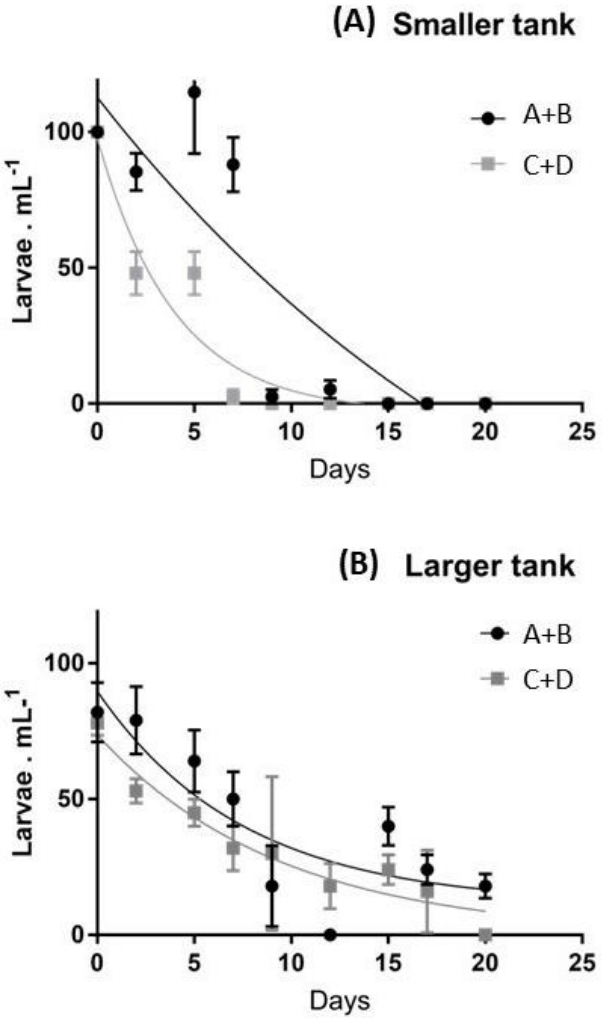


Figure 1. Average survival rates of larvae obtained from the two pools of individuals (A+B vs. C+D) in smaller (**A**) and larger (**B**) tanks.

In particular, pool A-B exhibited higher survival rates in the smaller tanks (Figure 1A), completing the settlement after 17 days. In contrast, pool C-D exhibited lower survival rates during the first two weeks and the number of swimming larvae was strongly reduced after two weeks, due to higher mortality. In fact, in the last two weeks, larvae were absent in both groups of females considered. These differences were less evident in larger tanks (Figure 1B), where the number of larvae continuously decreased in the first 20 days, exhibiting a low number of settled individuals in both A-B and C-D groups (Figure 1B). The larval stage progression was initially similar in the two pools and the embryonic stages developed synchronously through the pluteus stage. However, from the onset of feeding through the larval stages, the development became increasingly less synchronous between pools A-B vs. C-D, and even among tanks of different sizes. After the first week, larvae from the pool A-B exhibited faster development, showed dark-green guts and after 10–12 days reached a six-arm stage, while larvae from the pool C-D exhibited slower development, about half of them arrested their development at a four-arm stage, and were characterized by partially-empty guts. In particular, larvae cultured in smaller tanks reached the advanced rudiment stage after about two weeks, while larvae in larger tanks showed lower mobility, sank to the bottom more often and their guts exhibited a pale color.

To evaluate the effect of maternal influences and the effect of tank size on the survival of larvae (removing the influence of settlers on the density of swimming larvae) only the first week of culture was analyzed and the slopes of linear regressions were compared (Figure 2).

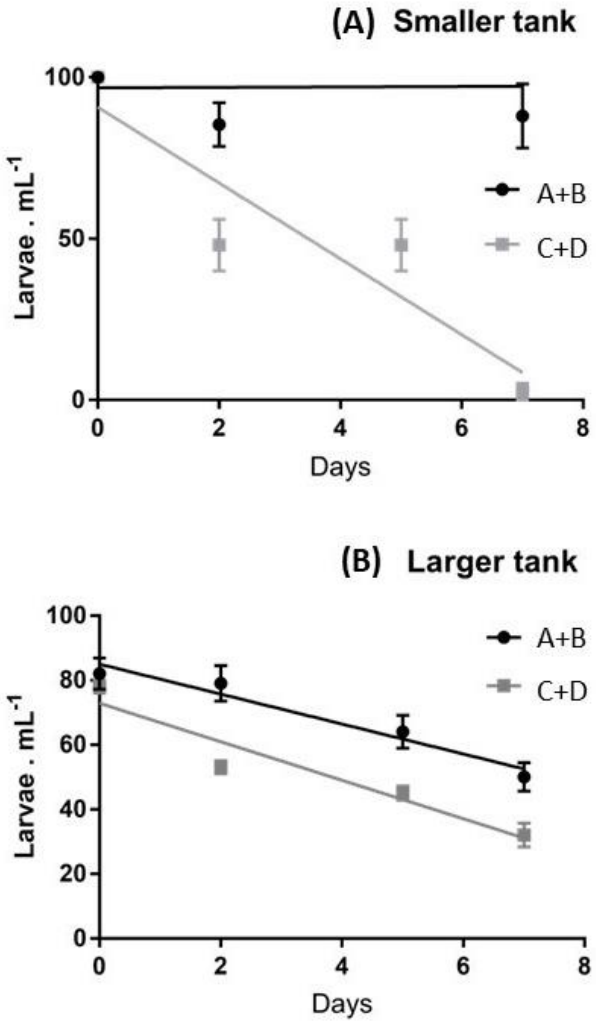


Figure 2. Average larval survival rates evaluated during the first week in (**A**) smaller and (**B**) larger-tanks, referring to the recruits of females A+B vs. those of females C+D. The linear regressions are superimposed.

This representation permits the evaluation that in smaller tanks, during the first week, an 100% survival was exhibited by larvae deriving from females A and B, whilst, in the same period, females C and D produced a decline of larvae, down to 2.6% of the initial pool. When slopes were compared, the differences in the survival patterns of the two larval pools were significant at $p < 0.05$. In larger tanks a continuous decrease of the swimming larvae was observed during the first week for both larval pools (Figure 2B) and the differences in the survival patterns of the two larval pools were not significant ($p > 0.05$).

2.2. Molecular Analyses of Embryos Deriving from Various Females

The variation of expression levels of twenty-three genes, involved in the stress response and skeletogenesis and previously analyzed [3,5,34–38] (see Table S3) was followed by *Real Time qPCR* (see Table S4).

2.2.1. Genes Involved in Stress Response

Concerning the 18 genes analyzed, the results show that the plutei deriving from female A did not have variations in expression for any of the genes analyzed. The same results were found for the plutei deriving from female B, with the only exception being *CASP8* gene, which decreased its expression level. In the case of plutei deriving from female C, fifteen genes decreased their expression levels: *ARF1* (−3.5), *CASP8* (−5.5), *caspase 3/7* (−3.5), *ERCC3* (−3.9), *GRHPR* (−2.4), *hsp60* (−2.4), *hsp70* (−6.4), *HIF1A* (−2.0), *PARP-1* (−3.0), *p53* (−4.8) and *14-3-3 ε* (−5.5). Only *cytb* gene showed an increase (2.5) of its expression level. Moreover, in the plutei deriving from female D, nine genes decreased their expression levels (*CASP8*, −2.7; *ERCC3*, −3.2; *hsp60*, −2.8; *hsp70*, −2.9; *p38MAPK*, −2.3; *SDH*, −2.7; *p53*, −4.4; and *14-3-3 ε*, −3.4).

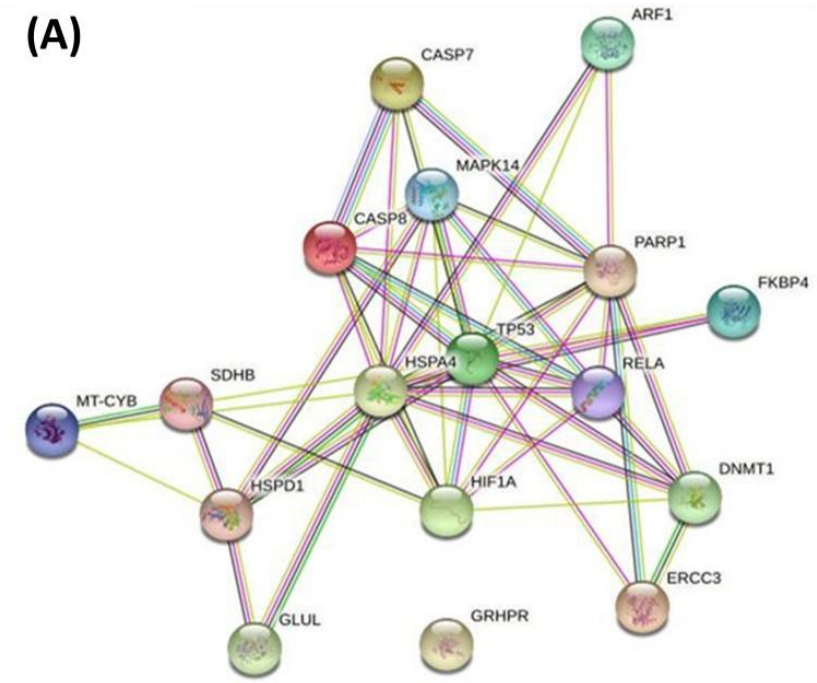
2.2.2. Skeletogenic Genes

None of the five genes analyzed was switched on in the plutei deriving from females A and B. Three genes were down-regulated in the plutei deriving from female C (*SM30*, −3.9; *SM50*, −4.0; *uni*, 4.0), whereas plutei deriving from female D showed a down-regulation of *SM50* (−7.3) and an up-regulation of *univin* (4.0).

2.3. Network Analysis

Interactomic analysis showed that eighteen genes of the twenty-three analyzed from *Real Time qPCR* are connected by functional point of view: ADP-ribosylation factor 1 (*ARF1*), caspase-8 (*CASP8*), Sp-Cspe3/7L (*CASPASE 3/7*), cytochrome b (*CYTB*), DNA-methyltransferase 1 (*MTase*), ERCC excision repair 3 (*ERCC3*), glutamine synthetase (*GS*), glyoxylate reductase/hydroxypyruvate reductase (*GRHPR*), heat shock protein 56 (*HSP56*), heat shock protein 60 (*HSP60*), heat shock protein 70 (*HSP70*), hypoxia inducible factor 1-alpha (*HIF1A*), *nuclear factor kappa light-chain-enhancer of activated B cells* (*NF-kB*), Poly(ADP-ribose) polymerase 1 (*PARP-1*), p38 mitogen-activated protein kinase (*p38 MAPK*), succinate dehydrogenase (*SDH*), tumor protein p53 (*p53*). Only the skeletogenic genes 14-3-3 epsilon protein (*14-3-3 ε*), bone morphogenetic protein 5-7 (*BMP5-7*), and nectin (*Nec*) weren't included in network analysis because they have no orthologous genes in humans. Moreover, GRHPR is not linked to any gene under analysis.

As shown in the network reported in Figure 3, the genes are correlated as follows: *ARF1* with *hsp70*, *p53* and *PARP1*; *Caspase 3/7* with *CASP8*, *hsp70*, *p38 mapk*, *PARP1*; *CASP8* with *Caspase 3/7*, *p38 mapk*, *hsp70*, *p53*, *NF-kB*, *PARP1*; *p38 mapk* with *CASP8*, *Caspase 3/7*, *hsp60*, *hsp70*, *p53* and *PARP1*; *Hsp70* with *CASP8*, *p53*, *NF-kB*, *MTase*, *HIF1A*, *hsp60*, *SDH*; *p53* with *p38 mapk*, *CASP8*, *hsp70*, *HIF1A*, *ERCC3, NF-kB, MTase, PARP1, hsp56; PARP1* with *ARF1, Caspase 3/7, p38 mapk, CASP8, p53, HIF1A, NF-kB, ERCC3* and *MTase; Hsp56* with *p53; NF-kB* with *PARP1, p38 mapk, p53, hsp70, HIF1A, ERCC3, MTase; MTase* with *PARP1, NF-kB, p53, hsp70, ERCC3; ERCC3* with *MTase, PARP1* and *p53; HIF1A* with *NF-kB, PARP1, MTase, p53, hsp70* and *SDH; SDH* with *hsp70, HIF1A, hsp60* and *CytB; CytB* with *SDH* and *hsp60; Hsp60* with *CytB, SDH, p38 mapk, hsp70, p53* and *GS; GS* with *hsp60* and *hsp70*. The corresponding human orthologous genes are also reported.



(B)

Gene	***P. lividus***	***H. sapiens***
ADP-ribosylation factor	*ARF1*	*ARF1*
caspase-8	*CASP8*	*CASP8*
Sp-Cspe3/7L	*caspase 3/7*	*CASP7*
Cytochrome b	*Cytb*	*MT-CYB*
DNA-methyltransferase 1	*Mtase*	*DNMT1*
ERCC excision repair 3	*ERCC3*	*ERCC3*
Glutamine synthetase	*GS*	*GLUL*
Glyoxylate reductase/hydroxypyruvate reductase	*GRHPR*	*GRHPR*
Heat Shock Protein 56	*hsp56*	*FKBP4*
Heat Shock Protein 60	*hsp60*	*HSPDI*
Heat Shock Protein 70	*hsp70*	*HSPA4*
Hypoxia inducible factor 1-alpha	*HIF1A*	*HIF1A*
Nuclear factor kappalight-chain-enhancer of activated B cells	*NF-Kb*	*RELA*
Poly(ADP-ribose) polymerase 1	*parp-1*	*PARP1*
p38 mitogen-activated protein kinase	*p38 MAPK*	*MAPK14*
Succinate dehydrogenase	*SDH*	*SDHB*
Tumor protein p53	*p53*	*p53*
14-3-3 epsilon protein	*14-3-3 ε*	*YWHAE*
Bone morphogenetic protein 5-7	*BMP5-7*	*BMP7*
Nectin	*Nec*	*Nectin-1*

Figure 3. (**A**) Interactomic analysis by STRING (https://string--db.org/; accessed on 30 April 2022). The network graphically displays the relationship between genes. The biological relationships between genes are indicated by different colors. Known interactions: reported by database = light blue and determined experimentally = pink. Expected interactions: gene proximity = green; gene fusion = red; and genes with similar pattern = light blue. (**B**) *Homo sapiens* gene names and the corresponding *P. lividus* orthologous genes. The most significant relations among genes (confidence score cut-off = 900) displaying experimental evidence are highlighted.

3. Discussion

Investigations of other echinoderms reported that the performance of one life history stage led to (positive or negative) carryover effects on the subsequent life stages [39]. In fact, carryover effects arising within a generation can influence next generations [40–42]. Carryover effects can also arise across a generation, an effect known as transgenerational plasticity (TGP) [43]. Due to environmental stresses [44] experienced by one or both parents during the development of their gametes [23,41], a transgenerational response can lead to phenotypic changes in the offspring [43,45]. Various evidence indicates that long-term exposure of adult sea urchins to thermal shocks impacts the embryo development of their offspring [39,46,47]. Here we aimed at checking and eventually confirming this hypothesis regarding *Paracentrotus lividus* embryos and larvae.

During the whole experiment the main abiotic descriptors were kept stable and did not achieve significant variations among replicates and treatments. Maternal effects on the survival rates of larvae were definitely observed in smaller culture vessels. Larger vessels prompt a negative influence on the survival, which is likely to be superimposed onto the material influence itself. This evidence indicates that both the maternal effects and the size of the culture tanks are critical to determine the reproductive success of this species, but the influence of the latter overwhelms that of the former.

Evidently the size of the tank matters, because the two experimental conditions produced significantly different results in terms of survival rates of larvae. The effect might be explained by taking into account that circulation of water and oxygen stratification change drastically according to the shape and the size of the culture vessels. In particular, deeper tanks exhibit a lower circulation of water and a higher stratification of gases, because the rate of aeration (air bubbling) cannot be proportionally changed to avoid damage to the delicate larvae swimming closer to the bubbling tubes [48–50]. We might also consider the need for larvae to remain in the upper levels and to avoid strong contact with the bottom of the tank, since it may contain algal and bacterial films which produce mucilage that promotes clogging and aggregation of larvae (personal observation; [51]). Since larger tanks require higher levels of aeration to guarantee sufficient gas exchanges, the fluxes produced by the air stone also promote the creation of sink areas where larvae aggregate close to the bottom. In contrast, the aeration may be very weak in the smaller tanks, permitting the larvae to swim towards the surface without interference. In fact, in smaller containers most larvae were continuously visible under the surface of the water, while in larger tanks they were actively transported by the currents and could accumulate in areas with lower perturbations, close to the bottom (personal observation). Attempts to reduce this effect by decreasing the flux of the air produced lower gas exchanges which were insufficient to guarantee consistency of the abiotic factors (including the saturation of oxygen in the seawater) that represented a pre-condition to evaluate other sources of stress [49,52].

As reported, the density of larvae deriving from females C-D exhibited a decrease in smaller tanks, as opposed to larvae deriving from females A-B, which performed best. In contrast, both groups of larvae showed mortality rates higher than 60% in the first week when cultured in larger tanks, and the differences were not significant in this condition. This may lead to the conclusion that the first factor determining survival rate is represented by the environmental conditions and that, in an optimal environment, maternal influences become determinant. The overlap of the growth conditions to the maternal influence may also explain the interannual differences in larval recruitment of this species observed in the field [53].

Molecular analyses were performed to understand if there were differences in the levels of gene expression associated with offspring deriving from different *P. lividus* females (see Figure 4). Molecular data strongly supported the low success of embryonic development for the plutei deriving from females 3 and 4. In fact, plutei deriving from females A and B did not show significant variations in their gene expressions.

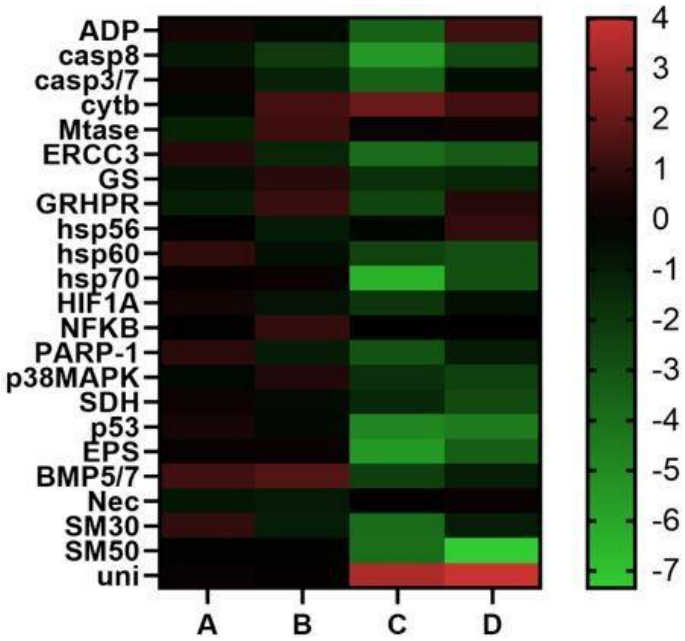


Figure 4. Heatmaps (Heatmapper available at www.heatmapper.ca, accessed on 28 July 2022) showing the expression profiles and hierarchical clustering of twenty-three genes analyzed by *Real Time qPCR* in *P. lividus* embryos deriving from females A, B, C and D. Color code: red = up-regulated genes with respect to the control; green = down-regulated genes.

In contrast, plutei deriving from females C and D exhibited a remarkable switch on in almost all the genes analyzed that are involved in stress response and in skeletogenic process, indicating negative maternal influences, as showed by the results of *Real Time qPCR*. In fact, the down-regulation of genes in the plutei deriving from *P. lividus* females C and D was evident compared to those deriving from females A and B. These effects in marine organisms are not new [23], because they have been widely investigated in various sea urchin species [54–56], but to the best of our knowledge such relationships have never been investigated in *P. lividus* [17].

Interactomic analyses showed that stress genes were functionally correlated among themselves. *GRHPR* was the only gene that appeared to have no correlation with others. Previous investigations indicated that this gene is correlated to *HSPA4* and *GLUL* through *CAT* [38], which was also analyzed in this research.

Changes of gene expression induced by environmental stressors on somatic cells can affect the physiology of exposed individuals. As a consequence, some alterations can be propagated to subsequent generations through the germline, occurring during the gametogenesis [57–59]. In particular, environmental stresses experienced by one or both parents may lead to aberrant patterns in F1, which can become evident from the early developmental stages [56,60]. It is known that a network of regulatory genes, the so called "defensome" [3,30,61], regulate the mechanisms of specification and differentiation during embryonic development.

Our findings show how changes in the levels of gene expression may be used as an early indicator of stressful conditions for sea urchins, and more generally for marine invertebrates and the marine environment itself. Furthermore, these findings demonstrate that changes in gene expression can help understand the health state of adult sea urchins. The identification of key genes and the molecular pathways in which they are involved represent fundamental tools for understanding how marine organisms attempt to protect themselves against toxicants in order to avoid deleterious consequences and irreversible damage. Furthermore, changes in the response to environmental stressors may contribute to an adaptive survival advantage of organisms through beneficial gene expression [39].

The findings herein demonstrated will be useful for the culture of the most common Mediterranean sea urchin, both for research purposes and for the needs of aquaculture. In fact, despite several established methods of commercially producing echinoderms from gametes [62,63], a scalable hatchery process for *P. lividus* has not yet been established. Attempts to culture this species over the last decades have been met with varying degrees of success, and speculative factors including environmental toxins, water quality and general culture methods precluded reliable development through metamorphosis [26]. This study was intended to develop *P. lividus* larval culture protocols within a laboratory or an industrial production plant capable of scaled production for aquaculture, restoration or scientific research. The culture conditions demonstrated are adequate for the larval development of *P. lividus*, but variables of interest, i.e., maternal influences and size of the culture vessels, are demonstrated to be critical factors to reach sufficient survival rates and complete development of this species. However, further investigations aimed at improving yields are necessary, as well as to understand how larval nutrition affects settlement and post-settlement success in order to further improve commercial production viability.

4. Materials and Method

4.1. Gamete Collection and Embryo Culture

Adult sea urchins were collected by scuba divers around the island of Procida (Italy) in February 2021, at 10 m depth. Collected specimens were transported in an insulated box to the laboratory and maintained in tanks with circulating sea water for ten days until testing. Sea urchins were injected with 1 mL of 2 M KCl through the peribuccal membrane to stimulate the emission of gametes. After the production of gametes, the specimens were restored in aerated recirculated tanks and released after a few days into the same area of collection. Eggs were collected in a glass dish, washed with filtered sea water (FSW) and kept in FSW until use. Concentrated sperm was collected from males by means of a plastic pipette and kept undiluted at 4 °C until use.

Eggs were fertilized and were kept at 20 °C in a controlled temperature chamber on a 12 h–12 h light–dark cycle. Forty-eight hours post-fertilization the embryos reached the pluteus stage, and these larvae were distributed in equal concentrations (1.5 plutei mL^{-1}) in triplicate tanks of two different volumes: (i) small scale, in 2 L beakers; (ii) larger scale, in 10 L tanks. Embryos deriving from individual females were paired two by two and kept separate, aiming at detecting any variation due to maternal influences and if the initial quality of the gametes influenced the larval development. In particular, the eggs deriving from four females were randomly pooled into two groups (females A-B and females C-D) prior to being fertilized with a pool of sperms deriving from five males. Four females were randomly chosen because TR-qPCR technique is useful to detect individual variations in the gene expression, avoiding the flattening of results due to variable gene expressions detected in larger populations.

Larvae were fed on *Dunaliella tertiolecta* (5000 cells ml^{-1}; [15]) along with a composed algal food (SHG Snow Reef, Super High Group, Ovada, Italy; www.superhigroup.com accessed on 28 July 2022) containing yeast, fish oils, *Chlorella* and various HUFAs reproducing the composition of natural marine snow in a suspension of particles sizing 5–10 µm [15]. Larvae were filtered every two days onto a 60 µm mesh sieve and transferred to a renewed culture medium using a Pasteur pipette. At the same time intervals, two 50 mL samples were collected to evaluate and record the survival rates, check the larval stage progression, the overall health conditions (mobility, malformations) and the gut fullness. During the experiment, seawater samples were collected biweekly and nitrites and phosphates were measured (using a photometer AL 450, Aqualytic, Alkimia Srl), as well as salinity and temperature (see Supplementary Table S1 in the Supplementary Materials).

4.2. Statistical Analyses

The survival rates of larvae deriving from two sets of females (pooling of individuals A-B vs. C-D) were calculated using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA, www.graphpad.com, accessed 1 September 2021) using the following equation for one phase decay:

$$Y = (Y0 - Plateau) * \exp(-K * X) + Plateau \quad (1)$$

where: Y0 is the Y value when X (time) is zero; Plateau is the Y value at infinite times; K is the rate constant, expressed in reciprocal of the X-axis time units. The survival rates obtained with A-B vs. C-D larvae, as well as those obtained in smaller vs. larger tanks, were compared and two-sided *p*-values were obtained by paired *t*-tests.

The slopes obtained from different individuals (A-B vs. C-D) in different types of culture tanks (smaller vs. larger) were compared by linear regression on the data limited to the first week, to avoid any influence of settlement (older larvae settle and the number of swimming individuals is reduced) and to determine the slope of a best-fit line. A value of $p < 0.05$ was chosen as a threshold level for significance. All data have been analyzed with the aid of GraphPad Prism 8.0 software.

4.3. RNA Extraction and cDNA Synthesis

About 5000 fertilized eggs from each of the four sea urchin females were collected at the pluteus stage, corresponding to 48 h post-fertilization (hpf). These samples were centrifuged at 3500 relative centrifugal force for 15 min in a swing-out rotor at 4 °C. Embryos were then placed in 60 microliters of the RNA-later (Qiagen, Hilden, Germany) and then frozen in liquid nitrogen. Samples were kept at −80 °C until use. Total RNA was extracted using *Aurum Total RNA Mini Kit* (Bio–Rad, Hercules, CA, USA), according to the manufacturer's instructions. The amount of total RNA extracted was estimated by the absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios, using a NanoDrop spectrophotometer (ND–1000 UV–VIS Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). The integrity of RNA was evaluated by observing the rRNA subunits (28S and 18S) on agarose gel electrophoresis. For each sample, 1000 ng of total RNA was retrotranscribed with an *iScript cDNA Synthesis kit* (Bio–Rad, Milan, Italy), following the manufacturer's instructions.

4.4. Real Time qPCR Experiments

The variations in the expression of twenty-three genes were followed by *Real Time qPCR* (see Supplementary Table S2 in the Supplementary Materials for their biological functions and Supplementary Table S3 for the sequence of the primers) in sea urchin embryos deriving from each of the four females collected at the pluteus stage, reached at 48 hpf. cDNA (1 µL) was used as a template in a reaction containing a final concentration of 0.3 mM for each primer and 1× FastStart SYBR Green master mix (total volume of 10 µL) (Applied Biosystems, Monza, Italy). PCR amplifications were performed in a ViiATM7 *Real Time PCR* System (Applied Biosystems, Monza, Italy) thermal cycler using the following thermal profile: 95 °C for 10 min, one cycle for cDNA denaturation; 95 °C for 15 s and 60 °C for 1 min, 40 cycles for amplification; one cycle for final elongation at 72 °C for 5 min; one cycle for melting curve analysis (from 60 °C to 95 °C) to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability, all *Real-Time qPCR* reactions were carried out in triplicate. Fluorescence was measured using ViiATM7 software (Applied Biosystems, Monza, Italy). For all *Real-Time qPCR* experiments the data of each cDNA sample were normalized with the mRNA level of the ubiquitin [5] and 18S rRNA [64] genes, used as housekeeping genes. The expression of each gene was analyzed and normalized against the internal control by REST program (Relative Expression Software Tool), based on the Pfaffl method, reporting the expression values of the genes of interest with respect to the housekeeping

genes, using each time one of four different females as the reference group [65,66]. Relative expression ratios above two cycles were considered significant. Experiments are means between duplicates. Statistical analysis was performed using GraphPad Prism version 8.00 for Windows (GraphPad Software, San Diego, CA, USA).

The network analysis on genes under analysis was performed by STRING [67], which aims to identify relationships on the basis of associated functions and data mining from experimental studies reported in the literature. Since sea urchin genes are not annotated in the STRING database, human orthologues were used to search for *P. lividus* genes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232112790/s1.

Author Contributions: S.F. performed the experimental work and the statistical analyses and produced the first draft of the manuscript. F.G. performed and supervised the experimental work in Procida. R.E. contributed to molecular experiments and aided the production of the first draft and its revision and contributed statistical analyses and molecular investigations. B.P. and M.G. contributed to the experimental work in Procida. A.D.C. planned the research and supervised the production of the manuscript. M.C. planned the research, performed molecular tests, supervised the students and contributed to the production of the first draft and its final revision. V.Z. planned the research, revised the manuscript and supervised the experimental work in Procida. All authors have read and agreed to the published version of the manuscript.

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Chapter 5

marine drugs

MDPI

Article

Two Benthic Diatoms, *Nanofrustulum shiloi* and *Striatella unipunctata*, Encapsulated in Alginate Beads, Influence the Reproductive Efficiency of *Paracentrotus lividus* by Modulating the Gene Expression

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Abstract: Physiological effects of algal metabolites is a key step for the isolation of interesting bioactive compounds. Invertebrate grazers may be fed on live diatoms or dried, pelletized, and added to compound feeds. Any method may reveal some shortcomings, due to the leaking of wound-activated compounds in the water prior to ingestion. For this reason, encapsulation may represent an important step of bioassay-guided fractionation, because it may assure timely preservation of the active compounds. Here we test the effects of the inclusion in alginate (biocompatible and non-toxic delivery system) matrices to produce beads containing two benthic diatoms for sea urchin *Paracentrotus lividus* feeding. In particular, we compared the effects of a diatom whose influence on *P. lividus* was known (*Nanofrustulum shiloi*) and those of a diatom suspected to be harmful to marine invertebrates, because it is often present in blooms (*Striatella unipunctata*). Dried *N. shiloi* and *S. unipunctata* were offered for one month after encapsulation in alginate hydrogel beads and the larvae produced by sea urchins were checked for viability and malformations. The results indicated that *N. shiloi*, already known for its toxigenic effects on sea urchin larvae, fully conserved its activity after inclusion in alginate beads. On the whole, benthic diatoms affected the embryogenesis of *P. lividus*, altering the expression of several genes involved in stress response, development, skeletogenesis and detoxification processes. Interactomic analysis suggested that both diatoms activated a similar stress response pathway, through the up-regulation of *hsp60*, *hsp70*, *NF-κB*, *14-3-3 ε* and *MDR1* genes. This research also demonstrates that the inclusion in alginate beads may represent a feasible technique to isolate diatom-derived bioactive compounds.

Keywords: encapsulation; microalgae; modulated genes; sea urchin development

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1. Introduction

Dietary uptake of organic and inorganic compounds influence the physiology [1,2], ecology [3] and the population dynamics [4] of several marine grazers. Besides pollution and other anthropogenic influences [5], which trigger specific effects on the physiology of planktonic and benthic consumers, algae are known to produce various defense metabolites,

characterized by a range of activities, from simple repulsive effects [6] to highly toxigenic effects against grazers [7] or their progeny [8,9].

The environmental impact of algal metabolites is often evaluated by forcing invertebrates to graze on given plants [10], or exposing small aquatic animals to the volatile organic compounds constitutively produced by algae, to record their behavioral reactions [11–13]. However, bioassay-guided fractionation is often required to isolate the active compounds triggering these reactions, and further elucidate their molecular structure, for biotechnological applications [14]. The administration of algal extracts and fractions may reveal some constraints, because it must be gathered through suitable feeds supplemented with polar or non-polar compounds. In seawater, some lipophilic compounds are stably stored in feeds, until their complete consumption by invertebrates [15]. Hydrophilic compounds, in contrast, are easily dispersed in the water after administration and cannot reach their target consumers if not ingested immediately [16].

A variety of encapsulation techniques may be adopted to stably conserve the secondary metabolites previously fractionated or isolated, and guarantee their correct vehiculation to the target organism. In particular, the development of encapsulation improves the efficiency of screening methods and offers a fast, simple and reliable tool (e.g., multiplexed screening methods) to test the effect of algal metabolites on their consumers. To this end, carboxylated microspheres [17] were used to immobilize various toxins on their surface and were easily vehiculated through compound feeds. An alternative approach is the adoption of microencapsulated feeds [18,19] since they may consistently and almost stably preserve various types of compounds avoiding contact with the seawater. Microencapsulated feeds, indeed, are commonly added to dry feeds and they can be stored in refrigerators maintaining their stable shapes. When they are added to humid feeds, or directly dispersed in the water (in the case of filter feeders or very small organisms), the lipidic capsules may slowly degrade and this may represent an issue, if experiments are supposed to last more than a few days.

For this reason, marine polysaccharides such as alginates and carragenates may be adopted to produce particles of variable size, able to preserve and deliver active compounds [20]. Marine polysaccharides are a large and quite complex group of macromolecules sharing some interesting biological properties. The field of marine polysaccharides is constantly evolving over the last decades, thanks to a wide variety of compounds extracted from marine organisms that are tailored for these applications. Red macroalgae are mostly used for the extraction of polysaccharides, but alternative sources may be green and brown seaweeds, as well as marine prokaryotes. The use of seaweed-derived alginates embodies an additional advantage when feeds are prepared for grazers, because their chemical properties are normally suitable for the physiology of various consumers. Moreover, these compounds are widely recognized for their taste and toughness, besides their well-known non-cytotoxic characteristics, biodegradability and biocompatibility. For example, algae-produced hydrogels, based on cross-linked polysaccharides, are often employed for drug delivery systems and tissue engineering. Polysaccharides derived from marine organisms may also be employed to produce superabsorbent/superporous hydrogels [20].

Marine polysaccharides are increasingly used for nutraceutical and cosmaceutical applications, particularly as tools for the incorporation of bioactive agents [21]. It is worth noting that sodium alginate, in combination with polyacrylics such as either poly(acrylic acid), poly(acrylamide) or both, forms interpenetrating networks that give rise to superabsorbent and superporous hydrogels [22], occasionally adopted to direct active compounds towards specific animal targets [23]. Alginate dressings maintain a physiologically moist microenvironment that is feasible for containing a variety of active compounds. Alginate is a naturally occurring polysaccharide of organic acids (guluronic and mannuronic), abundant in nature as a structural component of brown algae, but it may also be obtained from soil bacteria. Although the microencapsulation technique was initially developed for oral delivery of proteins and other compounds, which are quickly degraded in the acid gastric environment, this technique was demonstrated to produce interesting results

for bioassay-guided isolation of natural compounds [24]. In addition, sodium alginates may be modified with amine or acid moieties to optimize their efficiency for drug delivery applications. These modifications permit modulation of the erosion time, the release rates, and even their adhesion to specific substrates [21]. For example, using super-hydrophobic surfaces, polymer particles may be produced [25] allowing the loading of compounds into spherical structures (microspheres) with huge encapsulation efficiency [26]. Several benthic diatoms are commonly ingested by sea urchins, because they produce dense epiphytic layers on seagrasses and seaweeds, commonly grazed by these echinoderms. However, artificial feeds (e.g., agar blocks including specific diatoms) are needed to test the effect of individual microalgae on the reproductive physiology of sea urchins. Algae must be included when still fresh, to avoid rapid deterioration in the aqueous environment, prior to be ingested. Conserved algae (e.g., frozen diatoms) cannot be used for this purpose, because the loss of structural properties of their siliceous shells would immediately produce leaking of active compounds. This limitation imposes complex research procedures and various experimental limits.

Various diatoms contribute to toxic plankton blooms, including *Thalassiosira* spp., *Nitzschia* spp. and *Striatella* spp. [27] and their effects on planktonic consumers were extensively investigated [1]. Several harmful microalgae, known to produce biotoxins and cause fish and invertebrate deaths, were identified and documented. However, similar diatom genera are present in the periphytic benthos too [28], although their effects on benthic consumers are scarcely investigated. In particular, *S. unipunctata* is a benthic diatom often found in the gut content of various grazers and filter feeders [29] and its toxigenic properties are still to be investigated. This species appears in the list of most abundant taxa during harmful micro-phytoplankton blooms [30] but its direct effects on filter-feeders and grazers were never documented.

Previous investigations indicated that live marine benthic diatoms, *Nanofrustulum shiloi*, included in agar blocks produce clear physiological effects in the sea urchin, *Paracentrotus lividus* [8]. However, due to the several issues mentioned above, investigations may exclusively progress by using diets based on either frozen diatoms, diatom fractions, or both. Accordingly, this investigation attempted the administration of frozen benthic diatoms, *N. shiloi*, whose physiological effects were already demonstrated by simple inclusion of live diatoms, as a control for the inclusion in alginate hydrogel beads, never attempted before. In addition, *Striatella unipunctata* was tested in alginate beads because its physiological effects on sea urchins were never investigated. Consequently, we compared the known activity of *N. shiloi*, here considered as a positive control, to the effects of *S. unipunctata* and the seaweed *Ulva rigida*, adopted as control diet. Both diatoms may be seasonally abundant on the leaf surface of the seagrass, *Posidonia oceanica* [31–34], which represents a preferred food item for *P. lividus*. The toxigenic effects of encapsulated diatoms on sea urchin progenies were evaluated by morphological observations of plutei and gene expression analyses.

2. Results

2.1. Species Identification by Morphological and Molecular Analyses

The observation under SEM and optical microscopy revealed that the benthic diatom tested in the present study belonged to the araphid pennate species, *S. unipunctata* Agardh 1832 (Figure S1), whose first description was reported as *Fragilaria unipunctata* Lyngbye 1819. The diatom is about 70 µm in length, flat and rectangular in shape and with truncated corners. *S. unipunctata* was easily recognizable through its valve ornamentation that consists of septate girdle bands covering all the surfaces of frustules. Under light microscopy, it was possible to observe the typical reticulate chloroplasts and the mucilaginous stalk, which is secreted through a corner of the frustule allowing for attachment to the bottom (Figure S1).

Molecular data totally agreed with the morphological characterization obtained by spicule observations. BLASTn alignments revealed about 99% pairwise-sequence similarity

to four strains of *S. unipunctata* ribosomal 18S RNA gene (accession numbers: JX419383.1, AB430609.1, AF525666.1 and HQ912643.1) with 100% query cover (Figure S2).

2.2. Diatom's Encapsulation

As reported in Table S1, the alginate beads without diatoms showed a mean diameter of about 3.8 mm, after preparation. The encapsulation of diatoms led to a very slight reduction of the mean diameter of ~3.7 mm. As expected, dehydration strongly reduced the size of the beads resulting in 1.3 mm and 1.2 mm for alginate beads and diatoms/alginate beads, respectively.

2.3. Effects of Feeding Tests on Sea Urchin Progeny

2.3.1. Fertilization/Cleavage Rates and Embryo Development

After a one month of feeding with *U. rigida*, *N. shiloi* and *S. unipunctata*, eggs and sperms were collected prior to fertilization. The percentage of fertilized eggs and embryos at the two-cell stage resulted in 100% for all samples under analysis. These data were similar to those obtained for sea urchins collected from the wild and spooned at the beginning of the experiment (T_0). The morphological observations performed on sea urchin embryos 48 h post-fertilization (hpf) revealed that both diatoms induced several malformations affecting the apex and the arms [35] (Figure 1 and Figure S3). Firstly, *N. shiloi* exerted similar effects to those previously reported in Ruocco et al. [8], with a percentage of abnormal plutei of about 55% ($p > 0.05$) (Figure 1).

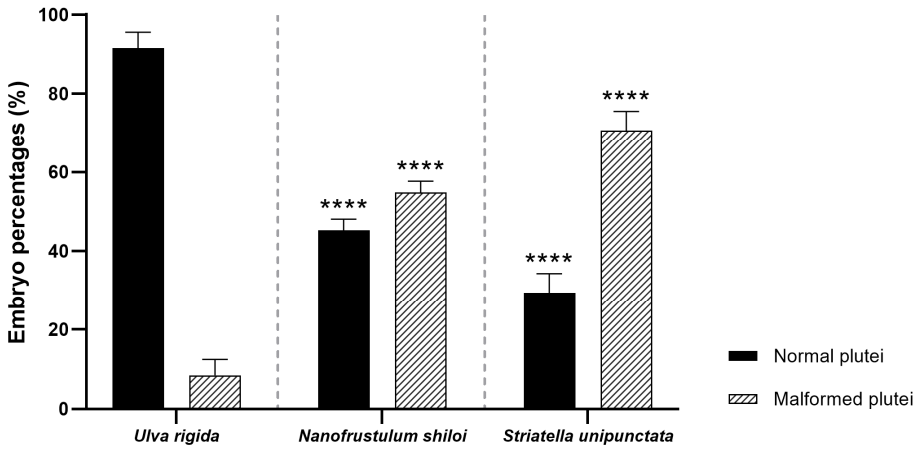

Figure 1. Percentages of normal and malformed plutei derived from sea urchins fed with the control diet (*U. rigida*), *N. shiloi* and *S. unipunctata* encapsulated in alginate beads. One-way ANOVA followed by Tukey post hoc for multiple comparisons: **** $p < 0.0001$. Pairwise comparison was reported between control (*U. rigida* diets) vs. samples treated with *N. shiloi* and *S. unipunctata*.

This result confirmed that diatom encapsulation, within the alginate beads, did not cause additional effects on embryonic development. Furthermore, *S. unipunctata* induced a significant increase in malformed plutei (70%), being statistically different from the percentages obtained in sea urchins fed on *U. rigida* (about 10%, $p < 0.0001$) (Figure 1). Interestingly, the toxicity of *S. unipunctata* diets on sea urchin progenies was found significantly higher than *N. shiloi* treated individuals ($p < 0.0001$) (Figure 1).

2.3.2. Gene Expression by Real Time (RT)-qPCR

Among the eighteen genes belonging to stress response, *N. shiloi* altered the expression of several genes, except for *Caspase 8*, *ERCC3*, *GRHPR* e *p38 MAPK* (Figure 2). On the contrary, *S. unipunctata* confirmed to induce the strongest effects by changing the expression levels of all stress genes under analysis (Figure 2). On the whole, both diatoms had a lot of common targets, down-regulating *ARF1*, *caspase 3/7*, *HIF1A*, *hsp56*, *Mtase*, *p53* and *SDH*, and up-regulating *cytb*, *GS*, *hsp60*, *hsp70*, *NF-κB*, *PARP1* and *14-3-3 ε*. Moreover, *S. unipunctata*

solely induced a variation of four stress genes with a decrease of *Caspase 8*, *ERCC3*, *GRHPR* and an increase of *p38 MAPK* (Figure 2).

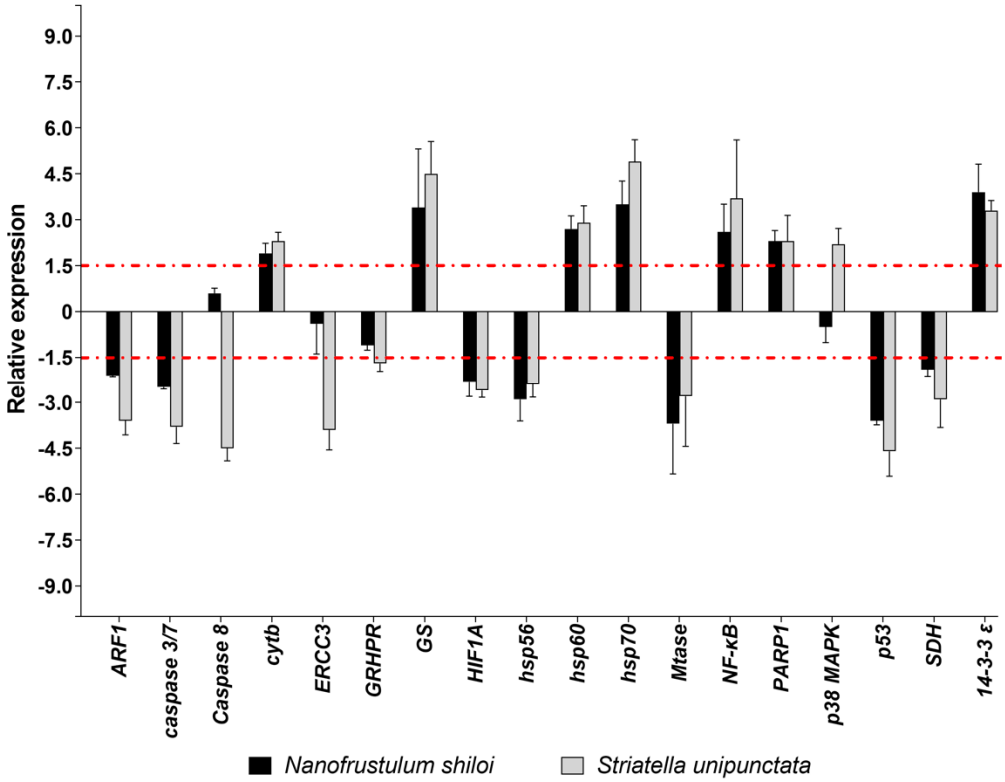

Figure 2. Fold changes of stress response genes in plutei from sea urchin adults fed with *N. shiloi* (black bar) and *S. unipunctata* (gray bar) encapsulated in alginate beads. The dotted red line represents the cut-off (1.5). Values are reported as the average fold changes ± SD (n = 3). Statistical differences were evaluated by nonparametric Mann–Whitney test. p-values < 0.05 were considered significant.

The functional class grouping development and differentiation processes was the most represented, with 28 genes analyzed. In particular, *N. shiloi* and *S. unipunctata* induced the up-regulation of *Bra*, *Delta*, *FoxG*, *Foxo* and *Wnt6*, and the down-regulation of *δ-2-catenin*, *GFI1*, *Goosecoid*, *H3.3*, *KIF19* and *TCF7* (Figure 3a).

In contrast, different results were detected in the cases of *FOXA*, *TAK1* and *VEGF* genes, whose expression increased with *S. unipunctata* and decreased with *N. shiloi*; and *nodal* and *OneCut/Hnf6*, revealing the opposite effect (Figure 3b). Moreover, benthic diatoms individually switched on the expression of *Blimp* and *Notch* (*N. shiloi*), and *ADMP2* (*S. unipunctata*) (Figure 3b).

Concerning the eight genes involved in the skeletogenesis of sea urchin plutei, *N. shiloi* and *S. unipunctata* had five common targets. Specifically, both diatoms up-regulated Nec and p19 and down-regulated *BMP5-7*, *SM30* and *uni* (Figure 4).

Different molecular effects were detected for *Jun*, since both the up- (*N. shiloi*) and down-regulation (*S. unipunctata*) was found (Figure 4). *S. unipunctata* also altered the expression of *p16* (up-regulation) and *SM50* (down-regulation) genes (Figure 4).

N. shiloi was able to change the expression of all genes belonging to detoxification processes, while half of them were significantly altered by *S. unipunctata*. Benthic diatoms shared the up-regulation of three genes, namely, *CAT*, *MDR1* and *MT5* (Figure 5).

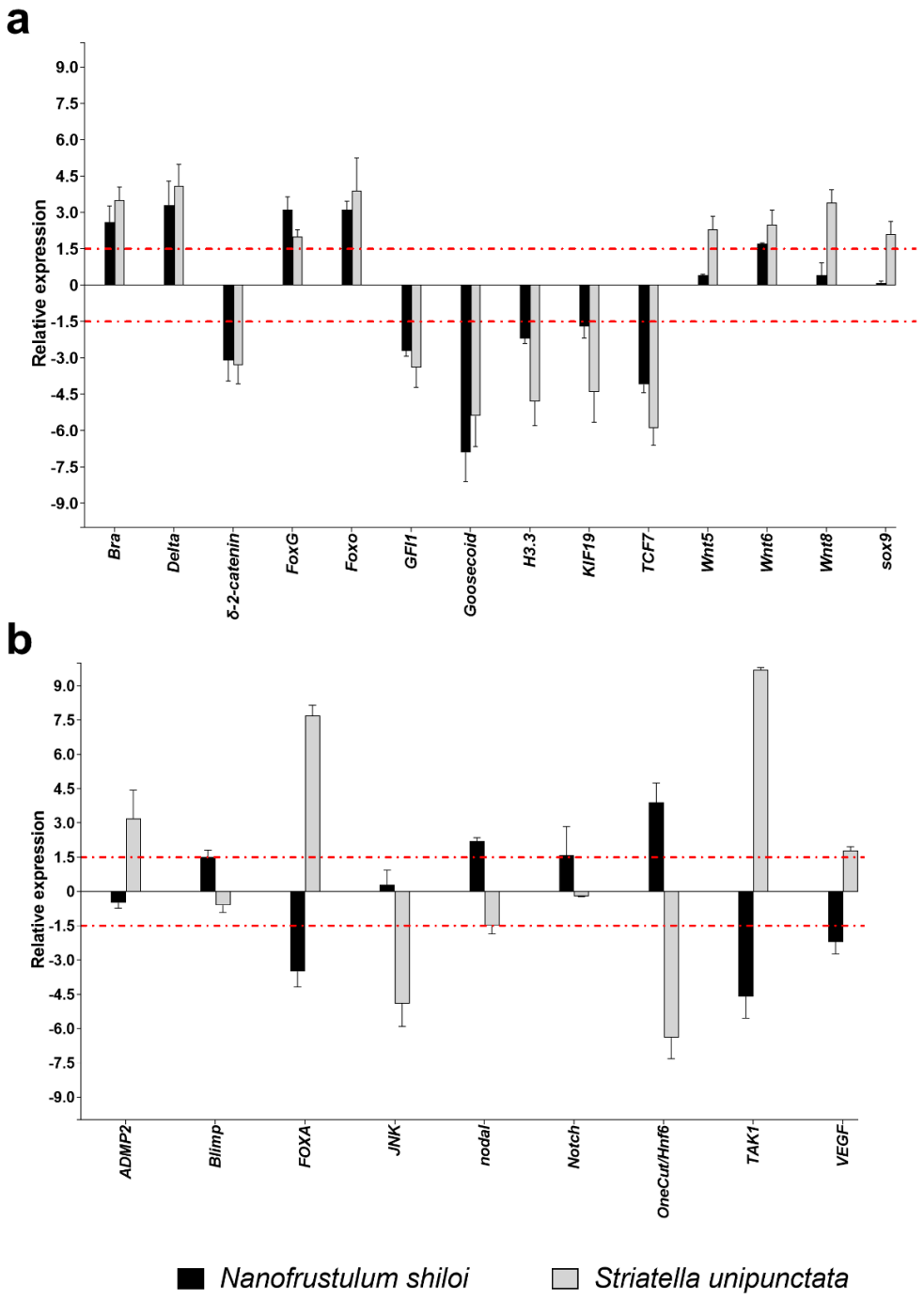

Figure 3. Fold changes of development and differentiation genes in plutei from sea urchin adults fed with *N. shiloi* (black bar) and *S. unipunctata* (gray bar) encapsulated in alginate beads where (**a**) the two diatoms induced the same gene expression (up- or down-regulation for both diatoms) or (**b**) different gene expression (up- in one diatom and down-regulation in the other diatom). The dotted red line represents the cut-off (1.5). Values are reported as the average fold changes ± SD (n = 3). Statistical differences were evaluated by nonparametric Mann–Whitney test. p-values < 0.05 were considered significant.

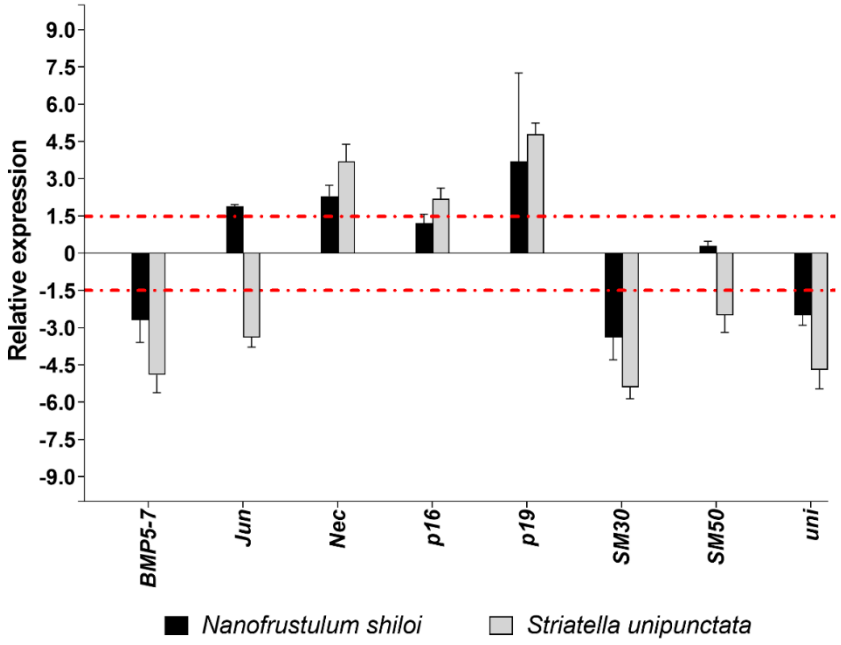

Figure 4. Fold changes of skeletogenesis genes in plutei from sea urchin adults fed with *N. shiloi* (black bar) and *S. unipunctata* (gray bar) encapsulated in alginate beads. The dotted red line represents the cut-off (1.5). Values are reported as the average fold changes ± SD (n = 3). Statistical differences were evaluated by nonparametric Mann–Whitney test. p-values < 0.05 were considered significant.

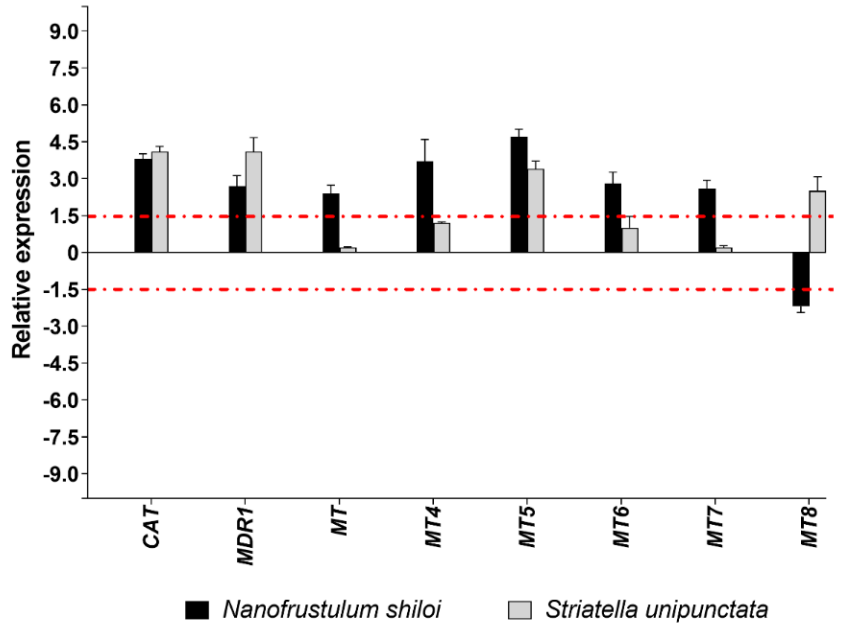

Figure 5. Fold changes of detoxification genes in plutei from sea urchin adults fed with *N. shiloi* (black bar) and *S. unipunctata* (gray bar) encapsulated in alginate beads. The dotted red line represents the cut-off (1.5). Values are reported as the average fold changes ± SD (n = 3). Statistical differences were evaluated by nonparametric Mann–Whitney test. p-values < 0.05 were considered significant.

Furthermore, the *MT8* gene was up-regulated by *S. unipunctata* and down-regulated by *N. shiloi* (Figure 5). Interestingly, *N. shiloi* was able to up-regulate all methallothioneins under study (*MT*, *MT4*, *MT5*, *MT6* and *MT7*), except for the *MT8* gene (Figure 5).

3. Discussion

In the present work we evaluated the harmful effects of diatom diets on the sea urchin P. lividus by encapsulating two diatom species, *N. shiloi* and *S. unipunctata*, in alginate hydrogel beads, combining morphological and molecular approaches.

3.1. Effects of Feeding Tests by Morphological Observations

Our results showed that *N. shiloi* induced several malformations in sea urchin progenies with a percentage of about 60% of aberrant plutei ($p < 0.0001$), confirming our previous investigations [8] (Figure 1; see also Figure S3). Moreover, *S. unipunctata* was also toxic to sea urchins, inducing stronger effects (75% malformed plutei) than *N. shiloi*, with a high statistical significance ($p < 0.0001$; Figure 1). Interestingly, *N. shiloi* was previously found to produce several toxic compounds, named oxylipins, that together with other unknown compounds, could induce such negative effects [36]. The accumulation of these bioactive secondary metabolites could be responsible for reducing gamete quality, as well as interfering with the fertilization and embryonic development processes, as demonstrated by Ruocco et al. [36] through Pearson correlation analysis. For this reason, we encapsulated diatoms in alginate beads to preserve the active molecules and avoid any dispersion into the surrounding water. In fact, the encapsulation of extremely sensitive compounds, such as proteins, vitamins and dehydrated extracts, within alginate hydrogel beads protect them from the external environment [37,38]. Furthermore, calcium-alginate beads were used in this work since they represent a biocompatible and non-toxic delivery system, which is very easy to prepare through cost-effective procedures without applying high temperatures [39,40].

3.2. Effects of Feeding Tests on Gene Pathways

Concerning the molecular investigation of 62 genes belonging to stress response, development and differentiation, skeletogenesis and detoxification processes, benthic diatoms were able to switch on almost all genes under analysis (Figures 2–5; Table S2). These molecular results corroborated our observation under light microscopy showing that feeding on benthic diatoms induced several malformations in sea urchin plutei (Figure 1).

Among the 62 genes analyzed, more than half were positively or negatively altered by both diatoms, revealing a similar variation in gene expression and a huge number of shared targets (Figures 2–5). On the other hand, a few genes were impaired differently, particularly those involved in development and differentiation events, such as *FOXA*, *nodal*, *OneCut/Hnf6*, *TAK1* and *VEGF* (Figure 3b). These results could indicate that benthic diatoms affect some common molecular pathways by changing the normal biological mechanisms, which, in turn, generate aberrant progenies in sea urchins.

Interestingly, several genes followed by RT-qPCR in the present study were previously found to be functionally interconnected [41–43]. In particular, Varrella et al. [41] showed that both diatoms altered the expression of all genes belonging to the network, except for *Alix*, whose relative expression was not significant (Figure 6).

The majority of these correlated genes were similarly affected by both diatoms with the only exception of Blimp, which was up-regulated in *N. shiloi* (Figure 6a) and *sox9/p38 MAPK*, whose expression was up-regulated in *S. unipunctata* (Figure 6b).

On the whole, a similar stress response pathway, mediated by *hsp60*, *hsp70*, *NF-κB*, *14-3-3 ε* and *MDR1*, was activated since this group of interconnected genes was significantly up-regulated after the feeding with both benthic diatoms. Some of these genes were previously proposed to be involved in the same molecular pathway in response to UV-B radiation and, recently, after the exposure of polystyrene nanoparticles [44–46]. Moreover, after analyzing gene expression data, we found that both diatoms induced the up-regulation of *NF-κB* signaling, which represent a fundamental cascade for the activation of the innate immune system following several stress events that causes DNA damage [47].

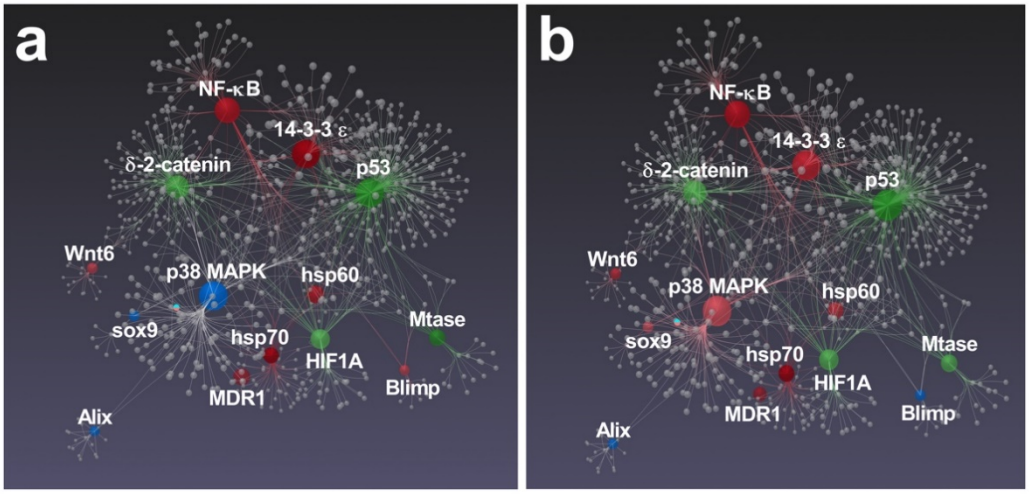

Figure 6. Gene network performed by STRING interactome of protein–protein interactions. Correlations with high confidence score cut-off (900) were reported. Among functionally correlated genes, those with up (red), down (green) and unchanged (blue) expression affected by *N. shiloi* (**a**) and *S. unipunctata* (**b**) were reported. Color shading depends on fold-change values. Gray spheres represent additional connections.

Concerning the gene network proposed by Ruocco et al. [42], in which mostly development and skeletogenesis genes were involved, interesting differences were brought to light (Figure 7).

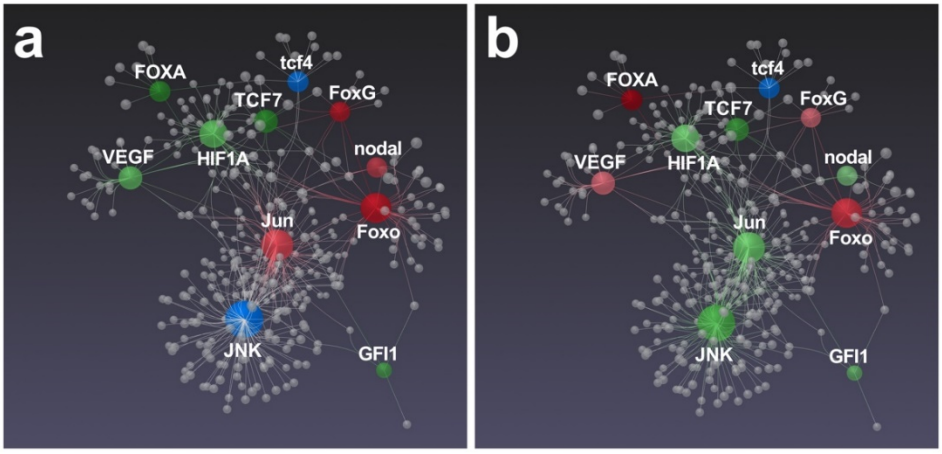

Figure 7. Gene network performed by STRING interactome of protein–protein interactions. Correlations with high confidence score cut-off (900) were reported. Among functionally correlated genes, those with up (red), down (green) and unchanged (blue) expression affected by *N. shiloi* (**a**) and *S. unipunctata* (**b**) were reported. Color shading depends on fold change values. Gray spheres represent additional connections.

In fact, a key transcription factor that controls skeletogenesis in sea urchins (*Jun*) [48] was significantly up-regulated by *N. shiloi* (Figure 7a). The connected *Foxo*, *FoxG* and *nodal* genes were also up-regulated, suggesting that a molecular pathway probably mediated through these genes could be activated by this diatom (Figure 7a). On the contrary, *S. unipunctata* induced the down-expression of *Jun* and *JNK* (Figure 7b) that, in turn, could trigger the aberrations in the sea urchin embryos, since both genes were reported to exert key roles in skeletogenesis and development [48,49]. In addition, *S. unipunctata* was able to increase the relative expression of the *VEGF* gene (Figure 7b), which is demonstrated to be involved in sea urchin spiculogenesis [50]. However, recent data on sea urchin gene regulatory networks (GRN) reported that hypoxia-induced stress is able to perturb the

expression of *HIF1A*, *nodal* and *VEGF* pathways, inducing severe effects on the structure of larval skeletons [51]. This is also the case with our results, reporting a significant variation of these latter genes following diatom feeding, that might explain the anomalies in sea urchin plutei observed under light microscopy (Figure 1; see also Figure S3). In fact, we found a significant down-regulation of *HIF1A*, which is known to limit the expression of *nodal* to the ventral side of sea urchin embryos for the activation of down-stream transcription factors [52]. The dysregulation of *HIF1A* and *nodal* genes could be responsible for such malformations (Figure S3).

Significant differences between *N. shiloi* and *S. unipunctata* were also found in the gene correlation analysis performed by Esposito et al. [43], since only *S. unipunctata* was able to target all connected genes, with the exception of only *Smad6* (Figure 8b).

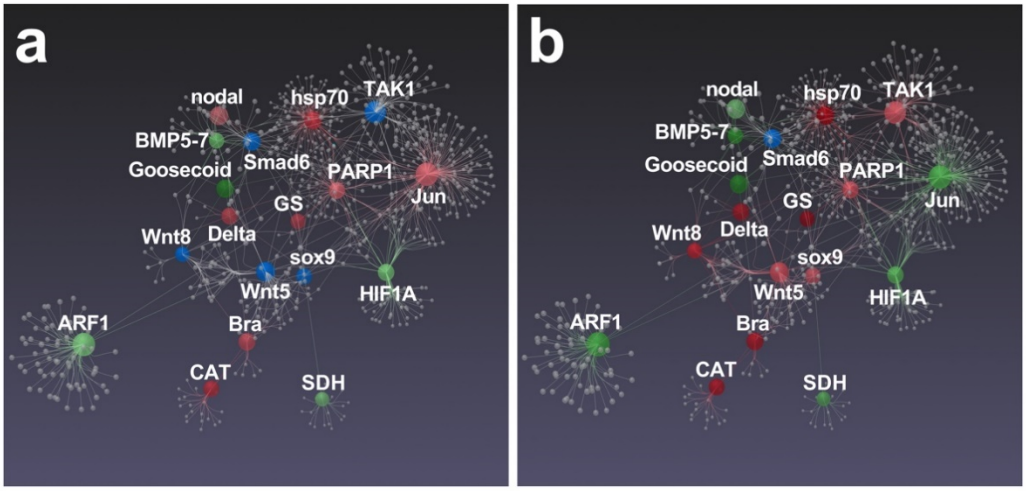

Figure 8. Gene network performed by STRING interactome of protein–protein interactions. Correlations with high confidence score cut-off (900) were reported. Among functionally correlated genes, those with up (red), down (green) and unchanged (blue) expression affected by *N. shiloi* (**a**) and *S. unipunctata* (**b**) were reported. Full color shows greater fold change values. Gray spheres represent additional connections.

Again, we found that *Jun*, strongly connected with several genes [48], was specifically up-regulated by *N. shiloi*. This gene was probably at the basis of huge molecular cascade activated by this diatom (Figure 8a). Within this pathway, we also noticed the involvement of *hsp70*, *PARP1*, *GS*, *Delta*, *nodal*, *Bra* and *CAT* genes, since mRNA levels significantly increased in sea urchin plutei deriving from adults fed with *N. shiloi* (Figure 8a). Then, *S. unipunctata* was able to up-regulate a great number of genes that displayed a connection with several genes in the same biological pathway, such as *TAK1*, *hsp70*, *PARP1*, *GS*, *sox9*, *Wnt5*, *Wnt8*, *Delta*, *Bra* and *CAT* (Figure 8b).

More specifically, PARP1 level increased in sea urchin plutei, deriving from adults fed on both benthic diatoms (Figure 8).

The up-regulation of genes involved in DNA repair may be due to a potential genotoxic effect triggered by unknown or known [36] (e.g., oxylipins) compounds that were supplied through diatom diets [53]. Moreover, a significant activation of the *CAT* gene was observed (Figure 8), probably induced by the accumulation of reactive oxygen species (ROS) in sea urchin eggs. The expression alteration of this gene might be connected to the clear aberrations of sea urchin embryos (Figure 1; see also Figure S3), since the mitochondrial redox signaling via H_2O_2 is well known to perturb *nodal* expression and the following oral-aboral axis specification [54].

Analyzing RT-qPCR data, we detected the alteration of *nodal*, *Delta*, *Bra* and *Goosecoid*, which are normally required for the early specification of ectodermal tissues [55]. Interestingly, *nodal* was found to influence the expression of BMP signaling and some dorsal marker genes, such as *Wnt5*, *Wnt8*, *smad6*, together with *sox9*, which is a transcription factor

involved in left-right asymmetry specification [55–57]. These experimental evidences were also corroborated by our results, since benthic diatoms induced the opposite regulation of *nodal* gene (up-regulated in *N. shiloi* and down-regulated in *S. unipunctata*) and, in turn, a different expression pattern in down-stream effectors (Figure 8).

Overall, the majority of genes, whose expression was modified by diatom feeding, are involved in the specification of ectodermal, endodermal and mesodermal cell fates during sea urchin embryo development. Despite several connections still being largely unknown due to the high complexity of molecular responses, some of them were experimentally demonstrated to join the same GRN [55–63].

4. Materials and Methods

4.1. Ethics Statement

Paracentrotus lividus (Lamarck) adults were collected from a site in the Bay of Naples that is not privately owned or protected in any way, according to Italian legislation (DPR 1639/68, 19 September 1980, confirmed on 10 January 2000). Field studies did not include endangered or protected species. All experimental procedures on animals followed the guidelines of the European Union (directive 2010/63/EU).

4.2. Isolation of S. unipunctata and Culturing

S. unipunctata was isolated from samples of *P. oceanica* leaves collected in the field from the meadows located in Lacco Ameno (Island of Ischia, Gulf of Naples, Italy). Once in the laboratory, the lamina of each leaf was rinsed with filtered seawater (FSW) and then gently scraped with a glass slide in order to collect the epiphytic communities. The diatoms of interest were isolated under inverted microscope (Leica Microsystems), through sequential transfer of single cells, by means of a micromanipulator (Leica Microsystems). The isolated diatom was deployed in multi-well plates filled with sterile seawater in order to obtain axenic cultures. The monoclonal strains thus obtained were gently renovated under a laminar flow hood and cultured in 12-multiwell plates with Guillard's f/2 medium (Sigma-Aldrich, Milan, Italy) and kept in a thermostatic chamber at 18 °C, with a 12 h:12 h light:dark photoperiod. Light was provided by Silvania GroLux (Osram Sylvania Inc., Wilmington, Massachusetts, USA) at 140 $\mu E \cdot m^{-2} \cdot s^{-1}$ irradiance.

4.3. Species Characterization

4.3.1. Morphological Analysis of Frustules

Diatom identification was firstly performed using a morphological approach, through the analysis of the frustule ultrastructure on images captured through a scanning electron microscopy (SEM, JEOL 6700F, JEOL Ltd., Akishima, Tokyo, Japan). Diluted monoclonal cultures were collected in graduated Pyrex glass tubes and cleaned through acid treatment by adding H_2SO_4 (96%) and HNO_3 (65%). After six washing cycles with distilled water, the pH was evaluated using litmus paper. Once neutral pH was reached, the cleaned samples were mounted on stubs and sputter-coated with platinum for SEM observations.

4.3.2. Molecular Identification of Diatom Species

Cell cultures were collected from the multi-well plates and concentrated by centrifugation for 20 min at 1800 relative centrifugal force (rcf) at 4 °C for 15 min, then frozen in liquid nitrogen until use. About 20 mg of pellet was treated with lysis buffer containing 2% cetyltrimethylammonium bromide (CTAB) and 2-mercaptoethanol (2-ME, Sigma-Aldrich). Then, DNA extraction was performed according the protocol reported in Ruocco et al. [8].

The total amount of DNA extracted was estimated by measuring the absorbance at 260 nm; purity was calculated using 260/280 and 260/230 nm ratios, using a NanoDrop spectrophotometer (ND-1000 UV-vis Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). The integrity of DNA was evaluated by agarose gel electrophoresis.

Polymerase chain reactions (PCRs) were performed using specific primers targeting the 18S rRNA region (528F/1055R [8,64]). Sequences were submitted to the NCBI (National

Center for Biotechnology Information) database through Basic Local Alignment Search Tool (BLASTn available at https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 1 February 2021 [65]) in order to identify the best hits with higher percentage of identity. In addition, PCR fragments were aligned to all 18S sequences found using the software MultiAlin (available at http://multalin.toulouse.inra.fr/multalin/, accessed on 1 February 2021 [66]).

4.4. Diatom Encapsulation in Alginate Beads

Monoclonal cultures of *N. shiloi* and *S. unipunctata* were grown as reported above. For experimental purposes, massive cultures were inoculated in 14 cm Petri dishes containing 100 mL of f/2 medium. At the end of the exponential phase, cells were counted under a Neubauer chamber and biomass was evaluated as logC (quantity of intracellular carbon in picograms) = $-0.541 + 0.811 \times \log V$ (cell volume in μm^3) [8,67]. The same biomass previously used for the diatom *N. shiloi* in Ruocco et al. [8] was then collected and lyophilized.

Dried diatoms were then encapsulated in alginate hydrogel beads [38–40]. Alginate beads were prepared as described by Zhang et al. [37], with some modifications. Briefly, a 2% alginate solution (w/v) was prepared by dissolving sodium alginate powder in deionized water under magnetic stirring for about 3 h. Then, freeze-dried diatoms were dispersed in the sodium alginate solution at the predetermined concentration. Contemporarily, a calcium chloride solution (5% w/v) was prepared by melting calcium chloride powder in double distilled water under magnetic stirring at room temperature. Diatoms/alginate solution was then poured, drop by drop, into the corresponding 5% calcium chloride solution under continuous and gently stirring. The resulting alginate beads were then collected, washed with deionized water and dried for 48 h at room temperature. Dry beads were stored at 4 °C before use.

The diameter of alginate beads was measured after preparation, in the hydrated form, and immediately after dehydration, to evaluate whether diatoms were perfectly encapsulated. Measurements were carried out by analysis of digital images of alginate beads, using open-source software, Image J (Java 1.8.0_112). The mean diameters were calculated as the average values over three different measurements ($n = 3$).

4.5. Feeding, Gametes Collection, Evaluation of Fertilization/Cleavage Success and Detection of Abnormal Plutei

Alginate beads were included into a 2% agar substrate and used as food for sea urchins with a daily rate of 1 g of agar per sea urchin [8,68]. Twenty adult (12 females and 8 males) *P. lividus* were reared in each experimental tank with a continuous flow-through system [8] and fed with *U. rigida* (3 control replicates) and the 2 benthic diatoms tested (3 replicates for each species). After 1 month of feeding, eggs and sperm were collected. Eggs were washed three times with filtered seawater (FSW) and kept in FSW until use. Concentrated "dry" sperm was collected and kept undiluted at +4 °C until use. Eggs were fertilized utilizing sperm-to-egg ratios of 100:1. Fertilized eggs were kept at 20 °C in a thermostatic chamber on a 12 h:12 h light:dark cycle. After 48 hpf, morphological malformations were determined for at least 100 sea urchin plutei from each female (fixed in 0.5% glutaraldehyde) using a light microscope (Zeiss Axiovert 135TV, Carl Zeiss, Jena, Germany).

4.6. Molecular Analysis on Sea Urchin Plutei

About 5000 eggs (in 50 mL of FSW) from each female fed on *U. rigida* and the two benthic diatoms were collected and fertilized. Embryos were then collected at 48 hpf by centrifugation at 1800 rcf for 10 min in a swing out rotor at 4 °C. Embryos were placed in at least 10 volumes of the RNAlater (Qiagen, Hilden, Germany), and then frozen in liquid nitrogen. Samples were kept at −80 °C until use.

Total RNA was extracted using Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions for RNA-seq experiments. For each sample, 600 ng of total RNA was retrotranscribed with an iScript cDNA synthesis kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions.

Gene expression analysis was performed on three biological replicates. The levels of each gene were followed by real time-qPCR. The relative expression ratios were calculated from quantification cycles (Cq) through an efficiency (E) corrected calculation method ($E_{target}^{\Delta Cq\ target\ (Mean\ Control–Mean\ Sample)}/E_{reference}^{\Delta Cq\ reference\ (Mean\ Control–Mean\ Sample)}$) [69,70], using REST software (Version No., Relative Expression Software Tool, Weihenstephan, Germany). *Ubiquitin* [71] and *18S rRNA* [46,72] were selected as reference genes, since no variation was assessed during the embryonic development of the sea urchin and between control and treated samples. Values larger than 1.5-fold were considered significant.

To elaborate the 3 gene networks previously published in literature [41–43], an interactomic analysis was performed by NetworkAnalyst 3.0 software [73] available at https://www.networkanalyst.ca/ (accessed on 1 February 2021), using STRING interactome of protein–protein interactions [74]. Human orthologs of selected genes were used to compute the network analysis. The most significant relations among genes (confidence score cut-off = 900) displaying experimental evidence were highlighted.

4.7. Statistical Analysis

Data-sets were analyzed by D'agostino and Pearson normality test to ensure that values were normally distributed. Statistical differences of normal and malformed embryos among testing groups were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons (n = 15). Regarding RT-qPCR data, a nonparametric Mann–Whitney test was applied to ΔCq (Cq gene of interest—Cq reference) values between treated and control samples (n = 3).

p-Values larger than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism Software (version 9.00 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com, accessed on 1 February 2021).

5. Conclusions

In the present work, we demonstrated that microencapsulation of diatoms for feeding purposes could be an efficient experimental system to vehicle toxic sensitive compounds, since we observed a clear negative effect on sea urchin progenies. The two benthic diatoms tested in the present work (*N. shiloi* and *S. unipunctata*), induced similar malformations, which were confirmed by the alteration of several genes involved in stress response, development, skeletogenesis and detoxification processes. The two diatoms shared several molecular targets thus supporting the hypothesis that they could probably activate the same molecular pathways. However, among those genes, previously found to be functionally interconnected [41–43], slight expression differences were observed. This result suggests that benthic diatoms under analysis did not necessarily trigger the same biological cascade, particularly in skeletogenesis and development processes.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/md19040230/s1, Figure S1: Images of *S. unipunctata* under the optical microscope (a) and SEM (b,c). The diatom shows a big chloroplast in the middle of the cell (a), a frustule with rectangular shape and truncated corners (b), a mucilaginous stalk (b) and characteristic ornamental striae on the surface of each valve (c). Scale bars = 15 µm (a), 10 µm (b) and 1 µm (c). Figure S2: Alignment of *S. unipunctata* to the four annotated 18S rRNA sequences found in the BLASTn search with accession numbers JX419383.1 (a), AB430609.1 (b), AF525666.1 (c) and HQ912643.1 (d). Forward and reverse primers were highlighted by a green and sky-blue rectangle, respectively; Figure S3: Images of sea urchin embryos under optical microscope. Normal (a) and malformed (b,c) plutei were reported (Zeiss Axiovert135TV microscope, 10×/0.30 magnification/numerical aperture). Scale bar: 50 µm; Table S1: Diameter in millimeters (mm) of alginate beads before and after dehydration; Table S2: Fold change values of 62 genes belonging to stress response, development and differentiation, skeletogenesis and detoxification processes analyzed by RT-qPCR. Up-regulated genes and down-regulated genes were highlighted in red and blue, respectively.

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Chapter 6

Emerging molecular approaches as an alternative to traditional bioassays

Introduction

Several species are widely used in scientific research as model organisms to understand biological processes (Murthy & Ram, 2015). Most of these organisms are widely used for several research areas such as ecotoxicology, molecular biology, and biotechnological applications (Brenner, 2003) and, for this reason, numerous tools and detailed protocols are already available in the literature. However, in some cases there is a constrain closely related to the model organism. In fact, despite their eventually complexity, not easy maintenance in a laboratory and the absence of standardized available protocols, certain species are used as models because they are the only targets for specific compounds or for particular pathways (Howe et al., 2013). This is the case of *Hippolyte inermis* (Leach), a marine caridean protandric shrimp living on the seagrass *Posidonia oceanica* (L. Delile) meadows. Decapod crustaceans are often used as models in the study of sex differentiation, which is the process that leads to the development of either males or females. This is because of the unique functions of their androgenic gland, which produces an insulin-like hormone called the androgenic hormone which acts as a switch to control sexual development ((Grilo & Rosa, 2017; Levy & Sagi, 2020; Short et al., 2014). But even if sex differentiation is well studied in crustaceans, *H. inermis* in particular undergoes to a singular process of food determining sex reversal (Reverberi, 2009; Zupo et al., 2008) in which has been proposed that an algal compound can trigger the early development of the female phenotype as a result of a coevolutionary process with a specific diatom; *Cocconeis* spp. (Zupo, 1994). So, this protandric shrimp is characterised by two reproductive cycles, one in spring and one in autumn but, at the

same time, it has developed a unique reproductive strategy that produces both immature males and females in the spring but only males in the fall, which eventually change their sex after a year (Zupo, 1994). According to Zupo (2000) it has been proposed that the early sexual shift from young male to female during the spring is related to a diet based on the *Cocconeis* spp. diatom (Zupo, 2000) that is abundant in their habitat in that period (Zupo, 1994, 2000). It has been suggested, in details, that this shift is consequence of an apoptogenic activity that act selectively on *H. inermis* androgenic gland and that is triggered by a specific compound present in this diatom (Zupo & Messina, 2007). This apoptogenic compound is actually under investigation, through bioassay-guided fractionation, to identify its molecular structure. Considering this strong correlation between this early sexual shift in this shrimp and the presence of *Cocconeis* apoptogenic compound, fractions obtained via various HPLC separations must be tested on *H. inermis* post-larvae in order to identify its presence (Nappo et al., 2012).

Unfortunately, the traditional bioassay consists in a long complex culture practice (Zupo, 2001) which needs heavy operational work. In fact, after around fifty culture days, the sex of the animals fed with different fractions is evaluated to detect the presence of the active compound. To obtain these data, it is necessary to sacrifice post-larvae of the proper size to discern the presence of masculine appendix on the second pleopods, by observation under the dissecting microscope (Levy et al., 2020; Mutalipassi et al., 2018; Zupo et al., 2008). To date, no molecular tools are available for *H. inermis*, with the only exception of the data reported in Levy et al. (2021), where eye and whole body RNA libraries of four representative stages during its protandric life cycle (immature, male, young female and mature female) was constructed. In details, the body libraries contained transcripts mainly related to the reproductive system, while the eye libraries contained transcripts related to the X-organ-sinus gland, which represents a central endocrine

complex that regulates crustacean reproduction. This is probably due to the fact that this shrimp is not a model organism commonly used. For these reasons, this work proposed a molecular approach, based on the identification of key genes and molecular pathways involved in sexual differentiation, to early detect this mechanism.

Firstly, we aimed to setting-up an RNA extraction method to obtain high quality and sufficient quantity of RNA, comparing two different RNA extraction protocols from post larvae after five days of their metamorphosis (PL_5 stage; Zupo et al., manuscript under publication). Then, housekeeping genes (to be used as control) and genes of interest (belonging to apoptosis, ferroptosis and insulin secretion) were identified from the transcriptome (Levy et al., 2021). Their expression levels were detected by Real Time qPCR and compared to the results obtained in the transcriptome for its validation.

Materials and methods

Samples collection and culture

Ovigerous females of *H. inermis* were collected in Lacco Ameno in the Ischia Island (Italy) in a *P. oceanica* meadow. Samples were identified in the laboratory under a Leica MZ6 stereomicroscope based on their morphological characters (Zupo, 1994). After the sorting, *H. inermis* ovigerous females were taken in culture in 1500 mL conical flasks until the release of the larvae. Larvae were then collected and moved in 800 mL conical flasks according to the standard culture protocol described by Zupo and Messina ((2007). After around 25 days, settled post-larvae were transferred into 500 mL crystallization dishes and passed from the alive feeding to the treatment diet, according to the culture protocol, for 5 days. In details, post-larvae were fed with basic food for the negative control and basic food plus lyophilized *Cocconeis* spp. for the positive control (Zupo & Messina, 2007), as previously done for all the traditional bioassays. All the

methodological tests for the optimization of the protocol were carried out on individuals fed with basic food. After five days from the settlement, post-larvae in PL_5 stage were sacrificed and fixed individually in RNAlater (RNA Stabilization Reagent, Qiagen, Germany).

RNA extraction and cDNA synthesis

Two different methods of RNA extraction were compared, using as starting material 1, 2, 3 e 5 PL_5.

i. RNeasy Mini Kit. Post-larvae were lysed with different quantity of Buffer RLT/2-ME (10 µl β-mercaptoethanol for each ml of Buffer RLT), according to the number of PL_5: 350 µL for 1, 400 µL for 2 , 450 µL for 3 and 600 µL for 5 PL_5. The samples were homogenized with TissueLyser (Qiagen, Austin, US) at 20.1 Hz for 3 minutes. RNA was extracted following the kit manufacturer protocol (Qiagen, Austin, TX, US) and eluted with 30 µL RNase-free water, then stored at -80°C.

ii. PureLink™ RNA Mini Kit

Post-larvae were collected from the RNA later and moved into a 2 mL eppendorf, each with a 3 mm sterile aluminium bead. Previously, 10 µL β –mercaptoethanol were added for each 1 mL Lysis Buffer than we added this mix, according to the manufacturer instruction, 350 µL Lysis Buffer/ βMe (350 µL for 1 individual pool, 400 µL for 2 individual pool, 450 µL for 3 individual pool and 600 µL for 5 individual pools, to promote the tissues disruption. The samples were than were homogenized using the TissueLyser (Qiagen, Austin, US) set at 20 Hz for 3 minutes. Following, RNA was extracted according to the kit manufacturer protocol. RNA was eluted with 30 µL RNase-

free water provided by the kit. The samples obtained were then stored again at -80°C. The total RNA quantity extracted with both methods was estimated by the Nanodrop (ND-1000 UV Vis, NanoDrop Technologies; Riesgo et al., 2012), measuring the absorbance at 260 nm and 260/230 and 260/280 nm ratios, to exclude the presence of proteins, phenol and other contaminants. For each sample, 600 ng of total RNA extracted was retrotranscribed with an iScript cDNA Synthesis kit (Bio- Rad, Milan, Italy), following the manufacturer instructions.

Identification of genes

The sequences of nineteen genes were retrieved from *H. inermis* transcriptome (Levy et al., 20221). In particular the following genes were selected belonging to different molecular pathways: Apoptosis, Insulin secretion and Ferroptosis. The two housekeeping were *Cytochrome oxidase subunit* (*Coi*) and *18S ribosomal RNA* (Table 1). For each gene, specific primers were designed on the basis of nucleotide sequences and used to amplify the selected fragments with Taq High Fidelity PCR System (Roche, Italy). in a 30 μL final volume with 3 μL 10× PCR reaction buffer, 3 μL 10× 2 mM dNTP, 1 μL 5 U/μL Taq, 100 ng/μL of each oligo, template cDNA and nuclease free water to 30 μL. The PCR program consisted of a denaturation step at 95°C for 5 min, 35 cycles at 95°C for 45 s, 54-60°C for 1 min and 72°C for 30 s and a final extension step at 72°C for 10 min. The fragments were then purified from agarose gel using the QIAquick Gel Extraction kit (Qiagen, Milan, Italy), and their specificity were checked by DNA sequencing. PCR products were then aligned with gene sequences by MultAlin Software.

	Acronym	**Gene name**	**Primer**	**Sequence 5′>3′**
Housekeeping	*Coi*	*cytochrome oxidase subunit*	Coi_Hi_F1	CTGAAGAGGTATAGTAGGAAC

	18S	*18S ribosomal RNA*	Coi_Hi_R1	CTCGGTGCCCCTGACATAGC
			18S_Hi_F1	CATGCATGTGTCAGTACAGGC
			18S_Hi_R1	CTTATCATATGAGAATCCAACC
Apoptosis	*Cyt-c*	*Cytochrome C*	CYT_Hi_F1	GTGCAGAGATGTGCTCAGTGC
			CYT_Hi_R1	ACATCCAGAGTGTCATCTGC
	Atfc	*Activating Transcription Factor*	ATFC_Hi_F1	GGCTGGAGTTCTGACAGAGG
			ATFC_Hi_R1	CAGCCCAGCTCTTCCAGATTG
	CatB	*Cathepsin B*	CATB_Hi_F2	GGATCTTGTGGATCATGCTGG
			CATB_HI_R2	GTTCCAGCCATCCGCCATTAC
	HtrA 2	*High-temperature requirement A serine peptidase*	HTRA2_Hi_F2	GACACAATGAAGCCAGAGCC
			HTRA2_Hi_R2	CGCCATCAGTTCTCTGCTAG
	Dronc	*Death regulator Nedd2-like caspase*	DRONC_Hi_F1	GGCATCATTATGACAGATATGC
			DRONC_Hi_F1	GTGTGATGATATCATGTAGAGC
	Tspo	*Translocator Protein*	TSPO_Hi_F1	GCAGGTGGCAAATGAAATGGAG
			TSPO_Hi_R1	CTGGCGTCCTCCTAACTGGATG
Insulin secretion	*Pclo*	*Protein scaffolding protein*	PCLO_Hi_F1	GGCTGGTGATGGACGAAGAC
			PCLO_Hi_R1	CCGCGATCTGGAAACGTCAG
	Ac	*Adenylyl cyclase*	AC_Hi_F1	GTGTCTTACGTGGCTGAGGC
			AC_Hi_R1	CTGCGGTGGGTATAGTCTGC
	M3R	*Auscarinic cholinergic receptor*	M3R_Hi_F1	GGAGTCGATCTCAATGGATC
			M3R_Hi_R1	CTAGCAGTGTGGCGATGGAG
	Cckar	*G-protein coupled receptor*	CCKAR_Hi_F3	CCCTCCTGATACCTGAAGATG
			CCKAR_Hi_R3	GGATTCTCTGGTATTCTTGAC
	Vamp 3	*Synaptobrevin/vesicle-associated membrane protein*	VAMP3_Hi_F1	CTAGTGCCAGTGACTGTGAC
			VAMP3_Hi_R1	CCACCTCATTCACCTCTCTC
	Plc	*Phospholipase C*	PLC_Hi_F1	GCTGAGAGTCTGAGAGACTG
			PLC_Hi_R1	CTAGTCACTAAACGTCGTCGG
	Snp25	*Synaptosome-associated protein 25*	SNP25_Hi_F1	GCAGAGCTGAGTGCCGTAGC

			SNP25_Hi_R1	GCAACGATCCGAACTACTAC
Ferroptosis	*Gshi*	*Gamma Glutamylcysteine Synthetase*	GSH1_Hi_F1	GCCGTGTGAAGTCCAGCTGA
			GSH1_Hi_R1	CATTCACGGACATCTGACTAG
	Stea 3	*Six-transmembrane epithelial antigen of prostate 3 metalloreductase*	STEA3_Hi_F1	GAGCATATGCAGATAACGTG
			STEA3_Hi_R1	GGCTATTCCTGATGAGCATC
	Gpx4	*Glutathione Peroxidase 4*	GPX4_Hi_F1	GCTGAGAGTCTGAGAGACTG
			GPX4_Hi_R1	CTAGTCACTAAACGTCGTCGG
	Sat	*Spermidine/spermine N1-Acetyltransferase 1*	SAT_Hi_F1	CTGTGGATGTGACTCAGAAG
			SAT_Hi_R1	GCAGATTCTTGCTGATGCGG

Table 1. List of the nineteen genes, divided according to the pathways with acronym, primer sequences and lengths of PCR amplified fragments is reported.

Gene expression by Real- Time qPCR

The specificity of amplification reactions for the different pairs of primers was verified by melting curve analysis. The efficiency of each primer pair was calculated according to standard method curves using the equation E=10−1/ slope, by using five serial dilutions to determine Ct values to generate standard curves using Ct values versus the logarithm of each dilution factor. PCR efficiencies were calculated for control and target genes and were found to be 2. Diluted cDNA was used as a template in a reaction containing a final concentration of 0.3 mM for each primer and 1× FastStart SYBR Green master mix (total volume of 10 μL) (Applied Biosystems, Monza, Italy).

The thermal profile chosen was in details: one cycle of 95 °C (10 min) for the cDNA denaturation; 40 cycles of 95 °C (15 sec) and 60°C (1 min) for the amplification; one cycle of 72 °C (5 min), for the final elongation; one cycle from 60 to 95 °C for melting curve analysis to verify the presence of a single product. Three duplicates of each RT-

qPCR reaction were performed. Fluorescence was measured using the ViiATM7 software. The relative expression ratios were calculated from quantification cycles (Cq). Undiluted cDNA (1:1) was than chosen as template to proceed to compare the expression of the genes of interest in samples deriving from animals coming from the positive and the negative control. The relative expression ratios were elaborated from Cq through Relative Expression method using REST software. Results that showed a difference above 2 were considered significant.

Statistical analysis

The difference among quantity and quality of all the samples of total RNA obtained from the two RNA extraction methods were evaluated using the paired Student t test. P value lower than 0.05 was considered significant. All the statistical analyses were performed using GraphPad PRISM v.7 software (GraphPad Software, CA, USA, www.graphpad.com, accessed 22 September 2022).

Results

Evaluation of quantity/quality of RNA

Different results were achieved using the two different kits according to the different number of PL_5 used. Trough the RNeasy kit significantly higher quantity of total RNA was extracted (paired t test p=0,0384), with all the samples analysed. A significant difference was also found considering the RNA purity according to the A260/230 ratio (paired t test p= 0,0143), while there was no significant difference for the A260/280 ratio. The minimum amount of total RNA requested for cDNA synthesis, according to the the iScript™ cDNA Synthesis kit (Bio-Rad, Milan, Italy), is ~30 ng/ µL. For this reason, we chose RNeasy kit as the most efficient method to produce sufficient RNA for our analysis.

	PL_5 number	ng/ µL	A260/280	A260/230
RNeasy Mini Kit	1	60,9	1,99	0.32
	2	84,5	2,03	0,82
	3	161,6	2,08	0,55
	5	176,8	2,02	0,93
PureLink™ RNA Mini Kit	1	29,9	2,03	1,47
	2	58,4	2,12	0,48
	3	87	2,13	2,07
	5	146,7	2,11	2,07

Table 2. Total RNA quantity (ng/ µL), purity (A260/280 and A260/230) and integrity obtained from the two extraction methods tested from four different pools (number of individuals).

After the specific primers were designed, PCRs reactions were performed to test their specificity. PCR bands obtained were then purified from agarose gel and checked by DNA sequencing. PCR products were aligned with gene sequences to confirm that the product obtained was corresponding to the target gene. All the products aligned to the original nucleotide sequences (Figure S2).

From the extraction with QIAGEN Kit derived from the pool with one individual, 600 ng and 1000 ng of total RNA were used for the retrotranscription resulting in: undiluted 20 µL of cDNA and diluted 50 µL of cDNA. Both were used to perform RT-qPCRs reactions with the housekeeping primers to test their efficiency. Efficiencies (E) for the *Coi* gene from samples obtained with the undiluted 20 µL of cDNA deriving from the synthesis from 600 ng showed an E= 2,1 while for the one deriving from 1000 ng of total RNA, E was 5,2. RT-qPCRs reactions resulting from starting diluted samples of cDNA (50 µL) from 600 ng had an E= Eff. 1,9 while for the one deriving from 1000 ng of total RNA, E was 1,6. These results demonstrate that good efficiency can be obtained with all tested dilutions. For this reason, we chose to proceed with the dilution of 50 µL deriving from

600ng of total RNA in order to optimise the amount of total RNA obtained from the replicates. Standard curves were generated for each primers pair using Cq values vs. the logarithm of each dilution factor (Figure S1).

Gene expression

The expression level of the genes of interest in PL_5 of *H. inermis* fed with basic food plus lyophilized *Cocconeis* spp. diatom was detected against the PL_5 fed only with basic food (prepared using lyophilized Artemia enriched, Spirulina powder, Micropearls "SHG" and flake feed "Super High Red", all feeds distributed by Super High Group, Turin, Italy) as previously done for the transcriptomic analysis reported in Levy et al. (2021). The expression of each gene was analysed and internally normalized against the negative control and then compared with the transcriptome data (Figure 1). In the apoptosis pathway all the analysed genes were significantly up regulated in both samples analysed in the present work and in the transcriptome. In details, the results from the samples for the validation of the transcriptome showed the following levels of gene expression: *Cyt* (5.25), *Atfc* (3.02), *Htra2* (4.65), *Dronc* (2.23), *Tspo* (2.79). Concerning the expression of the other genes involved in the insulin secretion pathway, excepting *Vamp 3* (4.5), all of them were downregulated. In details the results from our samples shows: *Pclo* (-3.7), *Ac* (-3.58), *M3R* (-3,04), *Cckar* (-4.6), *Plc* (-4.61), *Snp25* (-4.82).

Another important process that showed an alteration in expression triggered by the ingestion of the *Cocconeis* diatom is the ferroptosis. In details the results here obtained were congruent with the results obtained in the transcriptome: *Gshi* (5.38), *Stea3* (3.1), *Gpx4* (4.32), *Sat* (2.59). The difference between the results obtained from the samples of the transcriptome and the animals used for its validation are not significant (paired

Student *t* test), confirming that our result is in perfect agreement with those showed in the transcriptome analysis.

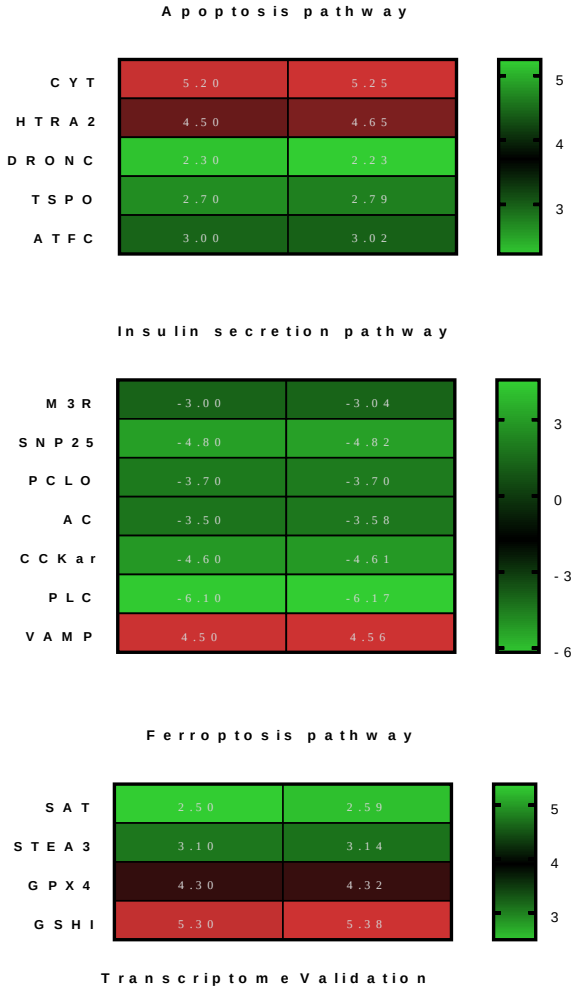

Figure 1. Heatmaps showing the expression profiles analysed by real-time qPCR deriving from the transcriptome and from the samples newly tested for its validation.

Discussion

This work aimed at optimizing of a new molecular approach for the investigations on *H. inermis* shrimp. This crustacean is of interest for the research because it undergoes a unique process of food determining sex reversal, in which an algal compound can trigger the early development of the female phenotype. This is a consequence of a coevolutionary

process with a specific diatom: *Cocconeis* spp. The traditional bioassays, which permits to identify the molecular structure of this algal compound able to trigger an early sexual shift in this animal, result in a long and complex culture practice. This necessary culture protocol leads to various problems, such as the difficulty in finding an adequate number of ovigerous females, given the serious anthropic threat that afflicts the *Posidonia* meadows where these animals usually live (Zupo, Buia, et al., 2006; Zupo, Mazzella, et al., 2006). Furthermore, culturing such a large number of sensitive larvae and post-larvae, risking that such a long culture protocol could influence their health and stress status, is extremely difficult. It involves the risk of achieve the end of the long culture period with a statistically insufficient number of available animals to proceed with the evaluation of the distribution of the sexes in the treatment replicates. Another concrete risk is that, at the end of culture, animals are too stressed, giving a possible altered biological response to the treatment (Harper & Wolf, 2009; Lorenzon et al., 2000). For all these reasons, the set-up of a new molecular approach, to be an alternative and a support to the traditional bioassay, is essential to optimize the investigations to identify the molecular structure of this apoptogenic compound. The final step in optimizing a protocol for RNA extraction, cDNA synthesis, identifying genes of interest, and designing and testing the efficiency of primers for RT qPCR analysis is to validate the results obtained in the transcriptome.

According to the results obtained in the transcriptomes, these genes were chosen among the ones that showed significant difference in expression induced by the ingestion of diatoms and that play key roles in the three pathways proposed as involved in *H. inermis* early sex shift (Levy et al., 2021). The three pathways were: Apoptosis; Insulin secretion; Ferroptosis (Zupo et al., unpublished manuscript).

Apoptogenic activity was proposed to be responsible in the premature destruction of the androgenic gland of *H. inermis* (Zupo, 1994) as selective mechanism of programmed cell

death. In parallel, insulin secretion pathway has been proposed to be fundamental for the regulation of the metabolism of insulin-like hormones, produced by the androgenic gland, acting as a switch to control sexual differentiation of the crustaceans (Ventura et al., 2012).

Finally, ferroptosis is another form of cell death, which is the subject of studies in the last years. It is based on a highly conserved mechanism, and it was discovered only recently in *Caenorhabditis elegans* (Deline et al., 2015). It has been proposed that ferroptosis can plays a crucial role in leading additional apoptosis events in invertebrates ((Deline et al., 2015; Zupo et al., unpublished manuscript).

According to these results, our validation confirmed the patterns of expression that were expected. We found the six chosen genes involved in apoptosis pathway (Table 1) influenced by the algal ingestion. In parallel, the seven genes involved in the insulin secretion pathway (Table 1), which are part of the metabolism of insulin like hormone produced by the androgenic gland of the shrimps, were differentially expressed. Another pathway influenced by the ingestion of this diatom was ferroptosis with all the four selected genes upregulated (Table1).

These results here obtained support the hypothesis that during the early sex shift of *H. inermis* induced by the *Cocconesis* diet, a cascade of pro apoptotic signals is promoted, triggering the programmed destruction of the androgenic gland of this shrimp. Also, the switch-down of genes involved in the insulin pathway support the hypothesis that a cascade effect is induced leading to the inhibition of an insulin-like androgenic hormone (Levy & Sagi, 2020; Ventura et al., 2012). The chance to track the presence of the active compound evaluating the gene expression in the PL_5 could significantly optimize the time and the number of bioassays needed to achieve the identification of the molecular structure of this apoptogenic compound(s). This point could possibly lead to medical

applications. In fact, previous *in vitro* investigations demonstrated that the crude extracts of these diatoms specifically activate a dose-dependent apoptotic process in human cancer cells (BT20 breast carcinoma) but not in human normal lymphocytes (Nappo et al., 2012). Once the molecular structure has been identified, the study and modulation of apoptotic processes in various types of cells will be possible, in order to develop new natural drugs useful for human anticancer therapies.

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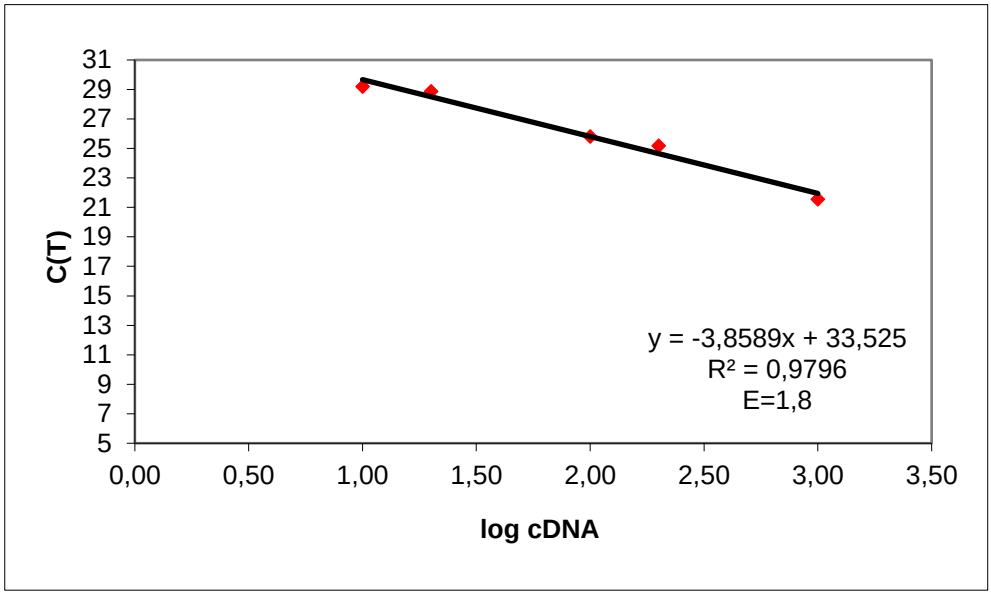
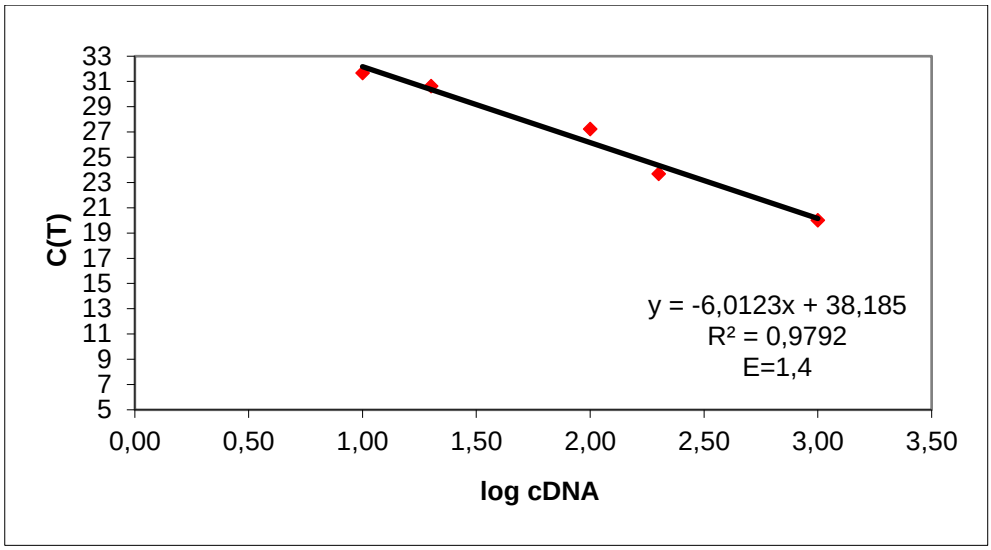
Supplementary materials

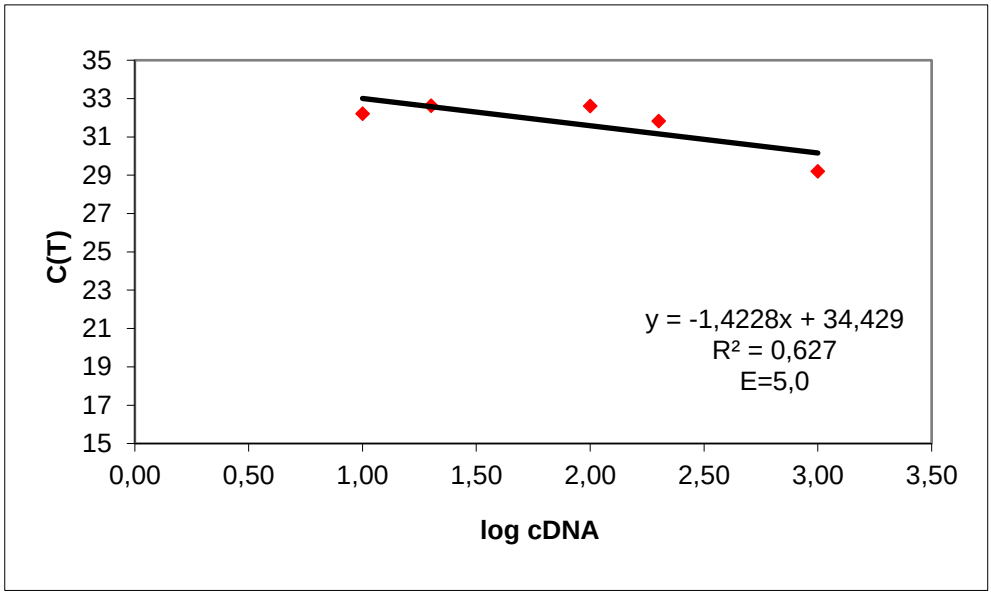
A)



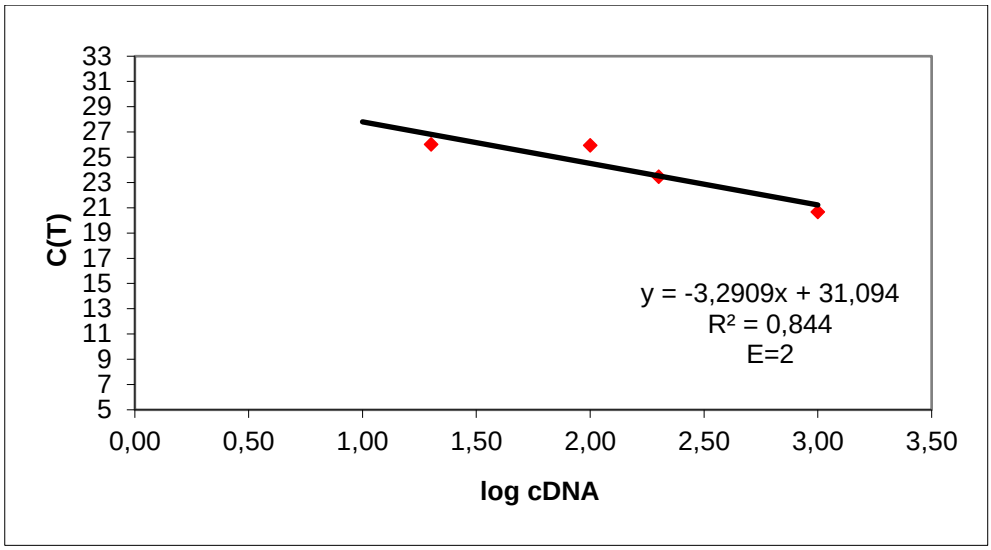
B)


C(T)
log cDNA
y = -6,0123x + 38,185
R² = 0,9792
E=1,4

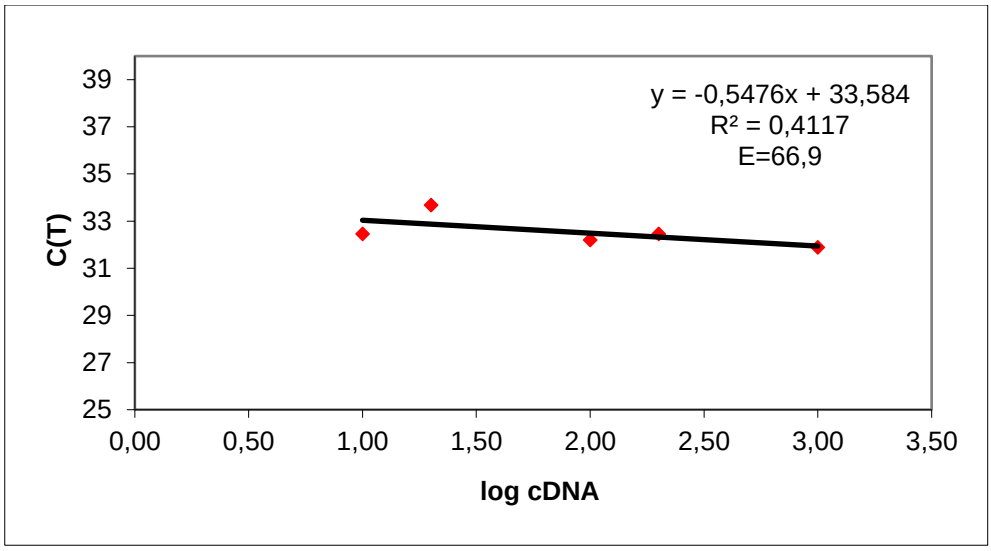
C)


C(T)
log cDNA
y = -1,4228x + 34,429
R² = 0,627
E=5,0

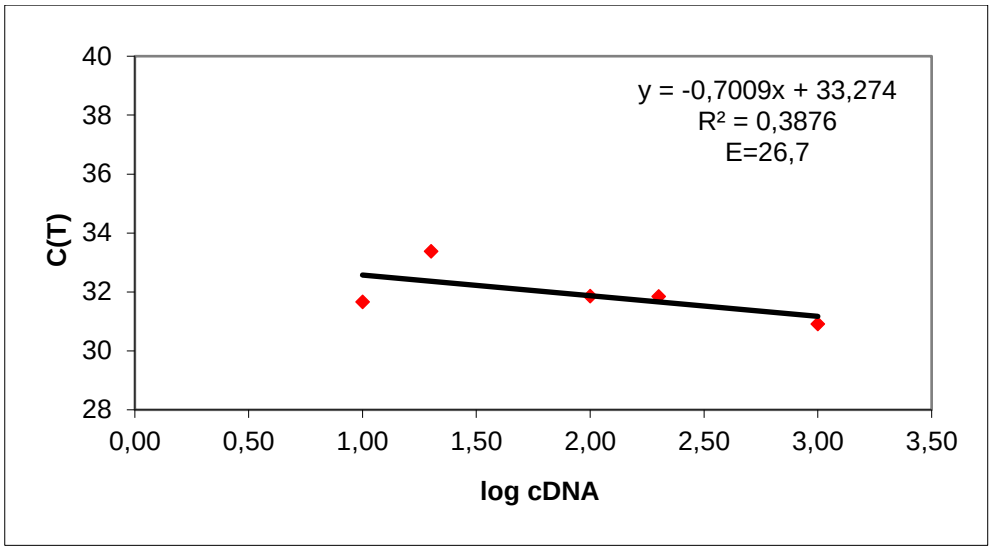
D)


C(T)
log cDNA
y = -3,2909x + 31,094
R² = 0,844
E=2

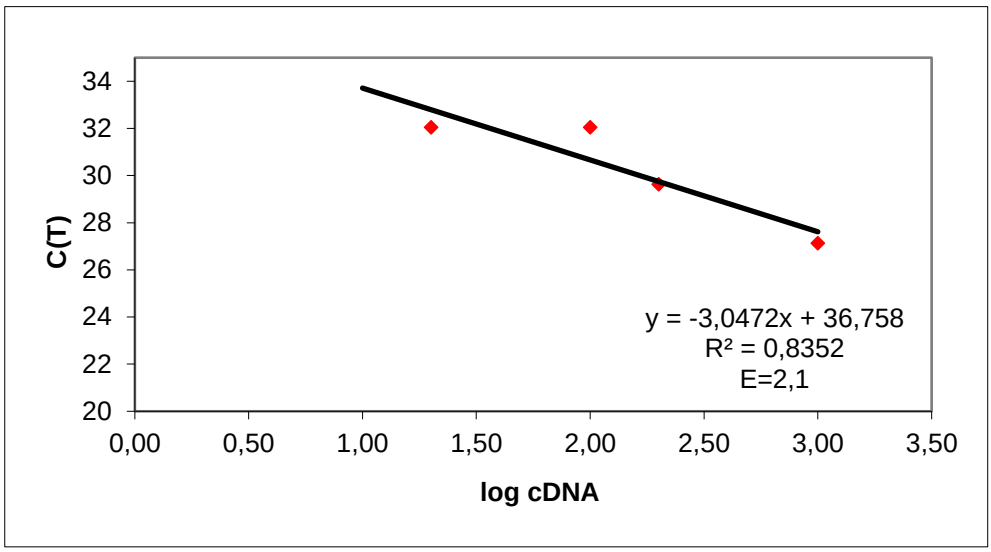
E)


C(T)
log cDNA
y = -0,5476x + 33,584
R² = 0,4117
E=66,9

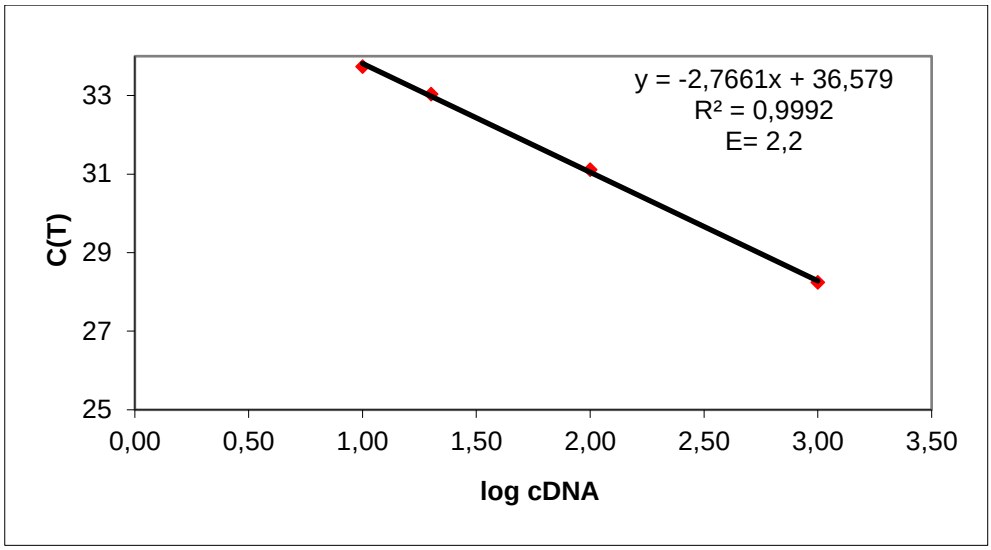
F)


y = -0,7009x + 33,274
R² = 0,3876
E=26,7
C(T)
log cDNA
40
38
36
34
32
30
28
0,00
0,50
1,00
1,50
2,00
2,50
3,00
3,50

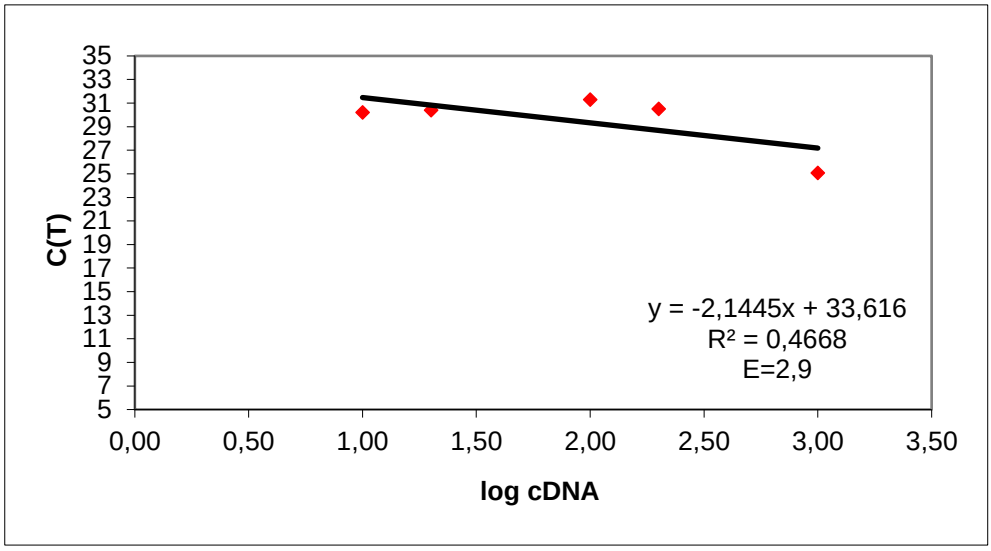
G)


y = -3,0472x + 36,758
R² = 0,8352
E=2,1
C(T)
log cDNA
34
32
30
28
26
24
22
20
0,00
0,50
1,00
1,50
2,00
2,50
3,00
3,50

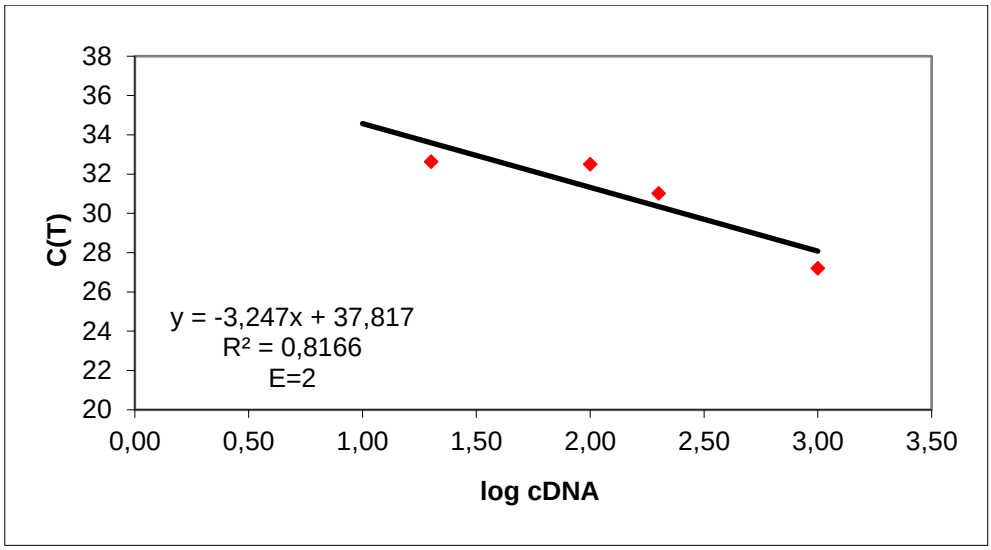
H)


y = -2,7661x + 36,579
R² = 0,9992
E= 2,2
C(T)
33
31
29
27
25
0,00
0,50
1,00
1,50
2,00
2,50
3,00
3,50
log cDNA

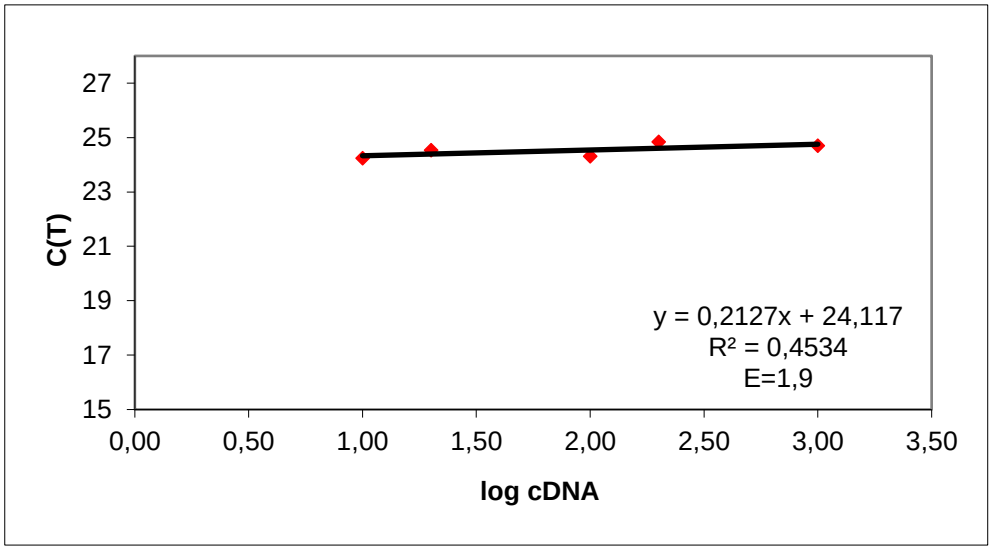
I)


y = -2,1445x + 33,616
R² = 0,4668
E=2,9
C(T)
35
33
31
29
27
25
23
21
19
17
15
13
11
9
7
5
0,00
0,50
1,00
1,50
2,00
2,50
3,00
3,50
log cDNA

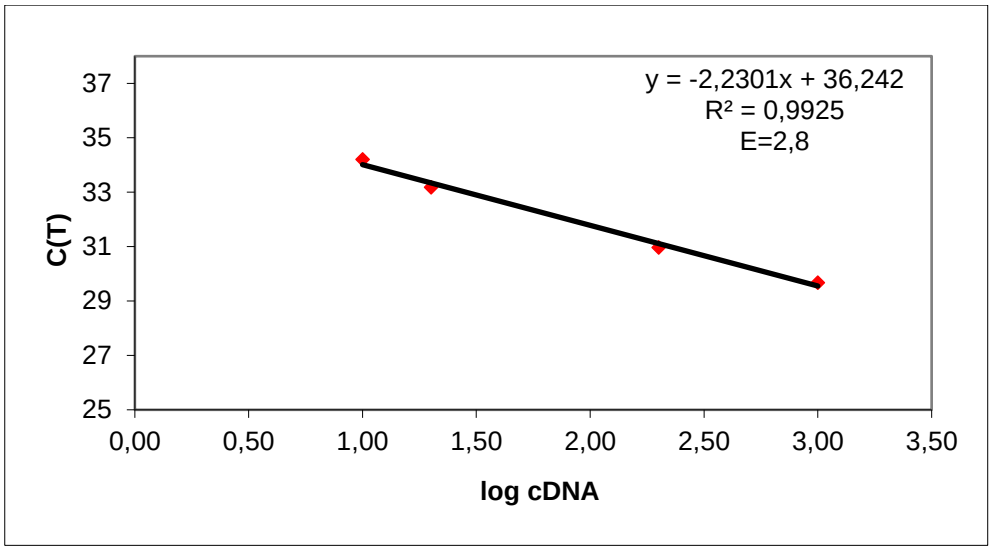
L)


38
36
34
32
30
28
26
24
22
20
C(T)
y = -3,247x + 37,817
R² = 0,8166
E=2
0,00
0,50
1,00
1,50
2,00
2,50
3,00
3,50
log cDNA

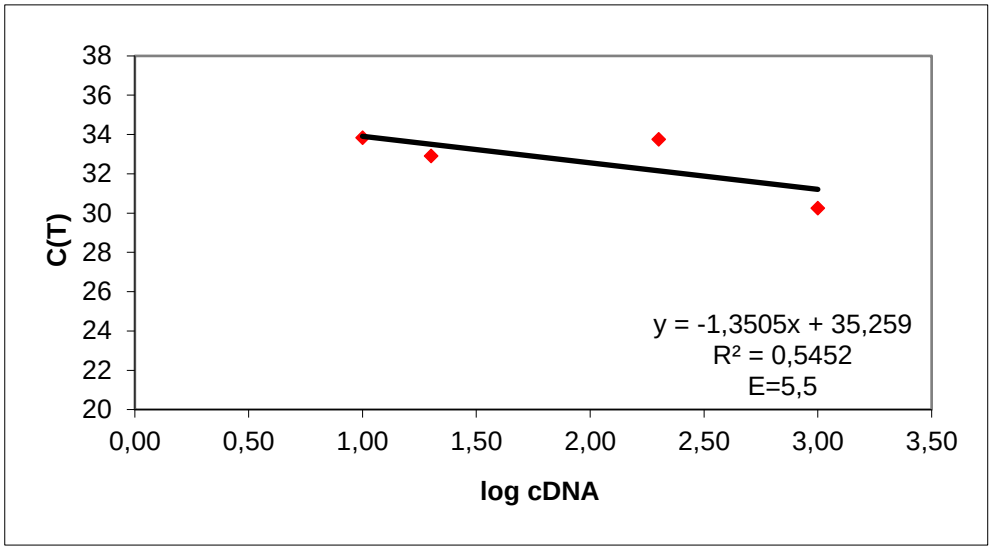
M)


27
25
23
21
19
17
15
C(T)
y = 0,2127x + 24,117
R² = 0,4534
E=1,9
0,00
0,50
1,00
1,50
2,00
2,50
3,00
3,50
log cDNA

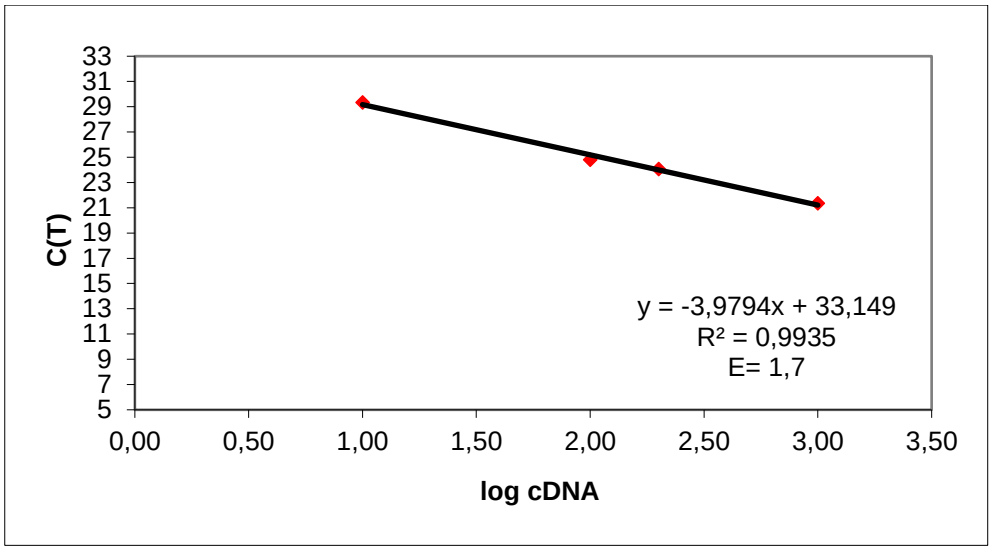
N)


y = -2,2301x + 36,242
R² = 0,9925
E=2,8
C(T)
37
35
33
31
29
27
25
0,00
0,50
1,00
1,50
2,00
2,50
3,00
3,50
log cDNA

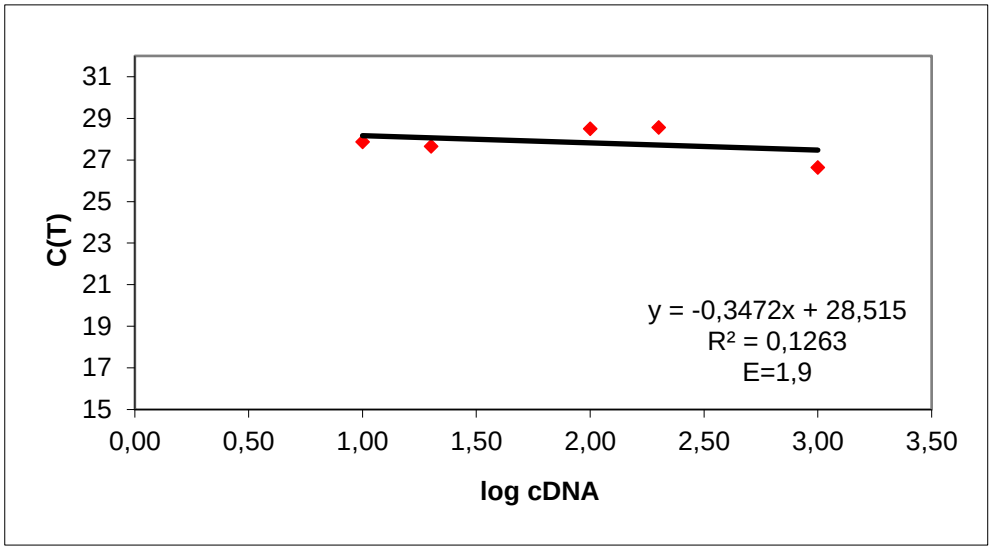
O)


y = -1,3505x + 35,259
R² = 0,5452
E=5,5
C(T)
38
36
34
32
30
28
26
24
22
20
0,00
0,50
1,00
1,50
2,00
2,50
3,00
3,50
log cDNA

P)


C(T)
log cDNA
y = -3,9794x + 33,149
R² = 0,9935
E= 1,7

Q)


C(T)
log cDNA
y = -0,3472x + 28,515
R² = 0,1263
E=1,9

R)

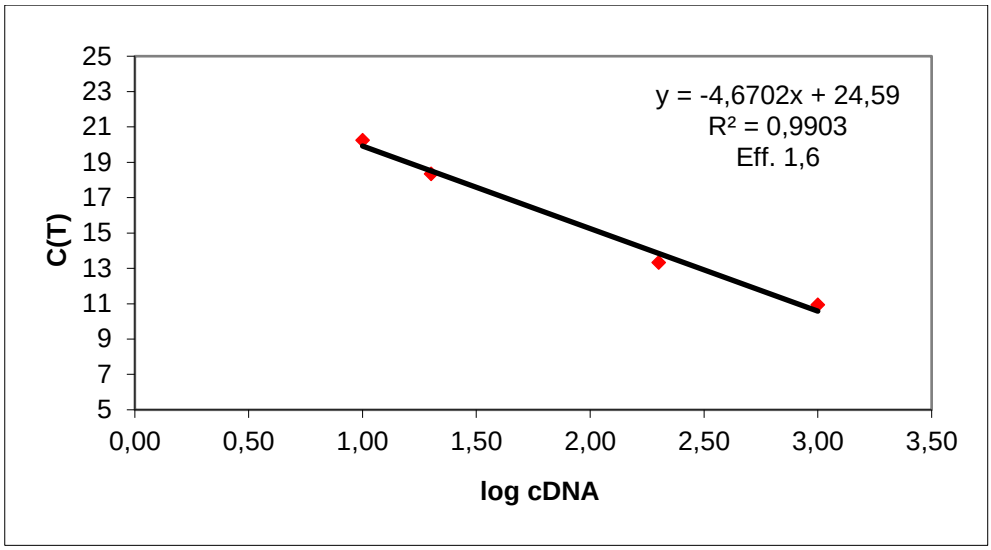
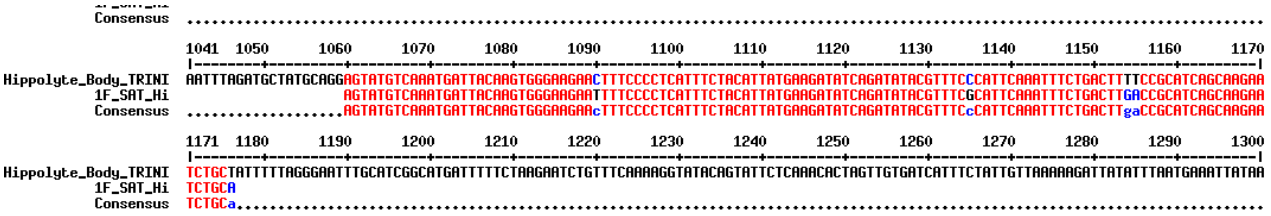
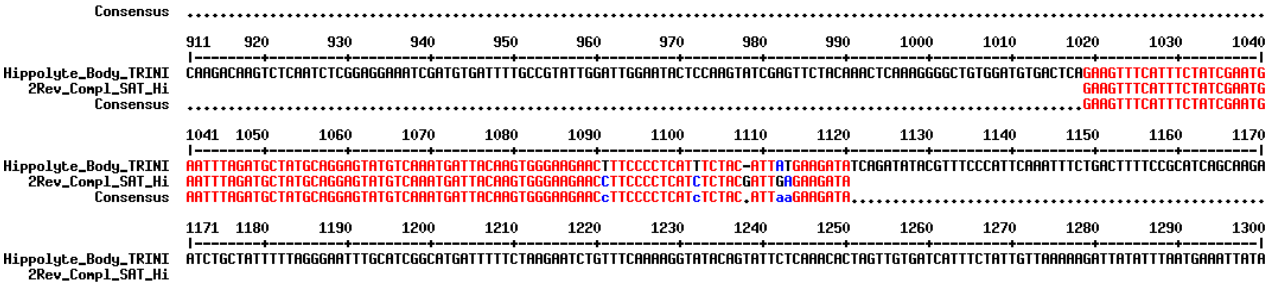


Figure S1. Efficiencies (E) for A) *AC*_Hi; B) *Atfc*_Hi; C) *CCKAR*_Hi; D) *Cyt*_Hi; E) *Dronc*_Hi; F) *Gpx4*_Hi; G) *Gshi*_Hi; H) *Htra*_Hi; I) *M3r*_Hi; L) *Pclo*_Hi; M) *Plc*_Hi; N) *Snp25*_Hi; O) *Stea3*_Hi; P) *Tspo*_Hi; Q) *Tspo*_Hi; R) *COI* genes. For those genes whose expression was detected significantly low, the efficiency was not measured.

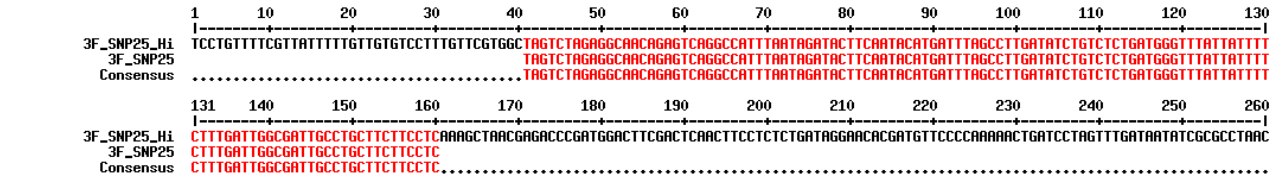
A)



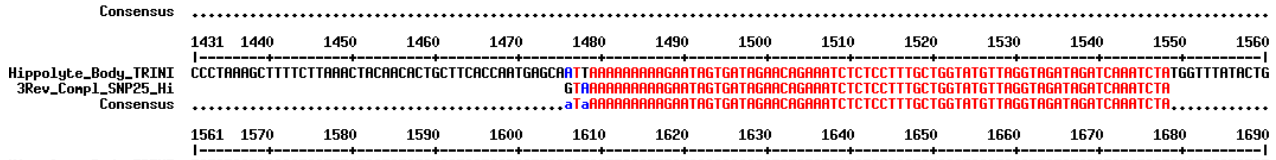
B)



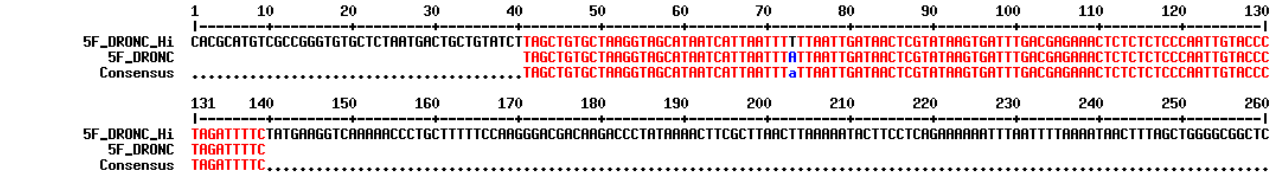
C)


3F_SNP25_Hi
3F_SNP25
Consensus

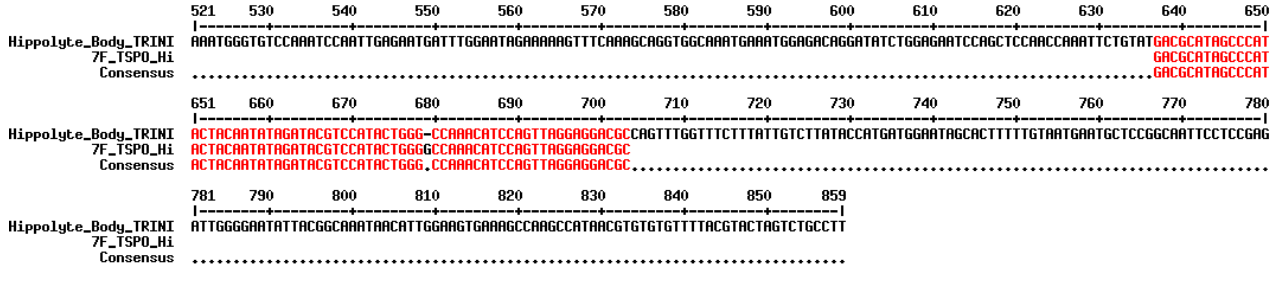
D)


Consensus
Hippolyte_Body_TRINI
3Rev_Compl_SNP25_Hi
Consensus
Hippolyte_Body_TRINI
3Rev_Compl_SNP25_Hi

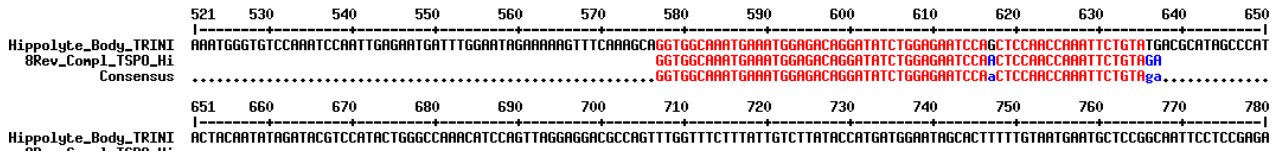
E)


5F_DRONC_Hi
5F_DRONC
Consensus
5F_DRONC_Hi
5F_DRONC
Consensus

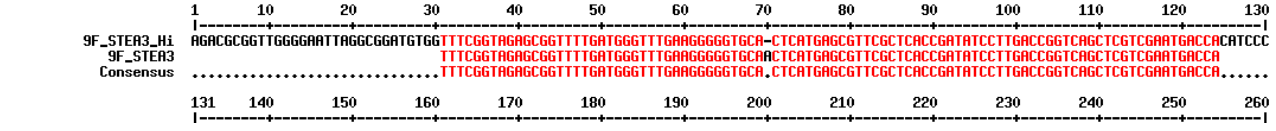
F)


Hippolyte_Body_TRINI
7F_TSPO_Hi
Consensus
Hippolyte_Body_TRINI
7F_TSPO_Hi
Consensus
Hippolyte_Body_TRINI
7F_TSPO_Hi
Consensus

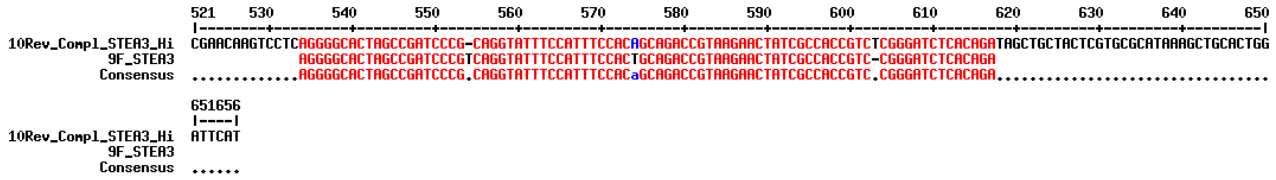
G)


Hippolyte_Body_TRINI
8Rev_Compl_TSPO_Hi
Consensus
Hippolyte_Body_TRINI

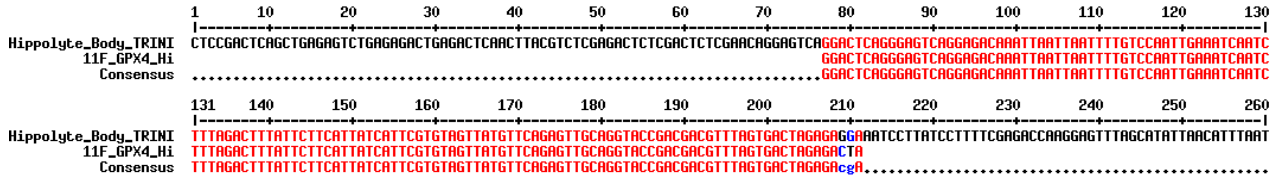
H)


9F_STEA3_Hi
9F_STEA3
Consensus

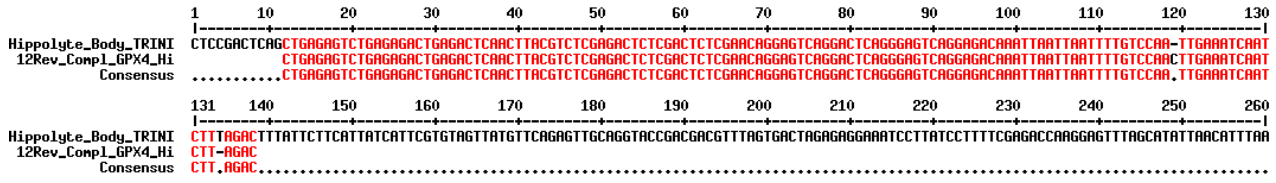
I)


521 530 540 550 560 570 580 590 600 610 620 630 640 650
10Rev_Compl_STEA3_Hi CGAACAAGTCCTCAGGGGCACTAGCCGATCCCG-CAGGTATTTCCATTTCCACAGCAGACCGTAAGAACTATCGCCACCGTCTCGGGATCTCACAGATAGCTGCTACTCGTGCGCATAAAGCTGCACTGG
9F_STEA3 AGGGGCACTAGCCGATCCCGTCAGGTATTTCCATTTCCACTGCAGACCGTAAGAACTATCGCCACCGTC-CGGGATCTCACAGA
Consensus .....AGGGGCACTAGCCGATCCCG.CAGGTATTTCCATTTCCACaGCAGACCGTAAGAACTATCGCCACCGTC.CGGGATCTCACAGA.....
651656
10Rev_Compl_STEA3_Hi ATTCAT
9F_STEA3
Consensus ......

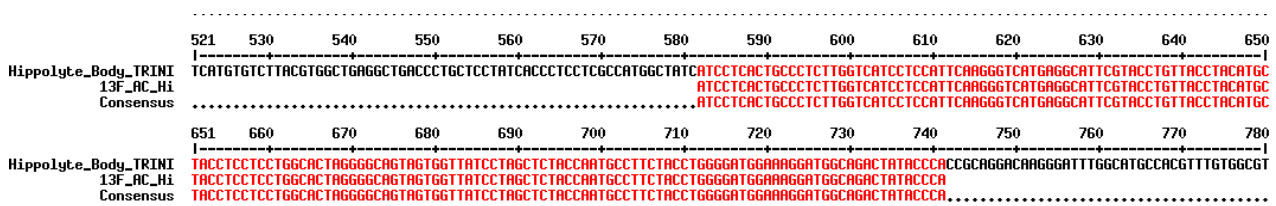
J)


1 10 20 30 40 50 60 70 80 90 100 110 120 130
Hippolyte_Body_TRINI CTCCGACTCAGCTGAGAGTCTGAGAGACTGAGACTCAACTTACGTCTCGAGACTCTCGACTCTCGAACAGGAGTCAGGACTCAGGGAGTCAGGAGACAAATTAATTAATTTTGTCCAATTGAAATCAATC
11F_GPX4_Hi GGACTCAGGGAGTCAGGAGACAAATTAATTAATTTTGTCCAATTGAAATCAATC
Consensus .....GGACTCAGGGAGTCAGGAGACAAATTAATTAATTTTGTCCAATTGAAATCAATC
131 140 150 160 170 180 190 200 210 220 230 240 250 260
Hippolyte_Body_TRINI TTTAGACTTTATTCTTCATTATCATTCGTGTAGTTATGTTCAGAGTTGCAGGTACCGACGACGTTTAGTGACTAGAGAGGAAATCCTTATCCTTTTCGAGACCAAGGAGTTTAGCATATTAACATTTAAT
11F_GPX4_Hi TTTAGACTTTATTCTTCATTATCATTCGTGTAGTTATGTTCAGAGTTGCAGGTACCGACGACGTTTAGTGACTAGAGACTA
Consensus TTTAGACTTTATTCTTCATTATCATTCGTGTAGTTATGTTCAGAGTTGCAGGTACCGACGACGTTTAGTGACTAGAGAcgA.....

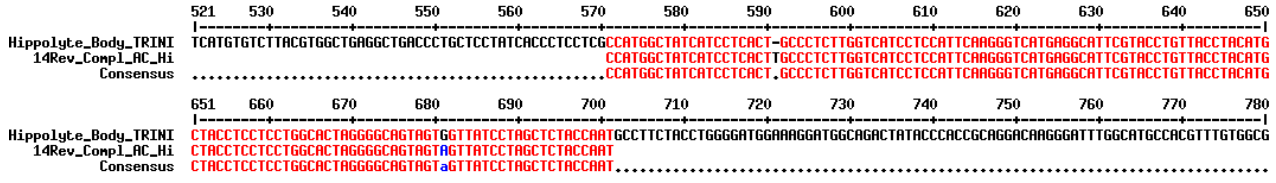
K)


1 10 20 30 40 50 60 70 80 90 100 110 120 130
Hippolyte_Body_TRINI CTCCGACTCAGCTGAGAGTCTGAGAGACTGAGACTCAACTTACGTCTCGAGACTCTCGACTCTCGAACAGGAGTCAGGACTCAGGGAGTCAGGAGACAAATTAATTAATTTTGTCCAA-TTGAAATCAAT
12Rev_Compl_GPX4_Hi CTGAGAGTCTGAGAGACTGAGACTCAACTTACGTCTCGAGACTCTCGACTCTCGAACAGGAGTCAGGACTCAGGGAGTCAGGAGACAAATTAATTAATTTTGTCCAACTTGAAATCAAT
Consensus .....CTGAGAGTCTGAGAGACTGAGACTCAACTTACGTCTCGAGACTCTCGACTCTCGAACAGGAGTCAGGACTCAGGGAGTCAGGAGACAAATTAATTAATTTTGTCCAA.TTGAAATCAAT
131 140 150 160 170 180 190 200 210 220 230 240 250 260
Hippolyte_Body_TRINI CTTTAGACTTTATTCTTCATTATCATTCGTGTAGTTATGTTCAGAGTTGCAGGTACCGACGACGTTTAGTGACTAGAGAGGAAATCCTTATCCTTTTCGAGACCAAGGAGTTTAGCATATTAACATTTAA
12Rev_Compl_GPX4_Hi CTT-AGAC
Consensus CTT.AGAC.....

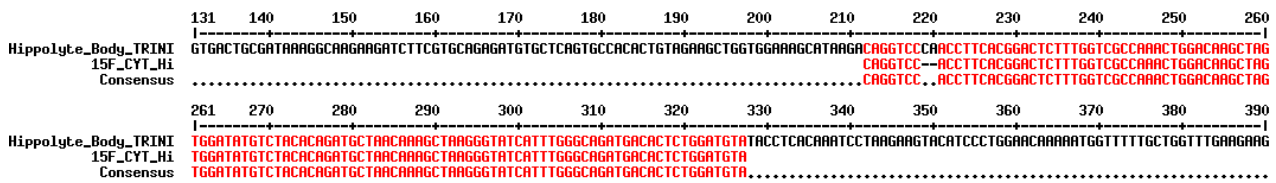
L)


521 530 540 550 560 570 580 590 600 610 620 630 640 650
Hippolyte_Body_TRINI TCATGTGTCTTACGTGGCTGAGGCTGACCCTGCTCCTATCACCCTCCTCGCCATGGCTATCATCCTCACTGCCCTCTTGGTCATCCTCCATTCAAGGGTCATGAGGCATTCGTACCTGTTACCTACATGC
13F_AC_Hi ATCCTCACTGCCCTCTTGGTCATCCTCCATTCAAGGGTCATGAGGCATTCGTACCTGTTACCTACATGC
Consensus .....ATCCTCACTGCCCTCTTGGTCATCCTCCATTCAAGGGTCATGAGGCATTCGTACCTGTTACCTACATGC
651 660 670 680 690 700 710 720 730 740 750 760 770 780
Hippolyte_Body_TRINI TACCTCCTCCTGGCACTAGGGGCAGTAGTGGTTATCCTAGCTCTACCAATGCCTTCTACCTGGGGATGGAAAGGATGGCAGACTATACCCACCGCAGGACAAGGGATTTGGCATGCCACGTTTGTGGCGT
13F_AC_Hi TACCTCCTCCTGGCACTAGGGGCAGTAGTGGTTATCCTAGCTCTACCAATGCCTTCTACCTGGGGATGGAAAGGATGGCAGACTATACCCA
Consensus TACCTCCTCCTGGCACTAGGGGCAGTAGTGGTTATCCTAGCTCTACCAATGCCTTCTACCTGGGGATGGAAAGGATGGCAGACTATACCCA.....

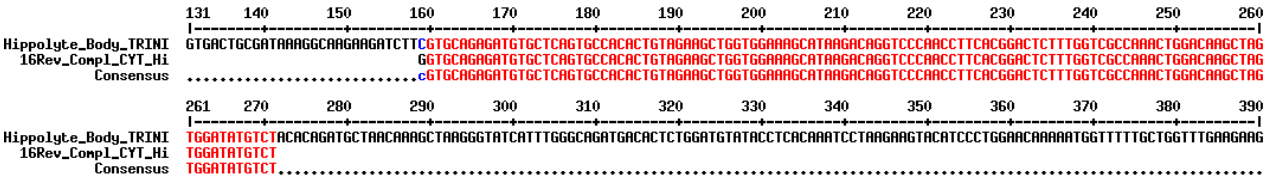
M)


521 530 540 550 560 570 580 590 600 610 620 630 640 650
Hippolyte_Body_TRINI TCATGTGTCTTACGTGGCTGAGGCTGACCCTGCTCCTATCACCCTCCTCGCCATGGCTATCATCCTCACT-GCCCTCTTGGTCATCCTCCATTCAAGGGTCATGAGGCATTCGTACCTGTTACCTACATG
14Rev_Compl_AC_Hi CCATGGCTATCATCCTCACTTGCCCTCTTGGTCATCCTCCATTCAAGGGTCATGAGGCATTCGTACCTGTTACCTACATG
Consensus .....CCATGGCTATCATCCTCACT.GCCCTCTTGGTCATCCTCCATTCAAGGGTCATGAGGCATTCGTACCTGTTACCTACATG
651 660 670 680 690 700 710 720 730 740 750 760 770 780
Hippolyte_Body_TRINI CTACCTCCTCCTGGCACTAGGGGCAGTAGTGGTTATCCTAGCTCTACCAATGCCTTCTACCTGGGGATGGAAAGGATGGCAGACTATACCCACCGCAGGACAAGGGATTTGGCATGCCACGTTTGTGGCG
14Rev_Compl_AC_Hi CTACCTCCTCCTGGCACTAGGGGCAGTAGTAGTTATCCTAGCTCTACCAAT
Consensus CTACCTCCTCCTGGCACTAGGGGCAGTAGTaGTTATCCTAGCTCTACCAAT.....

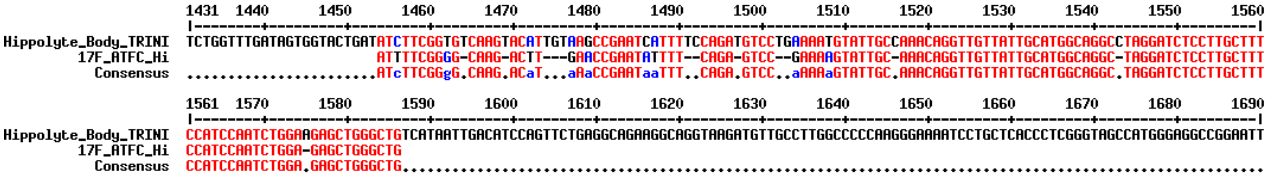
N)


131 140 150 160 170 180 190 200 210 220 230 240 250 260
Hippolyte_Body_TRINI GTGACTGCGATAAAGGCAAGAAGATCTTCGTGCAGAGATGTGCTCAGTGCCACACTGTAGAAGCTGGTGGAAAGCATAAGACAGGTCCCACCTTCACGGACTCTTTGGTCGCCAAACTGGACAAGCTAG
15F_CYT_Hi CAGGTCC--ACCTTCACGGACTCTTTGGTCGCCAAACTGGACAAGCTAG
Consensus .....CAGGTCC..ACCTTCACGGACTCTTTGGTCGCCAAACTGGACAAGCTAG
261 270 280 290 300 310 320 330 340 350 360 370 380 390
Hippolyte_Body_TRINI TGGATATGTCTACACAGATGCTAACAAAGCTAAGGGTATCATTTGGGCAGATGACACTCTGGATGTATACCTCACAAATCCTAAGAAGTACATCCCTGGAACAAAAATGGTTTTTGCTGGTTTGAAGAAG
15F_CYT_Hi TGGATATGTCTACACAGATGCTAACAAAGCTAAGGGTATCATTTGGGCAGATGACACTCTGGATGTA
Consensus TGGATATGTCTACACAGATGCTAACAAAGCTAAGGGTATCATTTGGGCAGATGACACTCTGGATGTA.....

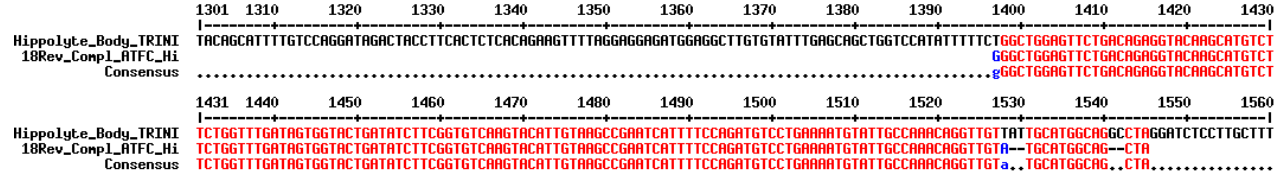
O)


Hippolyte_Body_TRINI
16Rev_Compl_CYT_Hi
Consensus

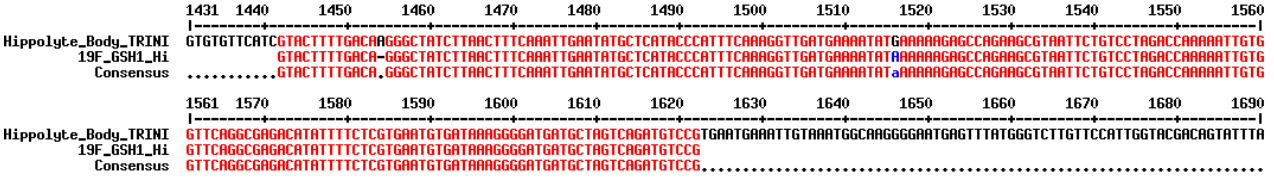
P)


Hippolyte_Body_TRINI
17F_ATFC_Hi
Consensus

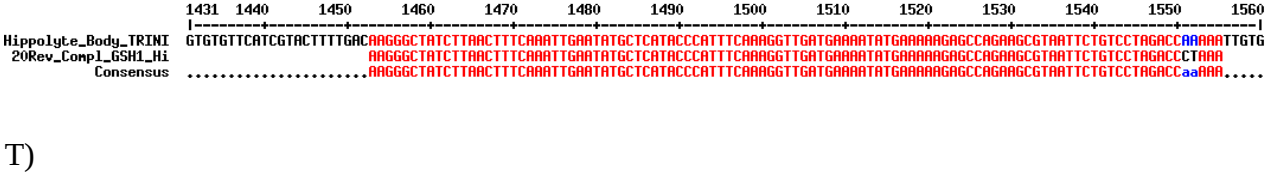
Q)


Hippolyte_Body_TRINI
18Rev_Compl_ATFC_Hi
Consensus

R)


Hippolyte_Body_TRINI
19F_GSH1_Hi
Consensus

S)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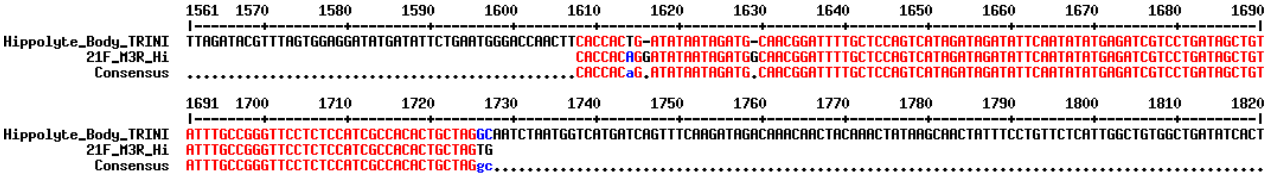
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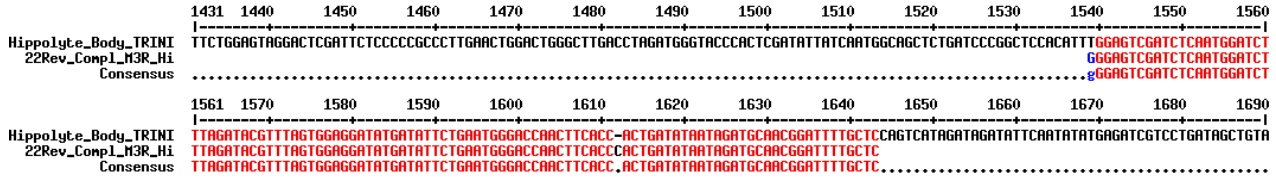
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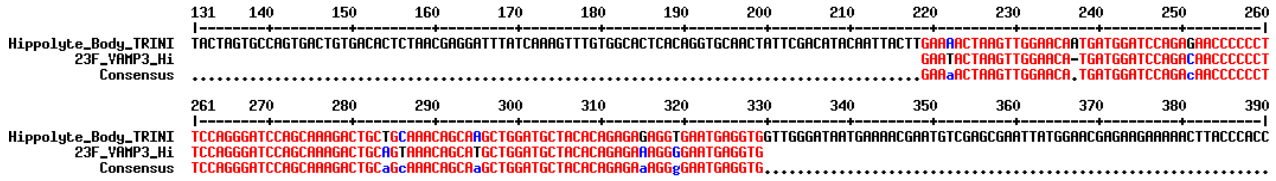
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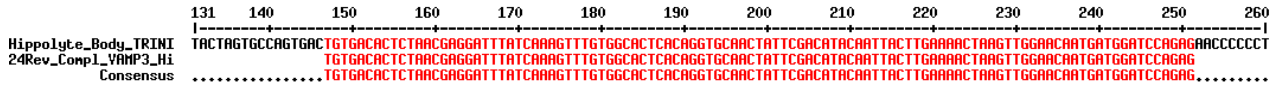
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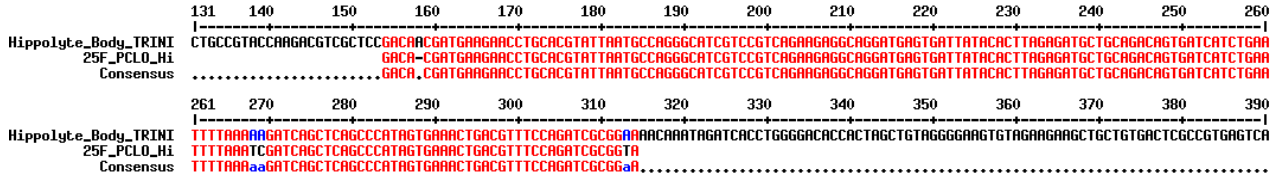
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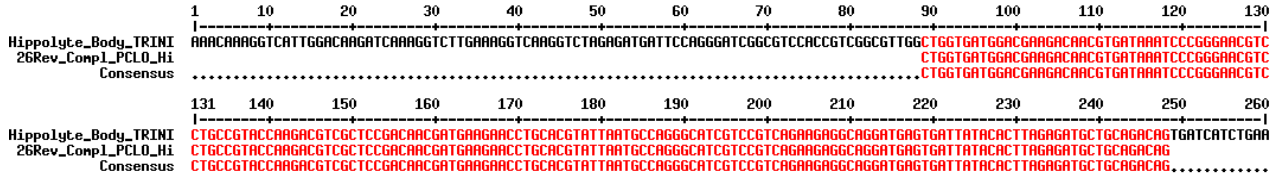
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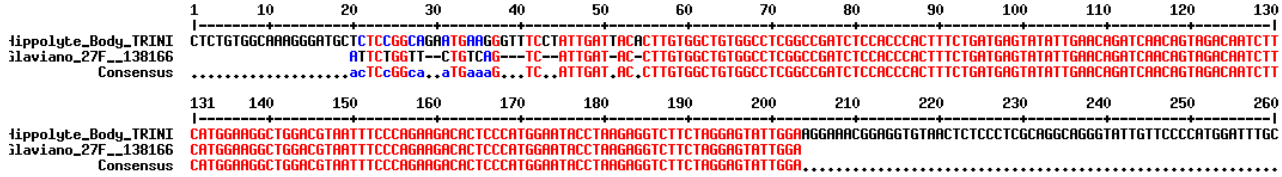
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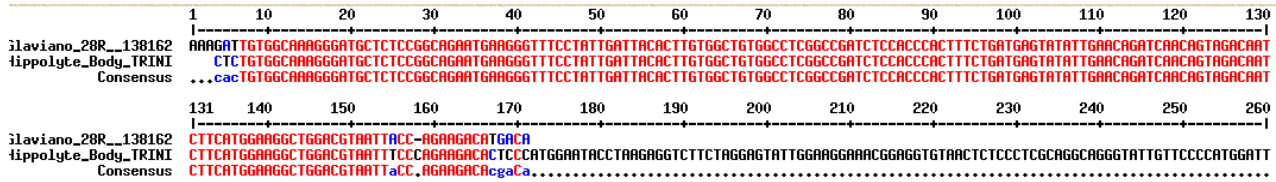
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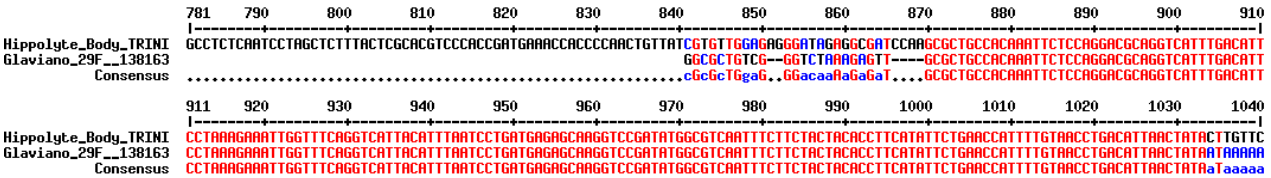

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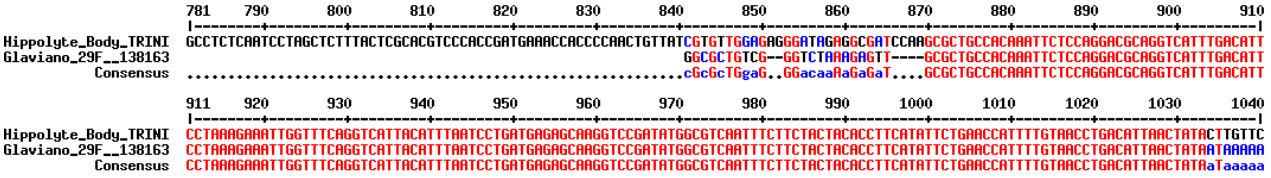

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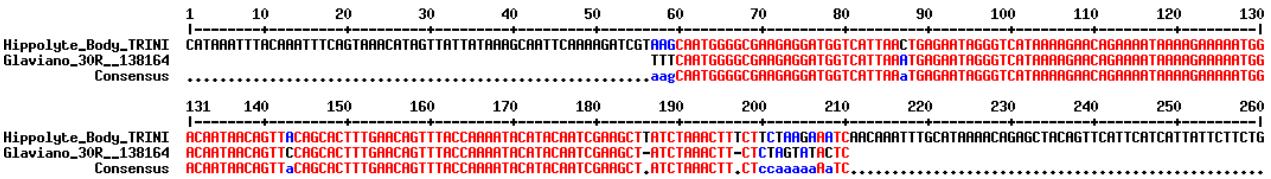

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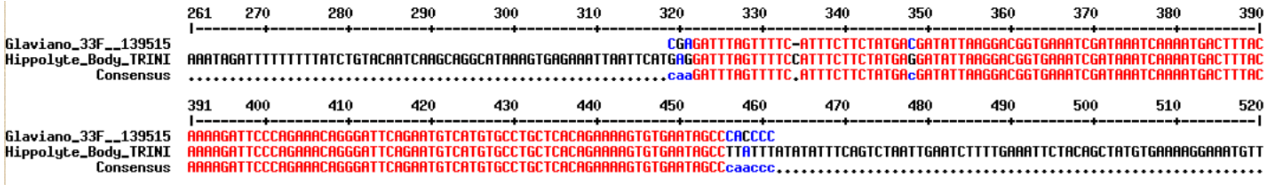

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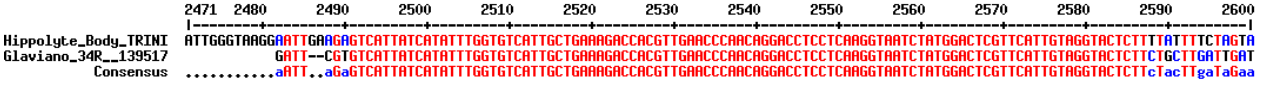


Figure S2. Alignment of primers to the original sequence of *SAT* F (A), *SAT* R (B), *SNP25* F (C), *SNP25* R (D), *DRONC* F (E), *DRONC* R (F), *TSPO* F (G), *TSPO* R (H), *STEA* F (I), *STEA* R (J), *GPX4* F (K), *GPX4* R (L), *AC* F (M), *AC* R (N), *CYT* F (O), *CYT* R (P), *ATFC* F (Q), *ATFC* R (R), *GSHI* F (S), *GSHI* T (T), *M3R* F (U), *M3R* R (V), *VAMP3* F

(W), *VAMP3* R (X), *PCLO* F (Y), *PCLO* R (Z), *CATB* F (AB), *CATB* R (AC), *HTRA* F (AD), *HTRA* R (AE), *CCKAR* F (AF), *CCKAR* R (AG) genes.

Chapter 7

Automated culture techniques applied to a continuous rearing of an ascidian *Botryllus schlosseri*

Introduction

Model organisms: rearing for scientific research

Model organisms (MOs) are commonly used in scientific studies to test biological, ecological, and evolutionary hypotheses (Brenner, 2003; Mutalipassi et al., 2019; Sahm et al., 2018). They range from bacteria to metazoans and are extensively employed as simpler systems to investigate complex scientific issues, providing solutions for challenging issues (Govind 2011; Bauermeister et al. 2018). Taking into account the relevance of model MOs for scientific research, several investigations focused on methods for improving culture procedures, involving practical aspects related to the proper maintenance techniques (Laudet & Ravasi, 2022; Mutalipassi et al., 2018). The increasing use of aquatic MOs in scientific investigations can be considered a challenge both for science and aquaculture, due to the need of optimising protocols and procedures that facilitate simpler, cheaper and more efficient facility management (Glaviano & Mutalipassi, 2022; Mutalipassi et al., 2018).

The need to monitor several chemical and physical parameters continuously, such as temperature, pH, salinity, photoperiod, light intensity, oxygen saturation, ammonia, nitrites and nitrates, imposes regularly the continuous contribution of human operators and this implies the need for a remarkable amount of skilled (consequently expensive) workers and resulting in time consuming operations (Calado et al., 2003, 2005; Mutalipassi et al., 2018). To reduce the need for personnel along with production costs, several investigations proposed automation processes. To this end, the use of sophisticated micro-controllers and programmable logic controllers (PLC) can be an

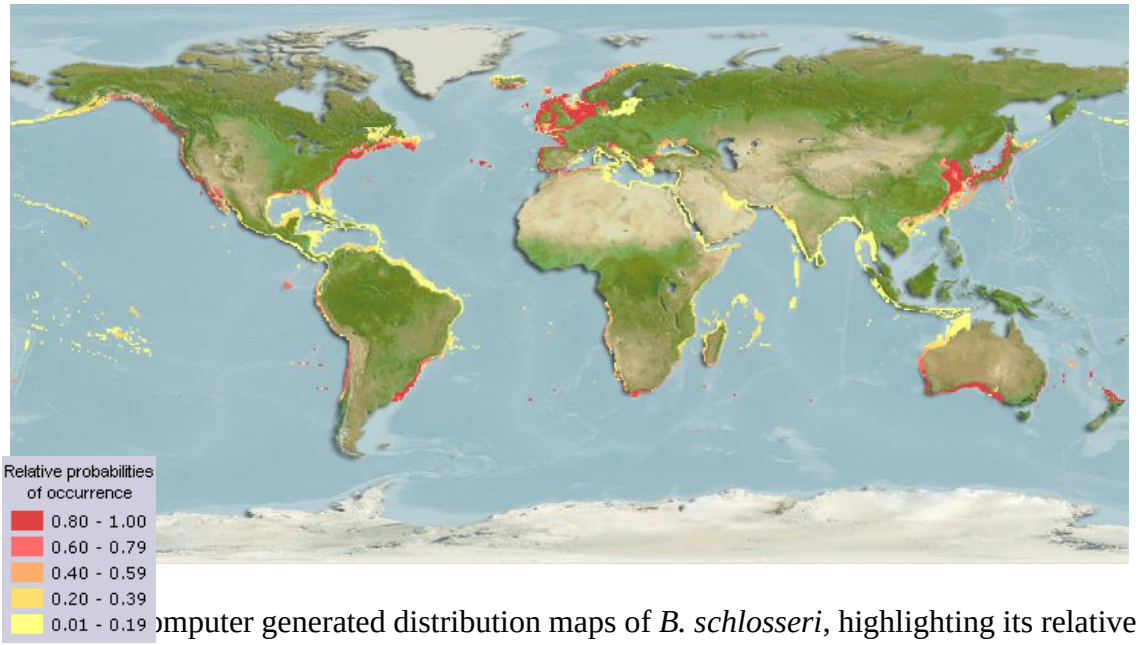
interesting alternative (Glaviano & Mutalipassi, 2022; Kolkovski et al., 2004; Mutalipassi et al., 2018). They permit to monitor several parameters at the same time (using specific probes), and to transmit collected data, by adding transmission modules to the PLC configuration. In general terms, specialised culture systems are set to meet the physiological requirements of target organism, also in order to control the concentration of pollutants and the abundance of decaying organic matter. However, they must also prevent the introduction of pathogens, and reduce stress that could alter behavioural and physiological patterns of MOs.

This study aimed at evaluating the opportunity to apply a patented programmable system (Patent W02016166696A1), developed at the Stazione Zoologica Anton Dohrn, for the rearing of the colonial ascidian *B. schlosseri*, due to its importance for the scientific research.

Botryllus schlosseri as model organism

Botryllus schlosseri is an ascidian belonging to the subphylum Urochordata. These last are considered a sister group of vertebrates (Craniata) since both are belonging to the Chordata clade Olfactores (Delsuc et al., 2006). Due to several unique characteristics, it is used by several research-groups as a model organism for several studies in the fields of genetic, physiology, age-related phenomena, developmental biology, and ecology. The class Ascidians includes both solitary and colonial species and they use several reproductive paths to produce an adult body. They have a swimming larva with structural traits that are in common with the chordates, such as the notochord, a segmented musculature, dorsal neural tube. After the embryonic development, it undergoes metamorphosis to become a sessile filter-feeding zooid, losing the above-mentioned morphological traits (Manni et al., 2007). Then, the zooid starts an ongoing,

cyclical asexual reproduction process called “budding” that can produce genetically identical individuals. After several synchronized waves of budding cycles, characterised by the regression and resorption of the previous filtering adults, the colony organised itself with all the clonal zooids (5-15) placed around a single cloacal syphon. All zooids are interconnected by a circulatory network and covered in a single tunic. On each adult zooid there are several buds growing, consisting in the next generation of juvenile budlets (Delsuc et al., 2006; Manni et al., 2019). In particular, *B. schlosseri* has emerged as an interesting species for experimental purposes also due to its peculiar regenerative plasticity (Tiozzo et al., 2006, 2008). It has become an effective model for research also for the possibility to follow in vivo long-lived primordial germ and somatic stem cells (Rosner et al., 2013). Moreover, *B. schlosseri* has also been explored as a promising model for in vivo investigations of apoptosis and toxicity because of its capacity to preserve colony homeostasis (Goldstein et al., 2021). Its wide distribution makes it relatively easy to find and be collected. In fact, these colonial ascidians are commonly found in the north-eastern Atlantic Ocean, including the coast of Europe and the Mediterranean Sea, but it is also found in the north-western Pacific Ocean, including the coast of Japan (Ben-Hamo & Rinkevich, 2021; Figure 1). It colonizes hard surfaces, such as rocks and shells, in the low intertidal and subtidal zones. It can be an ideal species for studying various aspects of marine biology and ecology. Scientists can easily collect samples of *B. schlosseri* using various techniques, such as manual sampling from harbours and cliffs as well as during underwater activities or also using a dredge from a small boat. Additionally, scientists can study the population structure and genetic diversity of the species in different regions, because of its wide distribution, and this can provide valuable insights into how different populations of this invertebrate adapt to different environmental conditions (Lee, 2002).



omputer generated distribution maps of *B. schlosseri*, highlighting its relative probabilities of occurrence (AquaMaps (2019, October, https://www.aquamaps.org.) using Sealifebase database (https://www.sealifebase.ca).

Culture systems for research purposes:

Several authors developed various culture systems for wide variety of model organisms characterized by different level of automation. These systems, ranging from ones dedicated to zooplanktonic organisms as in the case of the pelagic tunicate *Oikopleura dioica* (Bouquet et al., 2009) to the ones dedicated to vagile and sessile invertebrates, as in the case of the seagrass shrimp *Hippolyte inermis* (Mutalipassi et al., 2018), the starlet anemone *Nematostella vectensis* (Genikhovich & Technau, 2009) or the vase tunicate *Ciona intestinalis* (Zupo et al., 2020), respectively. In some cases, complex systems have been developed to the rearing and breading of vertebrate model organisms as in the case of the Anemonefish *Amphiprion ocellaris* (Ryu et al., 2022) or the Zebrafish *Dario rerio* ((Lawrence, 2007)). In the case of *Botryllus schlosseri*, due to its intrinsic traits and unique possible applications of this model organism, several attempts have been made to

improve procedures and protocols for its rearing. Culture systems dedicated to this colonial ascidians were described starting from 1955 with Sabbadin, that opened the possibility for other laboratories to adopt *Botryllus* as a model (Ben-Hamo & Rinkevich, 2021; Tiozzo et al., 2006; Voskoboynik & Weissman, 2015). Despite the increasing scientific interest in the use of *B. schlosseri* as a model organism, across various disciplines, only a limited number of laboratories worldwide maintain in continuous its colonies in captivity. These laboratories include facilities located in California at the Hopkins Marine Station, Stanford University (Boyd et al. 1986), in Italy, at the University of Padova(Brunetti & Copello, 2009) and in Israel, at the National Institute of Oceanography, Haifa (Rinkevich & Shapira, 1998). Most colonies are located in facilities having direct access to the seawater, but rearing methods vary among laboratories. Consequently, one of the major obstacles to developing a breeding stock for research is the lack of standardized methodologies and facilities for maintaining the species in captivity (Ben-Hamo & Rinkevich, 2021).

Aims:

Our primary objective was to evaluate and improve an existing programmable experimental system (Mutalipassi et al., 2018; Zupo et al., 2020) to establish an optimal culture protocol, in an automatic system dedicated to ascidians. Classic management of marine organisms in the laboratory involves simple tanks that entirely rely on constant maintenance by a human operator, providing frequent water exchanges, measurement of water parameters, and daily monitoring and supervision. In order to evaluate the performances of our automatic system, we compared the results obtained in “automated system” with “traditional protocols” involving the presence of human operators.

Mortality, growth, easy of management and healthy status were the parameters took into account to compare different systems.

Materials and methods

Collection of colonies

Colonies of *Botryllus schlosseri* were collected in the harbour of Ischia Island (Bay of Naples, Italy). They were carefully isolated, using a razorblade and a paddle from the surface of ropes and buoys. Further, they were transferred to the laboratory and the identification was confirmed observing the samples under the optical microscope. The identified specimens were gently laid down on a slide to let them attach on it, as an artificial substrate. After one week of acclimation in 15 litres tanks equipped with open flowthrough seawater, colonies were randomly sorted and distributed into replicates of two different culture systems. In addition, colonies that showed sexual maturity (visible presence of eggs, testes or embryos), were isolated in four different 15 litres tanks to obtain juveniles through sexual reproduction. For this purpose, all the surface of these tanks were covered with semi-rigid, atoxic, pvc plastic foils used as substrate for settlement of hatched tadpoles.

Due to the known effect of light stimulation on larval release (Bingham, 1997), the tanks with the mature colonies were kept in total darkness for 16h hours before exposure to intense light irradiation (Watanabe & Lambert, 1973) . After the settlement of tadpoles, the PVC plastic foils were cut and separated in the replicates of the two different culture systems (Table 1). Both adult colonies and juveniles were located in the tanks in vertical position, to avoid the precipitation of faecal pellet or algae on the specimens (BOYD et al., 1986).

	Number of tanks	Liters	Adult Colonies in each rep.	Juveniles in each rep.
Automatic system	2	60	24	27
Traditional tanks	2	60	24	27

Table 1. Schematic description of the experimental. Two different systems for cultivating *B. schlosseri* were compared. The first system was an "Automatic system" that uses two 60- Lt tanks. The second is a "Traditional system" that uses as well two 60- Lt tanks. Each tank holds 24 adult colonies and 27 juvenile colonies in each replicate The adult colonies and juveniles were divided among the replicates based on the initial number of individuals obtained from the samples and the same proportion of animals to water was maintained in each replication.

Culture systems

The "traditional" system used as a control consisted in rearing the colonies in two 60- Lt tanks with aeration into a thermostatic chamber (18 ◦C; 12:12h day: night cycle). Partial water changes (with filtered and sterilised natural sea water, prepared in dedicated tank) were administered every two days to reduce concentration of nitrogen compounds and other waste product of organic degradation. Differently, the automatic system was represented by the patented device controlled by the CCU Zelio Logic (model SRC2261BD; Schneider Electric, France). It may use both analogic and digital input channels to operate different sensors and probes, and several effectors connected via relay outputs. This culture system (Figure 2) is small, modular, and equipped with a central control unit (CCU), which can be programmed in Function Block Diagram (FDB) language to tune culture protocols and be adapted to the needs of several species (Mutalipassi et al. 2018). FDB programming permits: a) the use of "on / off" peripherals;

b) the use of analogic or digital inputs and outputs; c) the possibility to modulate the data obtained and re-elaborate them to create real complex programs; d) all of it combined with relative simplicity of programming and management as well as modification (Frey and Litz 2000). It was used to assess the feasibility of an easy and effective continuous culture of *B. schlosseri*. Using Zeliosoft programming system (Zelio Soft, Version 5.4.0, France), starting from the different input variables, it is possible to use several types of intermediate blocks to obtain the desired logic diagram to make the culture process independent from the constant presence and intervention of an operator. The culture system (Figure 2) itself consists of four 60 L tanks. The tank "A" receives continuously pumped seawater for the storage. In the tank "B" the water is sterilised and filtered. The tanks "C" and "D" are both dedicated to the culture of organisms, as replicates or also independently (if different organisms or internal conditions are imposed). To adapt the existing system, new inputs and outputs were added to pursuit this experiment after a proper reprogramming of the software. Each tank is connected to other tanks through several pumps (Multi 800, Sicce, Italy). Two water pumps placed in the tank "B" move the water to tanks "C" and "D", when operated by the CPU. In addition, each tank is equipped with a chiller (Tr-10 260 w, Teco srl, Italy) set at 18 ◦C, a canister filter (Whale 350, Sicce, Italy) loaded with perlon wool and activated carbon, two sensors of minimum and maximum level connected via 0-to-10-volt digital inputs, an aerator and a protein skimmer (Division 125, Seachem Aquavitro, Italy). Moreover, the tank "B" is equipped with an ozone generator (Ozonizer Certizon C200, Aqua-Sander, Germany) to assure sterilization of water.

Since *B. schlosseri* is usually found in low intertidal and subtidal zones (Zupo et al 2006), characterised by important tidal and wave motion, such a constant movement of water can help colonies keeping their surface properly clean (Ben-Hamo et al 2021). For this

reason, propeller pumps (XStream SDC, Sicce, Italy) have been positioned in both culture tanks (tanks A and B in figure X?) to ensure proper water movements. These pumps have the possibility of setting several programs, including the "custom wave profile", which has been applied, with sinusoidal increase and decrease of power. These pumps can be managed through any smartphone or smart device by using the app Sicce ContrAll (freely available on app stores), which connects the pumps and the smart device through a Wi-Fi connection. The app also provides real-time feedback on the status of the pumps and an alarm system in case of anomalies. Additionally, the system is equipped with fluorescent lighting over the culture tanks (12:12h day: night cycle) by means of a Sylvania Gro Lux light.

The system managed by the CCU was set up to operate automatically and coordinate all the pumps, skimmers, chillers, filters, light, and aerators. To fulfil daily partial changes of water, culture tanks were equipped with pumps, one for each tank, for the water discharge. The CCU controlled both the partial emptying of the culturing tanks “C” and “D” and their gradual refilling with previously filtered and sterilised water pumped from tank “B”. Moreover, this whole activity was continuously monitored by level sensors, to prevent the excessive draining or the overfilling of each tank. When a level sensor alerted CCU that tank “B” was emptied, using a Boolean logic subroutine activity, it was filled again with the water stored in tank A and then disinfected with ozone. The skimmer and ozoniser in tank “B” were turned on to begin the ozonation process. The ozoniser was switched off after a few hours, but the skimmer was kept running to enhance and assure the ozone removal. To ensure the disinfection and removal of any residual ozone, during the preliminary test running of the system, the redox potential was monitored three times a day, i.e., before, during, and after the planned automated ozonation operations.

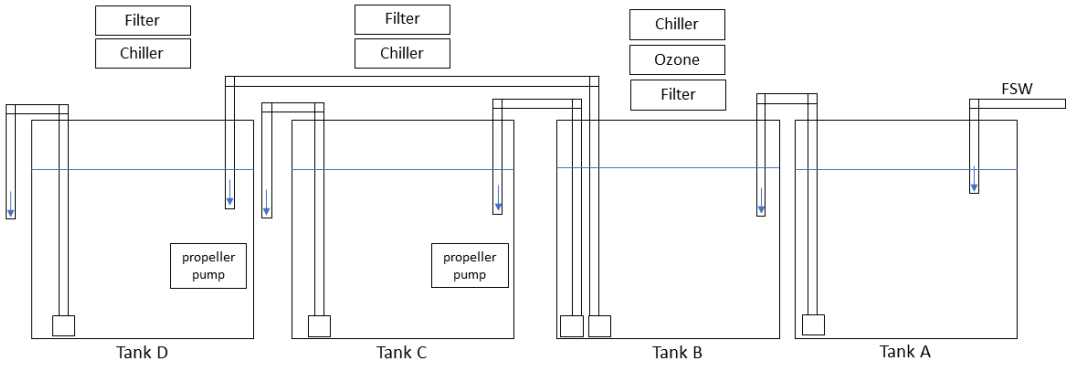


Figure 2 Schematic representation of the automatic culture system. It is composed of four 60-Lt tanks. Tank “A” is used for storing seawater that is continuously pumped in. In tank “B” the water is sterilized and filtered. Tanks “C"” and “D” are both used for the cultivation of organisms, either as replicates or independently. The tanks are connected to one another through pumps, with two water pumps in tank “B” moving water to tanks “C” and “D” as controlled by the CCU. Each tank is also equipped with a chiller, a canister filter, an aerator, and a protein skimmer. In addition, tank “B” is equipped with an ozone generator to ensure water sterilization.

Feeding

Alive microalgae, *Dunaliella tertiolecta* and *Isochrysis galbana*, obtained in axenic cultures set in the algal collections of the Stazione Zoologica Anton Dohrn and cultured in the laboratory, were used to feed the colonies (Rinkevich and Shapira 1998). Microalgae were cultivated in 5 L Erlenmeyer flasks containing Guillard's f/2 medium without silicates (Sigma-Aldrich, Milan, Italy) and kept in a thermostatic chamber (18°C, 12/12 h of photoperiod, ~1500 lux). Our batch cultures were weekly renovated to remain

in a stationary growth phase (Krishnan et al., 2015). This corresponded, for *Dunaliella*, to a density of 7×10^6 cells/ml cell (Fábregas et al., 1995) , while for *Isochrysis* it was 9×10^6 cells/ml (Fabregas et al., 1986). In the automated system, the feeding supply was administered by a peristaltic pump (G328, Grothen, China) controlled by the CCU delivering the microalgae mix (stored in the phytoplankton tank next to the system and refilled, when necessary, from the main batches) to culture tanks, to obtain 5×10^6 cells/ml final concentration in the tanks. Every 2-3 days, this concentration was monitored and adjusted manually by an operator, to maintain the required phytoplankton cellular concentration. However, also this measure could be automated by adding a turbidity sensor in future versions of the apparatus. A timer was set in the programme to deliver the algal mix to the culture tanks at a rate of approximately 50 ml/min for two minutes, two times a day. For the traditional system, the feeding was supplied by an operator in one administration every two days, in conjunction with manual water changes, following the amount of algal mix given to the automatic system, in the same ratio.

Experimental set-up

The experimental period lasted 20 days for juveniles and 60 days for adults. Every 2-3 days the specimens were monitored by an operator to evaluate growth rates and health status of juveniles. We evaluated survival rates in percentage, over time, while for adults we determined the growth rates as the increase in number of zooids of the colonies on each slide, deriving from asexual budding, during the experimental period. Specimens were observed every 2-3 days, under a MZ6 stereo-microscope (Leica, Germany), to count the specimens and monitor the health status of both juveniles and adults, as well.

To evaluate the health status of adult colonies, four categories were set, according to Brunetti et al 1980, to describe the physiological state of the specimens. A positive score

was set for each category according to a scale. According to Sharp (1984), the highest score was given to normal conditions, which could first disappear in case of adverse environmental conditions. A lower score was given to characteristics which would modify only with worse altered conditions. Lowest scores referred to different levels of abnormalities.

The features chosen to define the four categories were:

· Circulatory system; zooids are linked by a vascular network that exhibits well defined traits. We considered as anomalous every situation where the vessels connecting the zooids were collapsed or absent. Every situation in which the vessels linking the zooids were lacking or collapsed was considered as abnormal.

· Morphology of ampullae; they typically have bottle shape, so every other morphology was considered as abnormal.

· Cardiac contractions; By analysing how cardiac contractions affected blood flow, we were able to assess its effectiveness.

· General aspect of the colony; generally, the tunic is compact, but under some unusual circumstances, it may show hollows that are filled with water.

All the scores assigned to each condition are listed in Table 2.

Features	**Categories**	**Scores**
Circulatory system	normal	15
	abnormal	4
Ampullae	bottle-shaped	18
	spherical	6

	spindle-shaped	5.5
	atrophic	5
Cardiac contractions	normal	12
	not too efficient	3
	endocardiac vibrations only	1
	absent	0
General aspect of colony	normal	12
	swollen	2

Table 2. Description of the features and the categories indicating to the health status of the colonies (modified from Sharp, 1984).

Water parameters

Regular measurements of the biotic and abiotic conditions were taken throughout the experiment.

Every two to three days, measurements of the physical and chemical properties of the seawater from both the automatic and the traditional system were made. Beakers (50 ml) were used to collect water samples from each tank. Chemical tests for nitrites (NO^{2-} -N), ammonium (NH^3 -N) and phosphates were performed using a spectrophotometer and pre-prepared kits (DR/2010, Hach DR/2010, USA). Redox potential was measured using a portable ORP meter, (ORP57WP, Martini Instruments, Italy), pH was measured using a

pH portable tester (S62, Mettler Toledo, Italy) while salinity was measured through a refractometer model (Bioeropeak RFT PA Series).

Statistical analyses

Data for both the variation of number of zooids in the adult colonies, juveniles' survival rate and health status of specimens cultured in the automatic system and specimens cultured in the traditional tanks, were tested for normality and homogeneity of variances by the D'Agostino & Pearson normality test, then the significance was evaluated using paired t test performed using GraphPad Prism 8.0.0 (GraphPad Software, San Diego, USA). All the graphs and the regression lines were calculated through the same software.

Results

Water parameters

The temperature measured in the culture tanks of the automatic system during the eight weeks of the experimental period remained stable around 17.95°C (± 0.38) in the tanks of the automatic system, thanks to the chiller, and in the traditional tanks, 17.8°C (± 0.33), kept into the thermostatic chamber (Figure S1). There was no significant difference for all the replicates in each condition. Similarly, the salinity in the automatic tanks was 38.19 (± 0.4) while in the traditional tanks was 38.29 (± 0.4) with no significant difference (Figure S1). The pH, as well, in the automatic system was 8.16 (± 0.04) during the experiment while in the traditional tanks was more unstable with 8.09 (±0,8) and some drops till 7.9 in the 17^{th} and 39^{th} day (Figure S1). The unpaired t-test showed a significant difference ($p= 0.0007$). Redox potential in the automatic system was 268.96 (± 11.9) while in the traditional tanks was 261.83 (± 20.33). This difference was significant ($p= 0.0198$; Figure S1). The maximum phosphate, nitrite, and ammonium concentrations in

the automatic system were 0.15, 0.003 and 0.03 mg/l, respectively. In the traditional tanks they were 0.16, 0.004 and 0.04 mg/l (Figure S2). The t-test revealed no significant difference between replicates in each condition.

Juveniles' survival rate and health status

Our study demonstrated that both systems were characterized by a linear decrease of survival rates during the time. Specifically, the traditional tank system exhibited a rapid decrease in the number of individuals, with an average survival rate of 51.96% (±2.64) after 13 days and 26.65% (±2.02) after 20 days. Only 5.38% (±1.95) of juveniles survived until the end of the experiment. In contrast, the automatic system had significantly higher survival rates. After 13 days of culture, the replicates in the automatic system had an average survival rate of 61.8% (±3.47) and 58.86% (±2.76) after 20 days. At the end of the experiment, 41.59% (±2.24) of juveniles survived. The survival rates in the automatic system replicates were significantly higher compared to the traditional tank system ($p = 0.0034$).

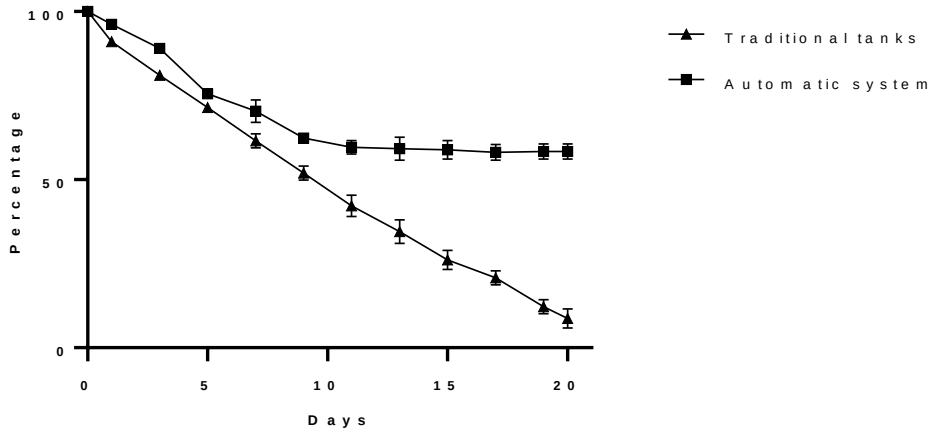


Figure 3 Juveniles' survival rate in percentage of *B. schlosseri* cultured in the automatic system and in the traditional tanks as control.

Juveniles' health status was evaluated by observations under the stereomicroscope, using the criteria outlined in Table 1. However, not all the features in the table were considered, as it was not possible to assess the circulatory system since the juveniles had not yet formed a colony. As a result, only the following criteria were considered throughout the experiment: morphology of ampullae, cardiac contractions, and general appearance of the juveniles. A score was assigned based on the different categories of each feature in Table 1, with the sum of all scores representing the general health status of the specimens in the two different systems (Figure 4). At the beginning of the experimental period, all the juveniles were in optimal condition with a score of 42. However, during the experimental period, scores for specimens reared in the traditional tanks showed a gradual decrease in all replicates. The score in the traditional tanks on the 10th day was 25 (±7.38) and on the 20th day, at the end of the experiment, it was 17.8 (±8.01). In contrast, in the automatic system, the score remained consistent throughout the experiment. On the 10th day, it was 39.6 (±5.36) and at the end of the experiment, it was 38.4 (±4.92). The difference between the two systems, according to a paired *t*-test, was significant (p=0.0168).

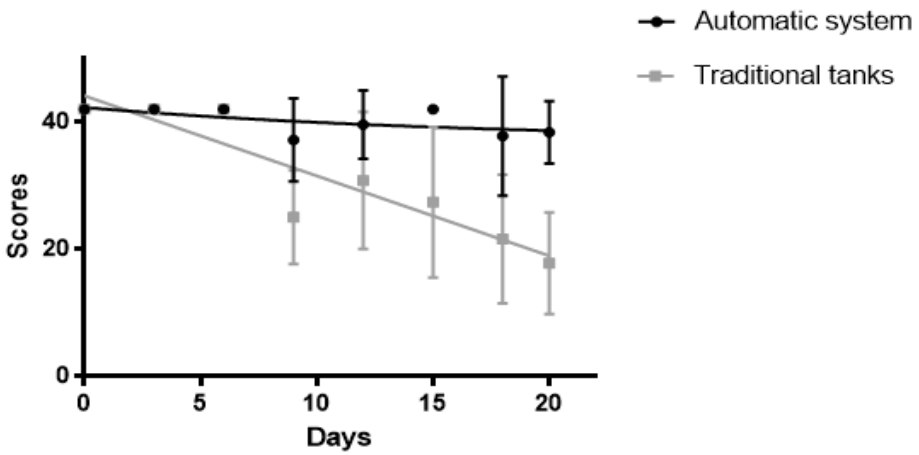


Figure 4. The health status of the juveniles in the two systems was assessed by the sum of the single scores assigned based on the categories in Table 1, according to Brunetti et al 1980. R^2 for the automatic tanks was 0.3742 while for the traditional tanks was 0.8419.

Adult growth rates and health status

The growth rates of adult colonies were evaluated by measuring the increase in the number of zooids (on average per slide). During the first week, both systems had an average of 5.1 (±1.2) zooids per slide. However, colonies cultured in the automatic system exhibited a rapid increase, reaching 17.2 (±4.54) zooids per slide by the fourth week and 34.8% (±4) zooids per slide by the eighth week (Figure 5). In contrast, the "traditional" tank system showed a relatively constant number of zooids without any significant increase, resulting in 8.4 (±1.9) zooids per slide by the fourth week and 7.6% (±3.4) zooids per slide by the eighth week. The difference between the two systems, according to a paired *t*-test, was significant (p = 0.0064).

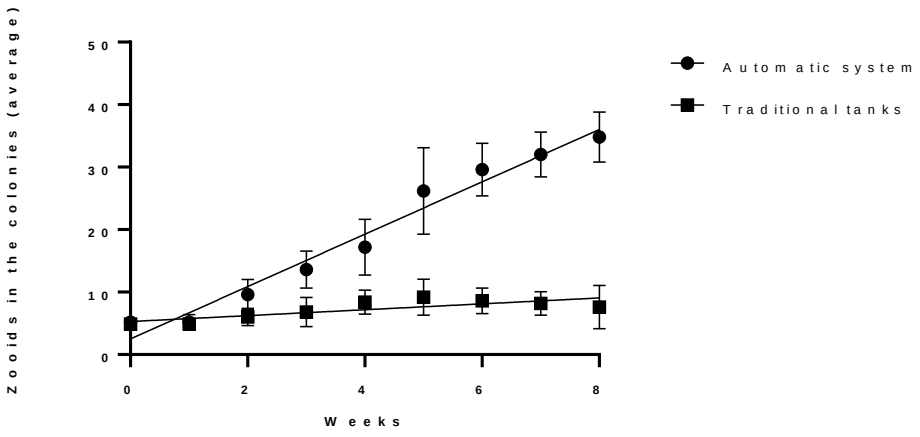


Figure 5 Time increases in number of zooids of *B. schlosseri* colonies, over each slide, cultured in the automatic system and in the traditional tanks, as controls. R^2 for the results of the automatic system is 0.9719 while for the traditional tanks is 0.6437.

The health status of colonies was evaluated by observing them under a microscope, using the criteria outlined in Table 1. A score was assigned based on the different categories of each feature in Table 1. At the beginning of the experimental period, all colonies were in optimal condition with a score of 57. However, during the eight weeks, scores for colonies reared in the traditional tanks showed a decrease in all replicates. The score obtained for the traditional tanks at 20 days was 36 (±3.95), at 40 days was 25 (±5.72), and at 60 days, at the end of the experiment, it was 12 (±3.96). On the other hand, in the automatic system, the score remained relatively consistent throughout the experiment, with a slight decrease mainly in the last 10 days (Figure 6). In detail, at 20 days, the score was 57 (±2.62), at 40 days it was again 57 (±2.94) and at 60 days, at the end of the experiment, it was 38 (±5.98). The difference between the two systems, according to a paired *t*-test, was highly significant ($p < 0.0001$).

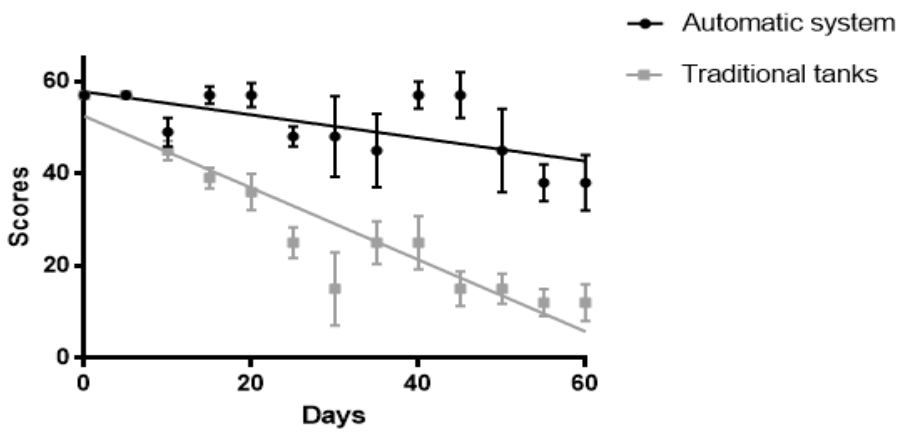


Figure 6. The health status of the adult colonies, in the two systems, was assessed through the sum of the single scores assigned based on the different categories of the features in Table 1, according to Brunetti et al 1980. R^2 for the automatic tanks is 0.446 while for the traditional tanks is 0.9342.

Discussion

This research was aimed at defining an automatic culture system sufficient to improve the rearing of small invertebrates in different life stages, in the laboratory. Automation has the purpose to lead to a type of culture that fulfils the necessities of a species while minimising the involvement of experienced biologists implied for the daily maintenance. According to this premise, we collected data on the growth and health conditions of *Botryllus schlosseri* juveniles and adult colonies, chosen as model organisms, cultured in two different systems. We compared an automatic culture system controlled by a CCU programmed *ad hoc* to optimise the culture of this species, to traditional systems consisting in simple tanks relying totally on the operator maintenance, resulting in a type of culture highly time consuming and with limited efficiency. In fact, considering the results obtained evaluating both the growth rates and health status of the specimens during two life stages, in both cases significant improvements were obtained. The survival rates of the juveniles raised in the automatic system were significantly higher than the ones cultured in the traditional thanks. Furthermore, the scores obtained observing their conditions reflected the gradual decrease in the numbers of specimens surviving in time with an important decrease, as well, in their health status.

Similarly, *Botryllus* colonies grew most rapidly in the automatic system, maintaining a constant good health status for the entire experimental period. The combination of daily automatic maintenance together with the reduced and temperature levels allowed for the growth of many contaminating organisms in this treatment tank, which apparently contributed to the eventual demise of the colonies.

Physical and chemical properties of the water recorded during the experiment to evaluate eventual influences on the specimens cultured even if, generally, *B. schlosseri* is considered a species with a wide range tolerance in chemical and physical values fluctuations in the water (Epelbaum et al., 2009; Gregorin et al., 2020). Temperatures and

salinity were stable in both the systems. On the other hand, variation in pH and ORP were significantly different.

Even if the statistical analysis provided evidence of not significant differences between the two systems, for phosphate, nitrite, and ammonium concentrations, it is still possible to observe that the values obtained in the case of the traditional tanks, characterized by lower frequency of water changes, had a larger fluctuation range, leading to a slightly worse quality of water. Nonetheless, there is still lack of data on this species susceptibility to nitrite and nitrate pollution, although its spread in the harbour and along coastlines (where river outflows release significant amounts of ammonium forms into the seawater) suggests that it is not particularly sensitive to these pollutants (Gregorin et al., 2020).

On the other side, the consideration that *B. schlosseri* is a species that lives in areas very affected by the movement of water (Young et al 2012), we can hypothesise that the reduction of individuals who survive over time and the reduction in their grow rate could be mainly related to the inadequate water movement in the traditional tank, supporting previous studies (Brunetti & Copello, 1978; MILKMAN, 1967). In fact, biofilms and debris on the surface of colonies can induce obstruction of the inhaling oral syphon, impeding normal feeding and even triggering premature death. For these reasons, Boyd et al., (1986) and Rinkevich & Shapira, (1998) proposed frequent zooids delicate cleaning using a tool like a soft, tiny paintbrush. This approach is frequently used in the culture of this species; however, it is extremely time consuming and potentially harmful if not done with adequate care and delicacy. On the other hand, our automatic system has managed to successfully carry out the culture of these specimens without the need for an operator to carry out this cleaning.

Moreover, in the system there was a continuous addition of feeding alternating with daily (partial) water changes without the specimen ever being handled. On the opposite, in the

classic tank the water change and feeding are administered by the operator once every two days and often these changes involve handling the specimens. Consequently, this automatic system may represent a possible solution to automate and simplify the rearing of small invertebrates in different life stages, in the laboratory.

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Supplementary materials

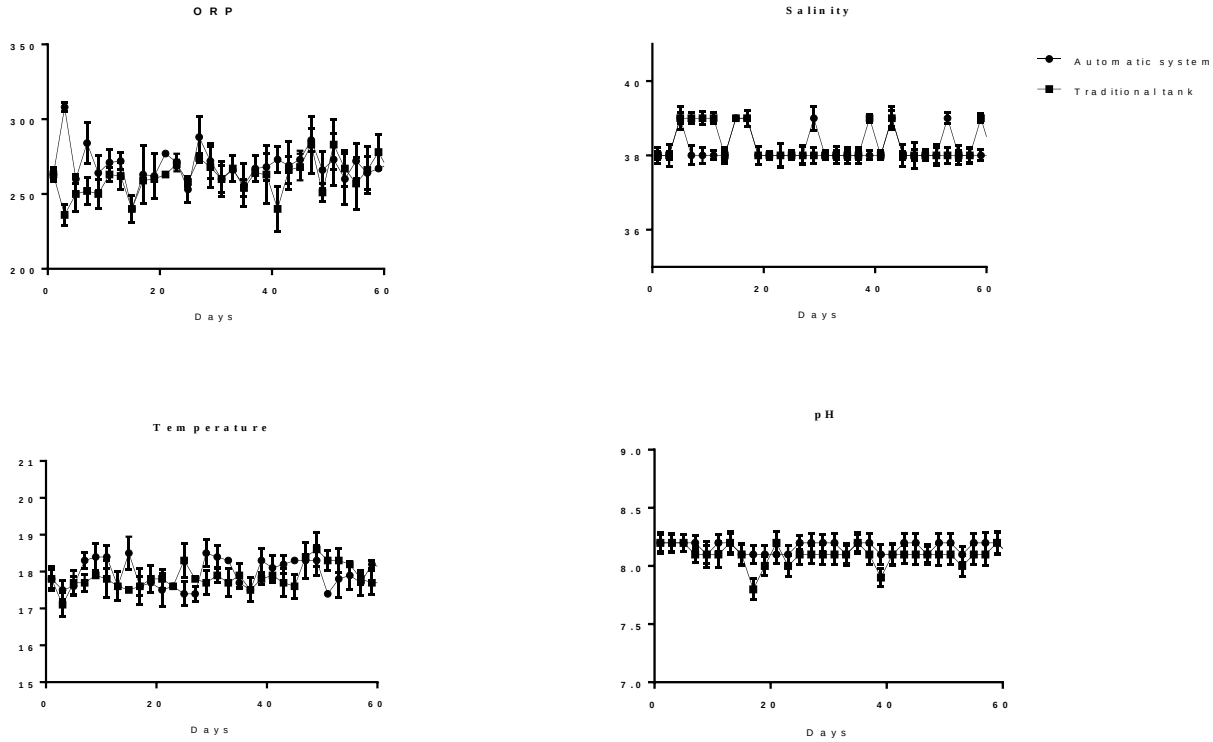

Figure S1. Average values of Temperature, Salinity, pH and ORP concentrations in the Automatic system (triangles) and in the Traditional tanks (squares).

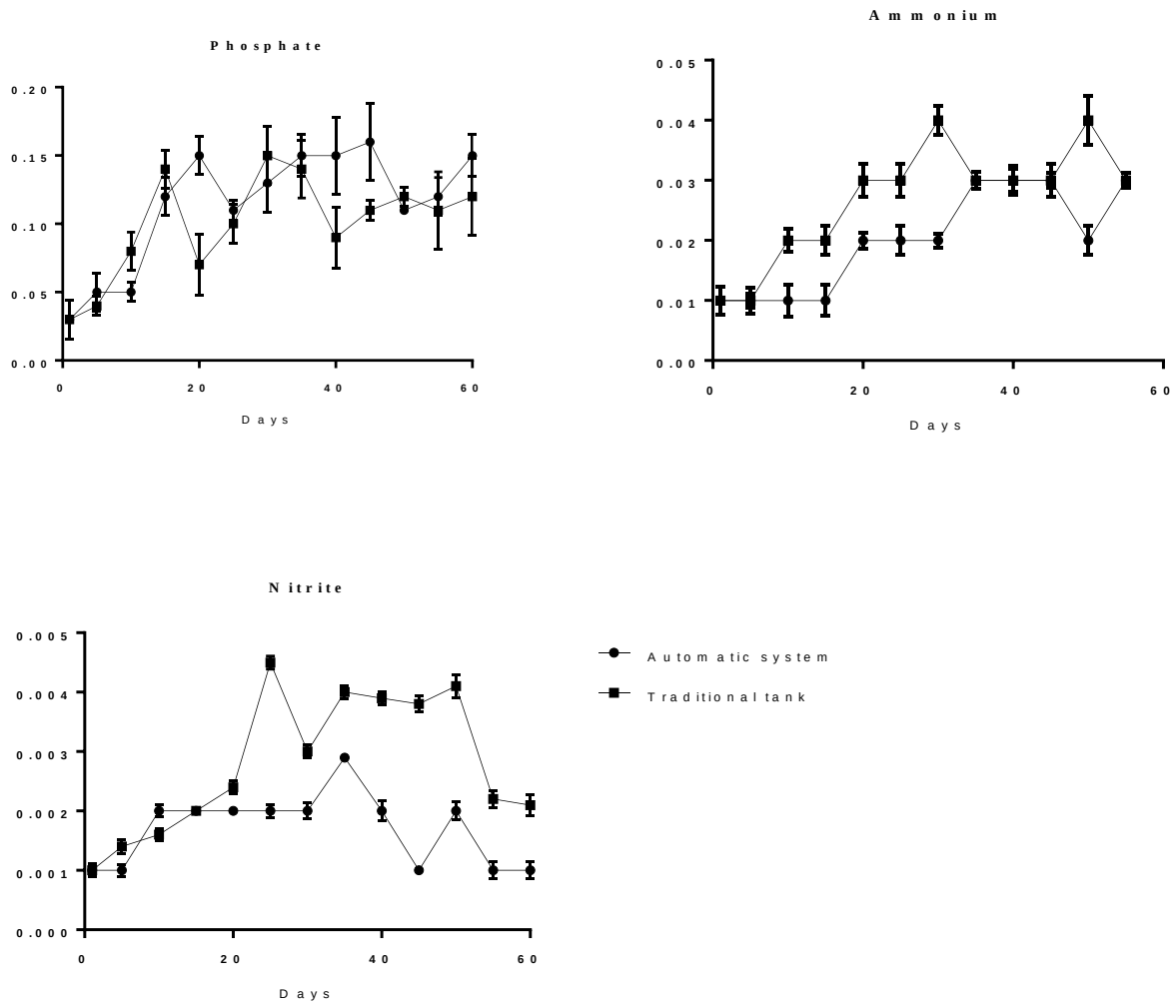

Figure S2. Weekly average of Phosphate, Ammonium and Nitrite concentrations in the Automatic system (triangles) and in the Traditional tanks (squares).

Chapter 8

A smart automated culture system for aquatic model organisms tested on the isopod *Idotea baltica basteri*

Abstract

The automation of culture systems for aquatic model organisms has the potential to transform the way scientific research is conducted. The ability to control and monitor the environment where these organisms are grown can improve the quality and the consistency of the data collected, as well as reduce the need for expensive daily care or the involvement of experienced operators. The use of a CR1000X data logger is a powerful tool in automating these systems, as it allows for the collection, analysis, and storage of data, as well as the control of various sensors and effectors.

Here, we investigated the potential set up of an automatic culture system for aquatic model organism, using a CR1000X data logger. The system was designed to control two tanks through the use of various sensors to monitor the tanks and effectors to control and react to changes in desired parameters. Our aim was to make this system fully automatic, with the ability to analyse data and adjust settings to optimize the culture and the set-up. The data logger was programmed to perform data analyses, such as calculating averages, determining maximum and minimum values, and more. This could allow for the identification of patterns and trends in the data, which can be used to optimize the conditions for the aquatic culture. Additionally, the system aimed to eliminate the need for significant daily care or the involvement of experienced operators, and to make it possible to monitor and control the system remotely. The data logger was configured to

send data via a communication protocol such as Ethernet, which allowed for remote monitoring and control of the system.

This chapter aimed at describing the details the system and its logic along with the results obtained applying it to the culture of a model species for the marine research. In order to investigate its potential use for scientific research, we tested the system with the culture of the isopod *Idotea baltica basteri*. However, this is intended to be a proof of concept, because it can be applied to other aquatic model organisms.

Introduction

Automatic culture of model organisms

Automatic culture systems have become increasingly popular in several scientific fields as a means for maintaining and studying model organisms in a controlled environment. They can offer several advantages over traditional manual methods of maintaining animals (Mutalipassi et al., 2018). These systems use computer-controlled equipment to monitor and adjust environmental conditions such as chemical and physical parameters. This ensures that the organisms are maintained in optimal conditions, which can improve the reproducibility and reliability of experimental results (Calado et al., 2008; Glaviano & Mutalipassi, 2022). One of the most significant advantages of automatic systems is their ability to automate repetitive tasks such as feeding, water changes and dynamic parameter adjustments. This reduces the need for constant human supervision and allows researchers to focus on other aspects of their research. Additionally, they can be programmed to perform complex experiments, such as exposing organisms to various environmental stressors, which would be difficult or impossible to accomplish manually (Zupo et al., 2016).

According to this evidence, researchers can use these systems to study the effects of pollution and climate change on the physiology, reproduction, and survival of specific model organisms. These studies can provide insights into the potential impacts of these environmental stressors on other, more complex, organisms, including humans (Mutalipassi et al., 2022). This is important for understanding the impacts of human activities on the coastal environment and for developing effective conservation and management strategies (Guinotte & Fabry, 2008).

Automation can be accomplished by using a combination of hardware and software tools (Vogel-Heuser et al., 2014). Hardware tools that may be used to include central control units (CCU), sensors to monitor water quality such as temperature and pH, as well as pumps and valves to control the flow of water and nutrients. Software tools that may be used include control systems to automate the process of adjusting the water flow and different parameter levels based on the sensor data (Jones et al., 2022).

The state of the art in automated culture systems for model organisms is constantly evolving, with new technologies and techniques being developed to improve their precision, efficiency, and versatility (Eckhause et al., 2015; Wilson & Sangster, 1992). One area of recent breakthrough is in the use of machine learning and artificial intelligence (AI) to optimize and control the culture system (Chang et al., 2021; Vo et al., 2021). For example, researchers are using AI algorithms to analyse sensor data and predict the growth and health of the organisms, which can be used to optimize the culture protocol over time, enabling it to learn from itself (Vo et al., 2021).

Another area of recent development is the use of microfluidic and lab-on-a-chip technologies to create highly miniaturized and automated culture systems. These systems are able to culture small numbers of organisms in very small volumes of liquid, which can be useful for applications such as high-throughput screening and toxicity testing

(Francesko et al., 2018). There are also new technologies for monitoring the organisms, such as imaging and tracking systems, which can provide real-time data on the growth and health of the organisms, and can also be used to study their behaviour and physiology (Glaviano & Mutalipassi, 2022; Ubina et al., 2021). Recent advancement is also the use of to create complex and customizable scaffolds for cell culture. This technology allows for the creation of highly porous and biocompatible structures that can mimic the microenvironment of the cells *in vivo*, which can be useful for applications such as tissue engineering (Li et al., 2014). In addition, the use of automation and robotic systems are becoming more common, with the goal of increasing the efficiency and reproducibility of experiments, and reducing the labour and expertise required to run them (Eckhause et al., 2015).

Finally, the innovation includes also the use of cloud-based data management and analysis platforms that are becoming more common, allowing researchers to easily store, share, and analyse large amounts of data, and to collaborate with others around the world (Schadt et al., 2010). Overall, the field of automated culture systems for model organisms is highly interdisciplinary and involves a wide range of technologies and techniques.

State of art

Various studies related to water quality monitoring have been conducted, as demonstrated by a study by Guerrero and Fernandez (2018) which discussed the main problems and alarms in particular in the aquaculture and water sector. Water quality is one of the critical criteria for the growth and survival of freshwater and marine life, but is often set aside by aquaculture farmers due to the lack of resources for water quality testing. A Real-time water monitoring and automation system created by Harun et al. (2018) focused on different parameters such as temperature, pH, and dissolved oxygen (DO) levels and

interfaced with aerating and water supply pumps utilizing Arduino. The data were later sent to the preferred communication or gadget at a certain period of time through the internet. Simbeye and Yang (2014) concentrated their study on temperature, dissolved oxygen, pH, ammonia, nitrates, salinity, and alkalinity that are the vital parameters needed to be monitored and regulated, since they directly affect animal's wellbeing, feed usage, growth rates and carrying abilities. A wireless sensor network (WSN) monitoring and control system was designed for this study. The research in references (Galido et al., 2019; L. K. Tolentino et al., 2017) employed ISFET and glass electrodes as devices for measuring water pH in their aquaponic systems (a combination of aquaculture and hydroponics). The smart sensors for Real-time water quality monitoring design by Cloeta et al. (2016) developed a system that can inform the user of the monitored water quality parameters in real-time setting. This system monitors various water parameters such as pH, water flow, temperature, conductivity, and ORP to detect various contaminants in water. Tolentino et al. (2019) designed a project that emphasized the importance of water quality to the community, bodies of water, and marine species. It is a profiling buoy network that allows the government and nearby industries to monitor the water quality. The network can coordinate and send information to a main station that records the data for monitoring by the use of LoRaWAN technology, employing a point-to-multipoint networking protocol utilizing the LoRa modulation scheme for its efficient and practical method of transmitting data. Nocheski and Naumoski (2018) focused on sustaining the maintainable setting of fishponds for particular fish types by doing the tasks quickly by an IoT (Internet of Things) based system. The IoT system monitors temperature, light intensity, and water level though sensors. It also uses an Arduino Mega2560 to analyse the parameters and provide sound and visual notifications to the user. For the study of Saha et al. (2018) the progression of IoT is applied and implemented for determining

water quality through the use of Raspberry Pi and Arduino (as data processors and IoT servers), different sensors, smart phone camera, and an Android app. The previous reference studies were analysed and taken into account in our study to create an innovative system that is, at the same time, easy and fully programmable, adaptable, modular and economically accessible, that can be useful for the automatic culture for research purposes.

Development of the smart automatic culture system

In our study, we aimed at investigating the potential of the Datalogger CR1000X (Campbell Scientific Inc., USA; Figure 1) as a central control unit (CCU) to enhance the performance of an innovative culture system for aquatic model organisms, that is at the same time, easy and fully programmable, adaptable, modular and economically accessible. The CR1000X (Figure 1) is a high-performance data logger that is capable of collecting and analysing a wide range of data. This makes it an ideal tool for monitoring and controlling the conditions of cultures.

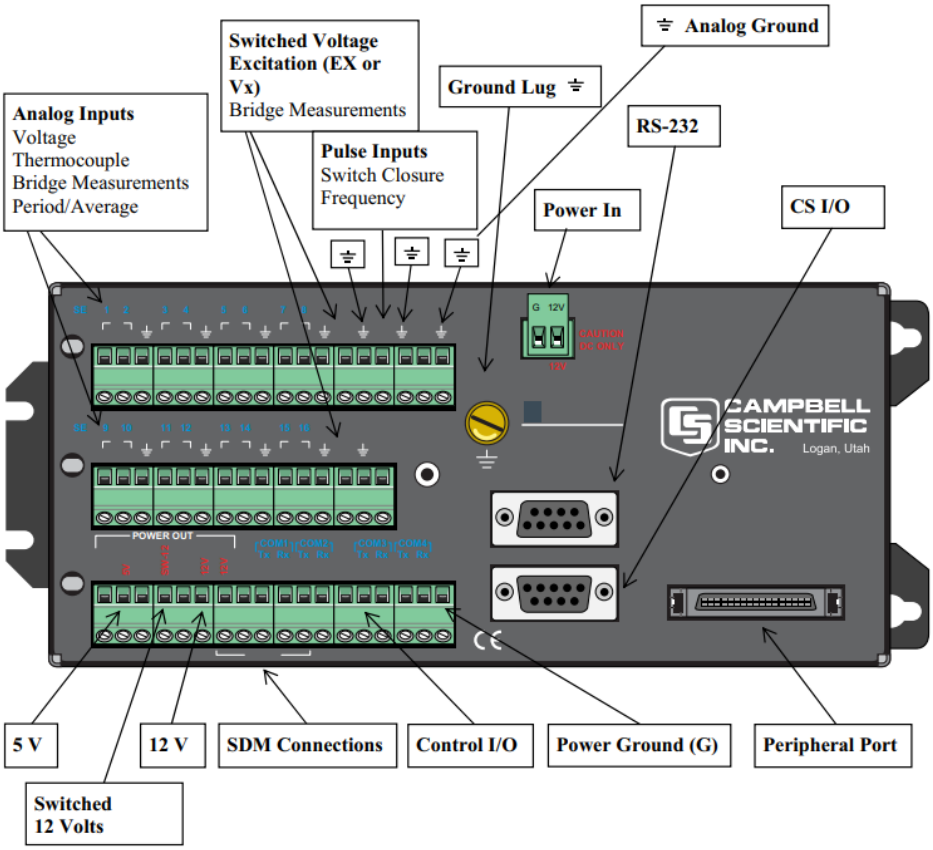

Figure 1. The Campbell Scientific CR1000X Datalogger. Image edited from the official datasheet.

The CR1000X (Figure 1) is a data logger manufactured by Campbell Scientific Inc. It features a central control unit (CCU), inputs for measuring analog and digital signals, outputs for controlling external devices, and memory for storing data. This data logger is operated by a firmware that coordinates its various functions in conjunction with its onboard clock and the CRBasic application program and it can measure a wide range of sensors, communicate with other devices, process and reduce data, perform calculations, and control external devices. Then it stores measurements in non-volatile memory and can also summarize data in statistical forms like averages and standard deviations, based on the instructions of the program. The application program for the CR1000X is written

using CRBasic, a programming language that includes measurement, data processing, and analysis routines.

Sensors convert physical phenomena into measurable electrical forms by changing voltage, current, resistance, status, or pulse output signals. This logger supports analog inputs, digital inputs, and pulse inputs. The analog inputs can be configured for voltage, current, resistance, and thermocouple measurements, while the digital inputs can be used to measure contact closures, frequency, and duty cycle. The pulse input can be used to measure frequency and period of a pulse signal. The CR1000X also has a variety of communication options, including Ethernet, Cellular, and Radio. It can be configured to transmit data to a remote location via these communication methods. Additionally, it has a built-in web server that allows users to access and configure the data logger remotely. It is also programmable using the proprietary datalogger programming language (CDL), which is a text-based language that allows users to create custom programs to control the data logger and process the data. Moreover, it also supports the use of various libraries (CRBasic, Python, and .NET) for programming and data processing.

The automatic culture system utilizes a hybrid power supply system to ensure efficient and reliable operation. The system is composed of two main components: the grid power supply and the AC-DC power supply unit (Figure 2). The grid power supply, which operates at 230VAC 50Hz, is responsible for providing power to a variety of system components such as the filter pumps, water motion pumps, chiller, and lights. This power supply is essential for the proper functioning of these components and ensures that they are able to operate at optimal levels. The AC-DC power supply unit is responsible for converting the alternate current from the grid power supply into direct current, providing 12VDC stabilized tension. This is achieved through the use of an electronic switching system, which is designed to ensure efficient and accurate power conversion. The direct

current output from the AC-DC power supply unit is then used to energize the CR1000X device.

The CR1000X is a very low energy consumption device that is used for data acquisition and sensor control. It is able to provide the right amount of energy to the sensors connected to the wiring panel, ensuring that they are able to function properly. The energy provided by the CR1000X is stabilized and can be distributed to the sensors with a controlled duration pulse. This technique, known as pulse energizing, allows the system to conserve energy and reduce sensor deterioration by only providing energy to the sensors when they are in use.

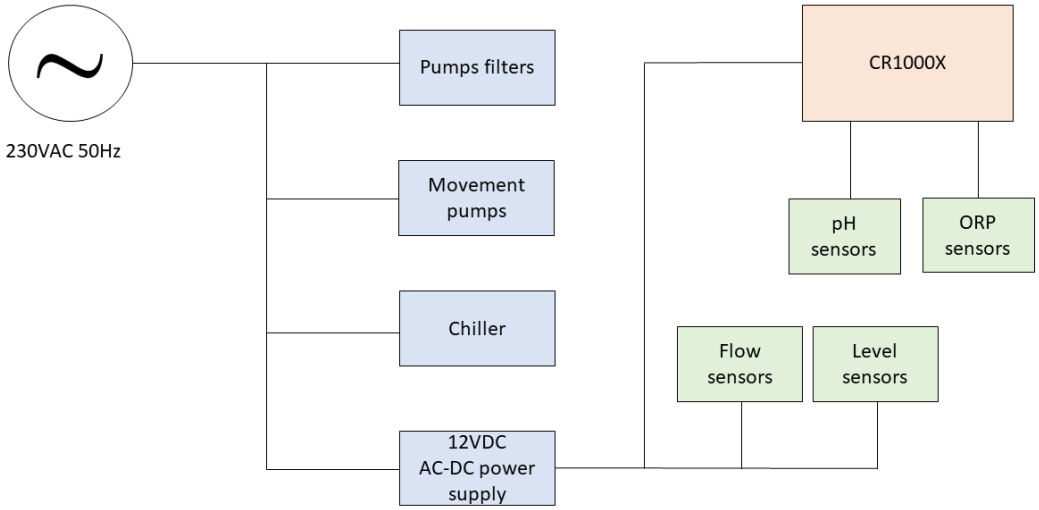

Figure 2. Block diagram of electric power distribution

Overall, the hybrid power supply system used in the automatic culture system is designed to ensure efficient and reliable operation, while minimizing energy consumption and sensor deterioration.

We equipped our automatic system with two CR1000X, one for each tank (Figure 2).

Programming with CRBasic

CRBasic is a proprietary programming language developed by Campbell Scientific for use with their data loggers. It is a text-based language with a simple syntax and a variety of built-in commands and functions. The language is designed to work seamlessly with Campbell Scientific data loggers, allowing for control and interfacing with various types of sensors and equipment, as well as data storage and retrieval. The language can support several communication protocols, such as RS-232, RS-485, and Ethernet, which allows the logger to communicate with other devices.

To program the CR1000X data loggers in our automatic culture system, we used CRBasic to control and monitor various aspects of the system, such as water temperature, pH, ORP, and water flow. Furthermore, the data logger was programmed to control equipment such as pumps, filters, and other equipment to maintain the desired conditions for the aquatic culture. In addition, CRBasic was used to log data to an external computer, for later analysis. The data logger was also configured to be able to send data (if requested) to remote locations via Ethernet communication protocol allowing: remote monitoring; remote control of the system; management of alarms via E-Mail that can be sent to a series of addresses. Moreover, the data logger can perform data analysis, such as calculating averages, determining maximum and minimum values. This allows for the identification of patterns and trends in the data, which can be used to optimize the conditions for the aquatic culture.

Culture system and sensors

The culture system consisted of two tanks of 50 L (Figure 3A): Tank A and Tank B. Both were filled with fresh sea water that had been previously sterilized and filtered. At the

center of both tanks, a cylinder (Figure 3B) dedicated to animal culture was installed. These cylinders were a modification of a standard tronco-conical larval rearing unit commonly used in aquaculture practices. It measured 350 mm in height and 180 mm in diameter, with a total volume of approximately 10 litres. It was equipped with two holes, one located laterally and measuring 36 mm in diameter, and the other located on the bottom measuring 50 mm in diameter. The lateral hole had a 50 µm net, while the bottom hole was connected to a pump (Multi 400, Sicce, Italy) in the middle of the tank that drew filtered water from the main tank, to promote water exchange in this culture area.

According to the Bureau of Fisheries and Aquatic Resources-National Inland Fisheries Technology Center (BFAR-NIFTC), the following parameters are considered necessary for water monitoring: pH Sensor, which measures the acidity or alkalinity of water and is commonly used for aquaponics, aquaculture, and environmental water testing; Oxidation-Reduction Potential Sensor, a combination sensor with a measuring electrode and a reference electrode; Water Temperature Sensor, used to measure the temperature of the water. For these reasons, each tank was equipped with a variety of other tools to ensure optimal water quality and conditions. Being an experimental project, to reduce the costs commercial sensors were used. These included:

- A chiller (Micro, Teco srl, Italy) set to 18 C° linked to a probe to constantly monitor and regulate the water temperature.
- An external canister filter (Whale 350, Sicce, Italy) filled with perlon wool and activated carbon to remove impurities and debris. It was equipped with a water flux sensor (YF-S201, Arceli, Italy) to monitor the flow of water through the filter, ensuring that it was operating at the proper level. This could help to prevent issues such as clogging.
- An under-gravel filter as an additional filtration system.

- Two level sensors (GP2Y0A21YK0F, Sharp, Deutschland) to monitor the water levels both in the tank and in the cylinder.
- A pump (Multi 600, Sicce, Italy) connecting the culture tanks to an additional extra tank filled with distilled water.
- Aerators (Askoll Holding Srl, Italy).
- A protein skimmer (Seachem Aquavitro, Acquariomania, Italy) which is linked to an ozone generator (Ozonizer Certizon C200, Aqua-Sander, Germany) to ensure that the water is clean and disinfected. A sock micron bag filled with activated carbon was placed in the outflow of the skimmer. This was to speed up the elimination of remaining, possibly harmful, ozone and to help filter out any remaining impurities from the water before it is returned to the tank.
- A pump (XStream SDC, Sicce, Italy), installed in the tank and positioned towards the lateral opening of the cylinder, dedicated to the cleaning of the net. This pump could be activated to perform a backwash by turning it on for several seconds at a time, as needed (for example, when the cylinder level sensor detected a water level too high). This allowed to continuously eliminate any debris or impurities that may accumulate on the net, ensuring optimal filtration performance.
- An ORP probe (Lab Grade, Atlas scientific, US)
- A pH probe (Lab Grade, Atlas scientific, US)
- A fluorescent light (12:12h day: night cycle).

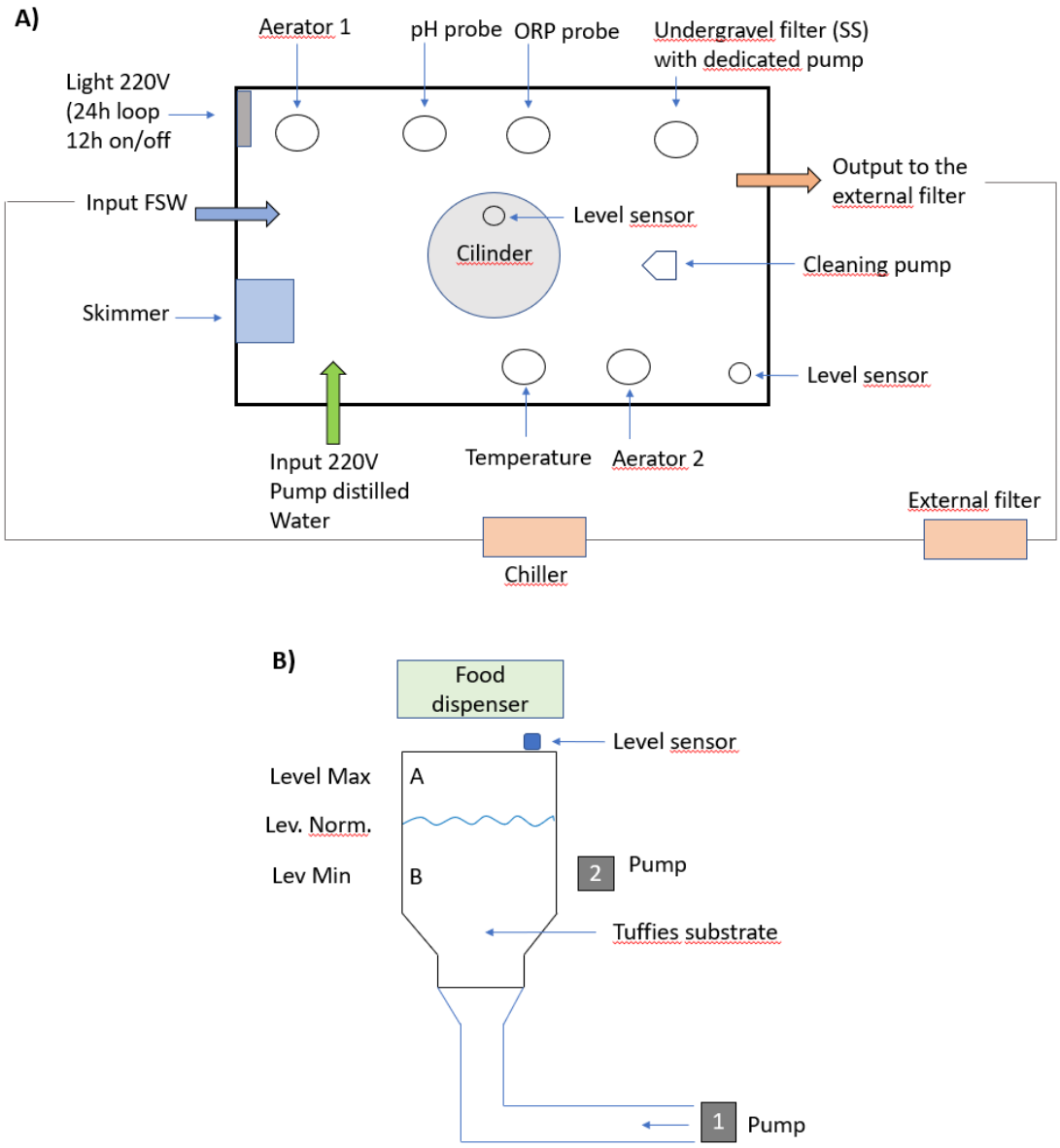

Figure 3. A) Automated culture system scheme B) Detailed scheme of the cylinder

During the culture, the system managed by the CCU was set up to operate automatically and coordinate all this equipment.

Logic configuration of the system

The data logger was programmed to follow a specific logic according to our culture protocol, to ensure optimal conditions of the cultured animals, of the tanks and the equipment present in the system. This scheme included specific parameters and variables

that needed to be monitored, as well as specific actions that needed to be taken (such as turning on or off certain equipment or adjusting settings). The logic of this scheme (Figure 4) is explained below.

The starting set-up refers to the initial configuration or arrangement of equipment and settings when a system is started. It may include turning on certain devices, adjusting settings, and configuring the system in a specific way to ensure that it functions properly and achieves the desired outcome.

The starting set-up of our system was defined as follow: The pump linked to the under gravel filter (SS) was turned on to circulate water through the tank; the external filter was also turned on to constantly filter impurities and debris from the water; the chiller was turned on to regulate the water temperature; the skimmer was turned on; the aerator was turned on; lighting was turned on and set to a 12:12 hours timer to mimic natural daylight cycles. Furthermore, the CCU received constantly inputs from various probes and sensors in the system. Based on these inputs, the CCU could determine the appropriate action to take. For example, if a sensor detected a problem, the CCU may have sent an alarm signal to alert the operator. Alternatively, if the input indicated that a certain effector should be activated, the CCU may have sent a signal to turn on that effector. This process was explained more in detail below, where the different types of input the CCU received were listed as well as the actions that the CCU took in response to each of them.

The logic followed by the CCU in explained in detail below:

- The input from the **level** sensor that monitors the level of water in the tank. If the sensor indicates that the level is normal or "OK" the CCU will continue with normal operation and no action will be taken. If the sensor indicates that the level is LOW (lower than 5mm), the CCU will activate the pump linked to the distilled water tank. The pump will continue to run until the level returns to the normal range, at which point the pump will

be turned off. If the sensor indicates that the level is below the lowest threshold, the CCU will trigger an alarm to alert the operator. Additionally, the CCU will turn off the filter, skimmer and pumps in order to prevent any further damage.

This way the CCU is able to manage the different actions needed to keep the system running and prevent any damage on it.

- The input from the **level** sensor that monitors the level of water in the cylinder. If the sensor indicates that the level is normal or "OK" the CCU will continue with normal operation and no action will be taken. If the sensor indicates that the level is HIGH (higher than 5mm from the normal level set), the CCU will activate the pump responsible for cleaning the net. The cleaning process, in backwash, is carried out by turning the pump on for a series of five cycles, each lasting three seconds. This should effectively remove any debris or impurities that have accumulated on the net, preventing the cylinder from overflowing. If the level of the cylinder is still not back to normal after the first series of cycles, the process will repeat three more times. If it still does not return to normal, the CCU will trigger an alarm to alert the operator.
- The input from the **pH** sensor. The lower limit is set on 7,9. If the sensor indicates that the level was over the threshold or "OK" the CCU will continue with normal operation and no action will be taken. If the sensor indicates that the level is LOW (lower than 7,9), the CCU will activate the second extra aerator and will trigger an alarm to alert the operator. Increasing the aeration in the aquarium can help to reduce the amount of carbon dioxide in the water. The aerator will continue to run until the level returns to the normal range, at which point it will be turned off.
- The input from the **ORP** sensor. The lower limit is set on 50 mV while the limit for the normal value is set on 100mV. If the sensor indicates that the level was over the normal value or "OK" the CCU will continue with normal operation and no action will be taken.

If the sensor indicates that the level is LOW (lower than 50 mV), the CCU will activate the ozone generator and will trigger an alarm to alert the operator. The ozone generator will continue to run until the level returns to the normal value (100mV), at which point it will be turned off.

- The input from the **Temperature** sensor. The highest limit is set on 25 °C. If the sensor indicates that the level is "HIGH" the CCU will trigger an alarm to alert the operator.
- The input from the water **Flux** sensor. If the sensor indicates that the level is normal or "OK" the CCU will continue with normal operation and no action will be taken. If the sensor indicates that the level is 0%, the CCU will turn off the filter, skimmer in order to prevent any further damage and additionally it will trigger another alarm to alert the operator.

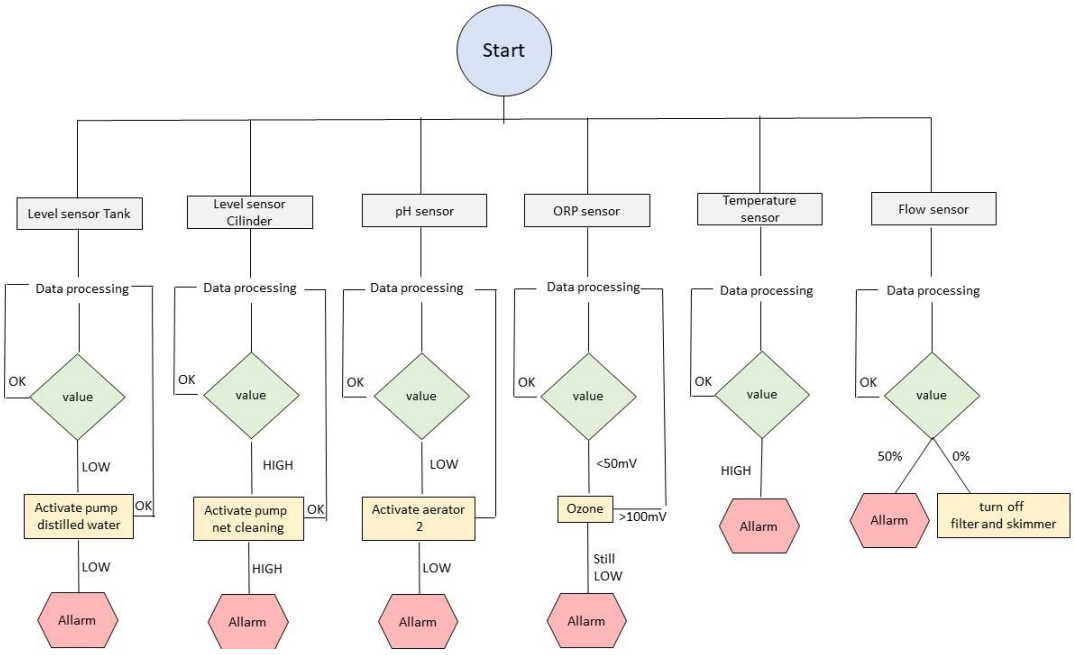

Figure 4. The overall Block Diagram of the proposed Logic in the automatic culture system.

Water parameters

Throughout the experimental time, regular measurements of the system were taken. Every three days, physical measurements of the seawater were made in both the automatic tanks. Water samples were collected using 50ml beakers from each tank. The redox potential was monitored using a portable ORP meter (ORP57WP, Martini Instruments, Italy), while pH and temperature were measured using a portable pH tester (S62, Mettler Toledo, Italy).

Experimental Evaluation

To demonstrate the reliability of the CCU sensors' data processing, we conducted an experimental evaluation where we compared the readings of the automatic sensor with those of laboratory sensors manually operated by an operator. The main objective of this comparison was to prove that the automatic measurements were providing accurate and reliable data. We chose to test both sensors in the same water environment (automatic system's tanks), this was done to eliminate any variation in the water conditions that could affect the readings. By doing this, we were able to ensure that any differences in the readings were a result of the sensors and not the environment. We conducted several concurrent measurements over the course of a month for a total of twelve measurements. The trends are illustrated in Figure 5. To confirm that the readings were comparable, we performed a series of statistical analyses. In details, data were tested for normality and homogeneity of variances by the D'Agostino & Pearson normality test, then the significance was evaluated using paired t test performed using GraphPad Prism 8.0.0 (GraphPad Software, San Diego, USA).

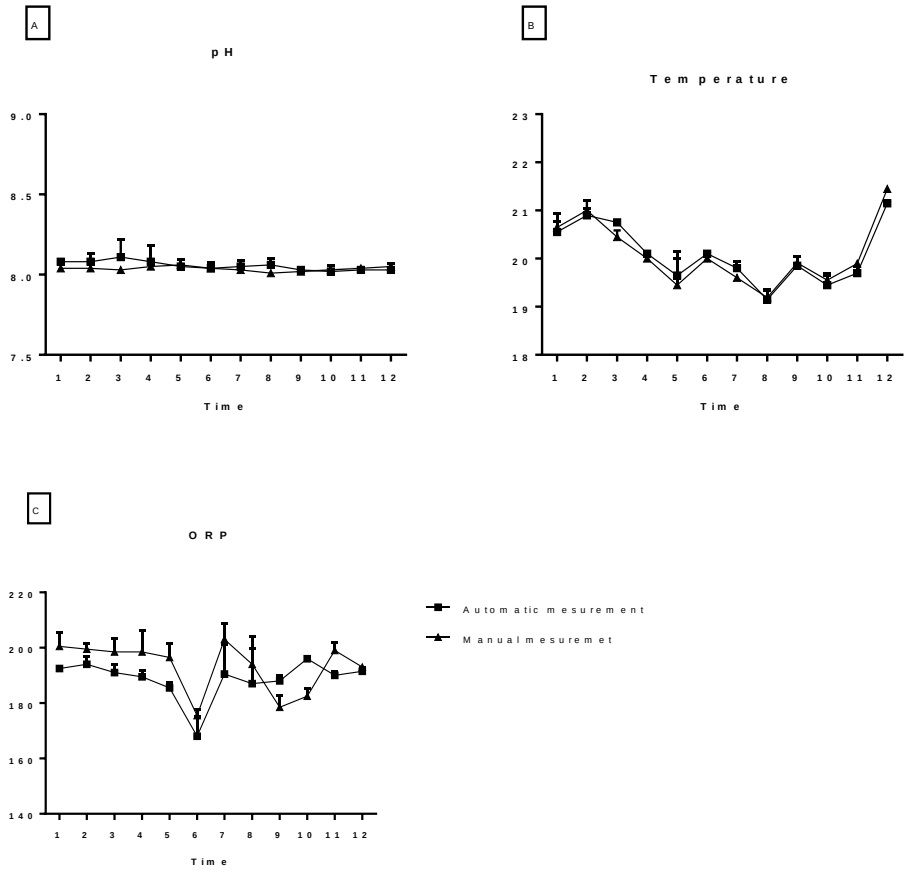

Figure 5. Trends to compare the data obtained through the automatic measurements of the system and the concurrent manual measurements of the operator.

The results of the comparison showed a high level of correlation between the readings of the automatic sensor and the laboratory sensors. This provided strong evidence that the automatic sensor was providing accurate and reliable data. The statistical analyses confirmed that the differences between the two sets of readings were not statistically significant, indicating that the automatic sensor was performing just as well as the laboratory sensors. Additionally, it was also found that the automatic sensor had several advantages over the laboratory sensors. For example, it was able to provide continuous monitoring of the water environment, whereas the laboratory sensors could only provide occasional readings. The automatic sensor was also much more convenient to use, as it

did not require an operator to manually take readings. Overall, the experimental evaluation provided strong evidence that the automatic sensor was a reliable and convenient tool for monitoring water quality.

Discussion

Automatic culture systems have become an important tool in the field of marine biology. They offer several advantages over traditional manual methods of maintaining and studying model organisms, including the ability to automate repetitive tasks, perform complex experiments, and study the effects of different environmental conditions on the physiology, reproduction, and survival of marine organisms (Glaviano & Mutalipassi, 2022; Mutalipassi et al., 2018). The use of these systems has greatly improved the reproducibility and reliability of experimental results and has allowed researchers to focus on other aspects of their research. The Datalogger CR1000X can be considered as a useful tool for monitoring and controlling the culture of aquatic model organisms. It's ability to collect and analyse data in real-time, as well as its ability to detect and alert to potential problems, makes it an essential tool for researchers working with aquatic model organisms.

Overall, our preliminary study shows that the Datalogger CR1000X is a potential valuable tool for automating and improving the culture of aquatic model organisms. Additionally, we found that the CR1000X was able to detect and alert us to potential problems, such as fluctuations in temperature or pH, before they became critical. This allowed us to take corrective action and avoid potential losses in the culture. The evaluation of the type of sensors used in the system is an important aspect of the study. In this case, commercial sensors were used due to their relatively lower cost. However, even if the results obtained from the measurements obtained both from the automatic system and the manual operator

were comparable it was found that these commercial sensors were more challenging to manage during the initial set-up from a programming perspective. This means that extra steps were needed to increase the accuracy of the measurements. This suggests that the use of commercial sensors, while cost-effective, may not be the best choice for experimental projects where fast and simple management of programming is a priority. Therefore, researchers need to be aware of the trade-offs between cost and accuracy when choosing sensors for their experimental projects.

In conclusion, even though the system described in this work is currently set up to work with two small tanks, it can be still considered a potential valuable tool for aquaculture as well. In fact, the size of the tanks does not affect the basic instrumental composition of the system. This means that the system is flexible and can be used to monitor fish farming tanks of different sizes. Additionally, it is noted that the system can be supplemented with other parameters, such as conductivity, salinity, and chlorophyll, which are important for the healthy development of the animal with are relevant for the market. These parameters are related to the water quality and can indicate the suitability of the environment for any aquaculture species. By monitoring these parameters, operators can ensure that the animals are living in conditions that are conducive to their growth and development, which is crucial for the success of the fish farming operation.

Further studies will be needed to explore the full potential of this technology and to identify the best practices for using the CR1000X in aquatic culture systems. We are currently working to test the efficiency of this automatic system on the cultivation of the *Idotea baltica* species in particular.

Idotea baltica **as a model organism**

Idotea baltica, commonly known as the Baltic isopod, is a species of isopod crustacean that is found in the Baltic Sea, in the North Sea and in other coastal regions of Europe, such as the English Channel and the North Atlantic coast (Leidenberger et al., 2012). They are generally found in rocky or sandy areas in the intertidal zone and can be found attached to rocks or other hard surfaces. They are known to feed on algae and other small organisms and are an important part of the coastal ecosystem (Orav-Kotta & Kotta, 2004). This species is a member of the family Idoteidae, which includes several other species of isopods found in coastal regions around the world (Leidenberger et al., 2020a). These isopods are known for their hard, protective exoskeleton and their ability to survive in harsh conditions such as high temperatures and low oxygen levels (Vetter et al., 1999). They are known to be able to tolerate a wide range of environmental conditions, including temperature fluctuations, waves, currents, and differing salinities, which allows them to live in a variety of environments (Wood et al., 2014). Moreover, this animal is important to the coastal ecosystem because it is a keystone species (Leidenberger et al., 2012). Keystone species play a crucial role in maintaining the structure and function of an ecosystem. They can have a disproportionate effect on the community and their loss can lead to a cascade of effects that can alter the ecosystem (Orav-Kotta & Kotta, 2004). In addition, *I. baltica* plays an important role in the food web of the coastal ecosystem as it is both a primary consumer and a secondary consumer. As a primary consumer, it feeds on algae, which are at the base of the food web, and as a secondary consumer, it is an important food source for a variety of organisms. Moreover, it is important for controlling the growth of algae in the intertidal zone, which can otherwise become overgrown and smother other organisms. They also help to control the growth of invasive species of algae that can outcompete native species. As a secondary consumer, it is an important food source for a variety of organisms, from many other invertebrates to birds such as seagulls,

oystercatchers, fish such as flounders and plaice, and other crustaceans (Leidenberger et al., 2012; Orav-Kotta & Kotta, 2004). In addition, *I. baltica* also plays a role in nutrient cycling by breaking down dead algae and other organic matter, which releases nutrients back into the ecosystem. It has also a role in maintaining the quality of the water removing the organic matter and prevents it from decomposing anaerobically, which can lead to the production of harmful compounds such as hydrogen sulphide (Leidenberger et al., 2020b).

This species is also important also for biofouling control, as they feed on microorganisms and other small organisms that attach to ships, pier pilings and other structures in the water. This prevents the build-up of these organisms, which can cause damage to the structures and promote the growth of other invasive species. *I. baltica* is often used as a model organism for scientific research due to its hardiness and ability to tolerate a wide range of environmental conditions. Moreover, it is a widely distributed species and can be found in many coastal regions around the world. In fact, model organisms are often chosen for their wide distribution in the wild (Leidenberger et al., 2012; Salemaa, 1979). This allows researchers to study the effects of pollution and climate change on organisms across different geographical regions, which can help identify regional variations in the impacts of these environmental stressors. Moreover, this widespread distribution makes it relatively easy to collect samples and, the fact that they are found in the intertidal zone which is easily accessible, facilitates even more their sampling and collection (Salemaa, 1979). This makes it possible for researchers to conduct field studies and experiments in a range of different habitats and environments, allowing them to investigate how variations in environmental conditions affect the physiology, reproduction, and survival of marine organisms. In addition, *I. baltica* is a relatively small and hardy species, making it easy to handle and maintain in laboratory conditions. All these factors make *I. baltica*

a valuable model organism and, for these reasons, it has been chosen for the tests of our smart system (Leidenberger et al., 2012, 2020b; Salemaa, 1979).

Experimental plan

Classic management of marine organisms in the laboratory involves simple tanks that entirely rely on constant maintenance by a human operator, providing frequent water exchanges, measurement of water parameters, and daily monitoring and supervision. In order to further evaluate the performances of this automatic system, we want to compare the results obtained in the continuous culture of *I. baltica* in the automated system and we will compare it with traditional protocol rearings involving the presence of human operators. Mortality, easy of management and healthy status of this species will be took into account to compare different systems. Health status in particular will be assessed through molecular approach. Several steps were necessary to optimise a molecular protocol for first obtain sufficient quality and quantity of RNA for Real Time-qPCR analysis. Then it was necessary to identify suitable housekeeping genes (for which the expression levels remain constant along all the different developmental stages of the organisms), as well as genes of interest involved in the stress response (Table 1).

Acronym	Gene name	OLIGO	Sequence 5'=>3'
ALDH	Aldehyde dehydrogenase	ALDH(NAD)_Ib_F1	GGTGGATTTGGGGGTTTCGG
		ALDH(NAD)_Ib_R1	CGGGAAACAATAGCTCTGAC
ALDH(NAD)	Aldehyde dehydrogenase	ALDH_Ib_F1	GCGAACGAGCTCGTGAGGAG
		ALDH_Ib_R1	GATGGTCTGACTTCTTTCGC
HSP 70	*Heat Shock Protein 70*	Hsp70_Ib_F1	GCCCAATCATAGCACTGAGAC
		Hsp70_Ib_R1	CGCAACAAAATGGTGTGGTC
HSP 90	*Heat Shock Protein 90*	Hsp90_Ib_F1	CTGGGAGGATTGAGTTACAG
		Hsp90_Ib_R1	CCACAGTCACTCTTCCTCCT
Casp	*caspase*	casp_Ib_F1	GCCTACGCTACAACAGTGTG
		casp_Ib_R1	CCCATCGTGCCTTCATCGATGC
Cytp450	Cytochrome P450	CytP450_Ib_F1	GGATCGCACAACTAGAAGACTG
		CytP450_Ib_R1	GATGAGAAGGTGTCTCTCGTCG
Cytc	Cytochrome	Cyt_c_Ib_F1	GATATCTCCCCGAGTGCTAG
		Cyt_c_Ib_R1	CAGGTGACATTCTGTCGCTCG

Met	Methoprene-tolerant	Met_Ib_F1	CACGCAAGGTATACTGGGATC
		Met_Ib_R1	GTGCCATACCCTGTCGATGC
GSS	*Glutamine synthetase*	GSS_Ib_F1	CATCGTACAGCAGACCCGAG
		GSS_Ib_R1	GCATTCCACACCTCTGCAGAG
GST	Glutathione-S-transferase	GST_Ib_F1	CCCATATGACGATCCTGCAAAAC
		GST_Ib_R1	GTTCCATACACAGAGCCCGTG
GSTter	Glutathione-S-transferase	GSTCter_Ib_F1	CTCCGTGATGCGAAGAGCAG
		GSTCter_Ib_R1	GAAGTCAGAGCAATGGGGCG
HIF1A	hypoxia inducible factor 1 subunit alpha	HIF1A_Ib_F1	CGCTGGATCTCGCCAGATTTG
		HIF1A_Ib_R1	CATGATCATCCGCCTCCAGCAC
NFAT	NFAT nuclear factor	NFAT_Ib_F1	GAACGCGATGATCTGGACGAG
		NFAT_Ib_R1	CGCAAGCGCAGCAGAAAGCG
Parp	Poly(ADP-ribose) polymerase 1	PARP_Ib_F1	CAGATTCCCGCACCCGCGCAG
		PARP_Ib_R1	GATTTCTGTCCTCCCACGAC
MAPK	mitogen activated kinase-like protein	MAPK_Ib_F1	GAAGATCAACATCTGCGCAGTC
		MAPK_Ib_R1	GCCCTTTTTGTCTTCGACCTC
SDH	Sorbitol dehydrogenase	SDH_Ib_F1	GTGGCAATCGCGATGGAGGC
		SDH_Ib_R1	CCGACAACGACAAGCGCTGC
TNF	Tumor necrosis factor alpha	TNF_Ib_F1	GATCGTCGTTTGACCGTGCG
		TNF_Ib_R1	TTGCAGCCACTCTGTGCATG
P53	*Tumor protein p53*	p53_Ib_F1	CGACACGCATGATTTTGCCG
		p53_Ib_R1	GTGGGTAGTGTTGGACGAGG
ABC	ATP_bind transport system	ABC_Ib_F1	TCGTGGATTCGTACAGCGAG
		ABC_Ib_R1	GAAGGTTTTCGGTAGGGGCATC
Perm	permease	Perm_Ib_F1	CCCTCGACATCTCCAAAGCA
		Perm_Ib_R1	CATCGATAACAACGGCGATGG
KIF	KIF-binding protein-like	KIF_Ib_F1	CTATCGCGCATCGAATCAGC
		KIF_Ib_R1	GCCACGATTTGCTCCACTTCG
18S	18S ribosomal RNA	18S_Ib_F1	GGTTCTGGCACAGGTCGTATAC
		18S_Ib_R1	CGGTGCAGGATGTCGTTCTG
Ubi	ubiquitin	Ubi_Ib_F1	GCGATCTCGGCTATCTCAGG
		Ubi_Ib_R1	CGGATATCGATGAACGGGGT
28S	28S ribosomal RNA	28S_Ib_F1	CTCGACGATGCGGTTATGG
		28S_Ib_R1	GGGAGGTCGGTAAAACGGAG

Table 1. Genes name, acronym, function and reference.

For each gene, specific primers were designed on the public transcriptome nucleotide sequences and PCRs (Taq High Fidelity PCR System, Roche, Italy) were performed to test the specificity of the product. PCR fragments were then purified from agarose gel using the QIAquick Gel Extraction kit and are going to be checked by DNA sequencing.

PCR products will then be aligned with gene sequences by MultAlin Software. Once verified, through all the previous steps, that the performance of the RT-qPCR reactions will be tested.

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Section 3

During my Ph.D., I spent three months abroad in Portugal collaborating with Professor Rui Rosa, who is based at Laboratório Marítimo da Guia, in Cascais and a Professor at the University of Lisbon. The goal of Professor Rosa research is to comprehend the effects of climate change on marine life, encompassing everything from cells to ecosystems, through a multi-disciplinary and comprehensive approach. His team is actually investigating the collective impact of climate change stressors such as ocean warming, acidification, and hypoxia on marine invertebrates and vertebrates of ecological significance.

In line with the objective of my Ph.D. project, I took advantage of this opportunity to engage with a new field of biological research and work with a new model organism (*Sepia officinalis*). My background allowed me to interface and optimize the experimental system for simulating hypoxic conditions to expose the model organisms for experimental purposes through programming an Arduino with C. The results shown here are preliminary and will be reviewed with additional analysis planned in our collaboration.

Lower hypoxia threshold can affect behaviour in early life stages of cuttlefish

Introduction

The global temperature is rising at an alarming rate and it is expected to increase between 3°C and 6°C by 2100 (Meehl et al., 2007). This temperature increase is expected to have negative effects on organisms at the individual level, as well as on phenology, diversity, and biogeography at the community level (Meehl et al., 2007; Rosa & Seibel, 2008). In coastal areas, many organisms already live close to their thermal tolerance limits, and ocean warming will negatively impact their performance and survival. Carbon dioxide levels in the atmosphere have also increased since the industrial revolution and are expected to continue to rise to 730–1020 ppm by 2100 (Rosa et al., 2012; Rosa & Seibel, 2008). This increase in carbon dioxide reacts with seawater, resulting in decreased pH levels, and is projected to decrease the pH of surface waters between 0.14 and 0.5 units by the end of the century. This process, known as ocean acidification, is expected to pose problems for key calcifying organisms (Rosa et al., 2012). In parallel, higher temperatures induce a decrease of the saturation concentrations for most gases, including oxygen and this may lead to hypoxia, in conjunction with other physical and biological effectors. Marine hypoxia has also become a major ecological concern in recent decades (Figure 1). Low oxygen levels in the ocean are a growing problem in many coastal and open ocean regions around the world. It is caused by a variety of factors, including excessive inputs of organic matter from human activities, as well as climate-related changes (Breitburg et al., 2018). When oxygen levels in the ocean become too low, this can lead to major declines in local biodiversity, with some areas becoming "dead zones" where the marine

life is highly impacted resulting in a very low surviving level (Levin et al., 2009; Pörtner & Farrell, 2008; Pörtner & Knust, 2007; Vaquer-Sunyer & Duarte, 2008). One of the main causes of marine hypoxia is the excessive input of nutrients and organic matter into coastal ecosystems, often from human activities such as agriculture and sewage discharge. These nutrients can lead to phytoplankton blooms, followed by death of microalgae and sink to the ocean floor, where they are decomposed by bacteria (Grantham et al., 2004; Siikavuopio et al., 2007). This process consumes large amounts of oxygen, leading to further decreases of dissolved oxygen. Additionally, climate-related changes such as ocean warming and changes in ocean circulation patterns can also lead to marine hypoxia. Warmer water holds less dissolved oxygen, and changes in ocean circulation can lead to the formation of low-oxygen zones in certain regions (Vaquer-Sunyer & Duarte, 2008).

Marine hypoxia may have several negative impacts on marine life. Many species are unable to tolerate low oxygen levels and die or migrate to other areas. Those species that can tolerate low oxygen levels may still suffer from reduced growth and reproductive potential, and early life stages are often more sensitive to oxygen stress than older life one(Levin et al., 2009). In addition to impacts on marine life, marine hypoxia can also have negative economic and societal impacts, such as decreased fish catches and reduced tourism opportunities in affected areas.

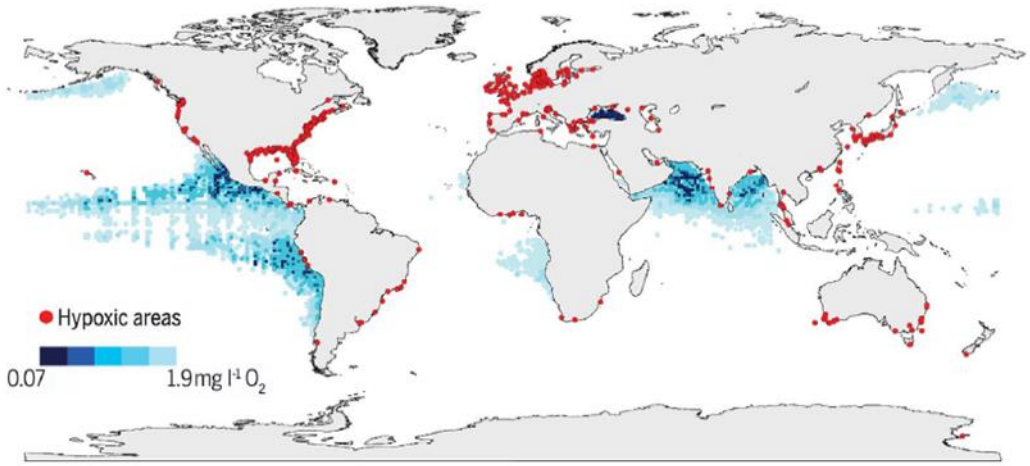

Figure 1. Local representation of zones of reduced oxygen concentration (mainly in the coastal zone of the Pacific Ocean and coastal zone of the Indian Ocean) and zones of hypoxia (distributed along the coastal zone of the continents) throughout the globe (Breitburg et al., 2018).

The combined effects of temperature and elevated CO_2 on hypoxic thresholds of marine biota are currently not well understood (Breitburg et al., 2018).

Biodiversity response to climate change: physiology and behaviour

Several studies have proven that the concentration of CO_2 has doubled compared to pre-industrial times, affecting marine life (Hoegh-Guldberg et al., 2007). According to recent studies, several species among echinoderms, bryozoans, and cnidarians showed a reduction in calcification, growth, and survival due to the decrease in pH (Kwiatkowski et al., 2020) as well as inhibition of aragonite formation (the main crystalline form of calcium carbonate deposited in coral skeletons; Hoegh-Guldberg et al., 2007). With the increase in atmospheric temperature, also the ocean has increased its annual medium temperature. This heating results in negative effects due to the increase in ocean stratification leading to a decrease in nutrient availability and subsequently a reduction in

primary production affecting phytoplankton and all primary and higher trophic levels such as zooplankton and large predators (Kwiatkowski et al., 2020; Levin et al., 2009). With climate change, the concentration of O_2 will decrease and influence the physiological and behavioural responses of various organisms, primarily obligate aerobic organisms, causing; i) a decrease in growth rates; ii) a reduction in fertility (due to the reduction of energy for the production of gametes) and iii) an increase in mortality (Hughes et al., 2020).

Exposure to different types of stress in organisms can have very negative effects, such as the case of temperature, whose increase causes a decrease in oxygen availability. This combination of phenomena will cause a change in the distribution of various fish and invertebrate species that will tend to migrate towards the poles. The increase in acidification together with the reduction of O_2 can cause a decrease in various species of fish important for human consumption (Fabry et al., 2008; Kroeker et al., 2010).

Different organisms have different thresholds of resistance to lack of oxygen, resulting these effects lethal or sublethal (Diaz & Rosenberg, 2008). These limits can be balanced with the regulation of enzymatic activity through energy metabolism, such as the negative regulation of ion pumps (Na+/K+-ATPase) (Farhat et al., 2022). The key of the adaptation, found in various organisms exposed to hypoxia phenomena, is their ability to reverse metabolic depression, reducing the production and consumption of ATP (Farhat et al., 2022).

The limitation of oxygen availability can lead to severe damage in the eye development of individuals, especially when this limitation occurs during embryonic development. Mollusca have a high level of visual complexity, but in contrast, they have less tolerance to oxygen limitation (Jereb et al., 2015). The decrease in available oxygen is an important factor for physiological limits in the visual level, specifically damaging the photoreceptor

function. This damage can affect the behaviour of individuals, their distribution, and interaction with other species. The decrease of oxygen led to a depolarization of the cellular current as a result of insufficient ATP production in squid (*Loligo pealii*), reduces the size of the eye in mussels (*Mytilus edulis*) and damages eye development in zebrafish (*Danio rerio*) leading to the absence of eyes (McCormick & Levin, 2017).

Sepia offiicinalis

Sepia officinalis, known as common cuttlefish, is a nekto-benthic decapod that is predominantly found on sandy and muddy bottoms. The species can be found from the coastline (2-3 m depth) to approximately 200 m depth, with the greatest abundance in the upper 100 m (Guerra, 2006). It has a broad distribution, ranging from the North Atlantic, throughout the English Channel, and South into the Mediterranean Sea to the coast of West Africa (Guerra, 2006; Jereb et al., 2015; Schaeffel et al., 1999).

This species is relatively tolerant to environmental changes, including salinity (Gutowska et al., 2008; Mattiello et al., 2012). Younger specimens have greater ecophysiological plasticity and can tolerate greater environmental instability, allowing them to colonize the upper zones, avoiding intraspecific competition (Pérez-Losada et al., 2002). However, they are not very tolerant of low oxygen concentrations and their temperature limits range from 10 ºC to 30 ºC. Temperatures below 10 ºC induce not feeding behaviour in the individuals, with consequent inactivity and resulting in their death in a couple of days (Guerra, 2006).

The common cuttlefish life cycle can last between 12 to 24 months but can vary depending on environmental conditions (Domingues et al., 2006). This species is gonochoristic (separate sexes) and the mating process begins with an elaborate and ritualized courtship, which includes stereotyped visual displays and "mate guarding"

(Sykes et al., 2014). During the mating season, cuttlefish undertake seasonal migrations between inshore waters (spring and summer) and offshore waters (autumn and winter) to depths of about 100 m (Sykes et al., 2014). Mature large females are typically the first to leave deeper waters and spawn, followed by mature large males. Smaller mature individuals arrive and spawn later, throughout the summer. This leads to two distinct spawning periods (Domingues et al., 2006; Guerra, 2006).

In general, the spawning period of this species occurs between late March and early December, with peaks at water temperatures between 13 °C to 18 ºC (these parameters can vary depending on location). After mating, the eggs are laid one by one through the funnel with a ring-shaped elongation of the envelope (Sykes et al., 2014) and in grape-like clusters, which simplifies the process of attaching them to seaweed, shells, debris, and other surfaces like drowned trees, cables, or nets (Reid et al., 2005; Guerra, 2006). The encased eggs are typically black or dark brown due to the ink layers added to the gelatinous envelopes, but can be translucent if there is a lack of this black pigment (Jereb et al., 2015; Schaeffel et al., 1999). The gelatinous and opaque capsule provides physical and chemical buffering between the embryo microenvironment and the surrounding, ensuring protection against microbial attack and predation. After spawning, females die, resulting in a massive post-spawning mortality and a predominance of males in the population (von Boletzky, 2003). The remaining and new individuals return to offshore waters, but the youngest animals only go to depths of about 50 to 80 m because their cuttlebones cannot withstand high water pressure (Reid et al., 2005).

Hiding in the substrate, changing colour, and changing arms disposition, with the dorsal arms stretched upwards and ventral arms downwards are the primary responses that cuttlefish has to protect itself (Messenger, 1968; Sykes et al., 2014; von Boletzky, 2003).

Another way of defence is by ink ejection, creating a cloud to push away the attacking predators (Palumbo et al., 1997, 1998, 2000).

Among the defensive responses, there is also the freezing behaviour, defined as temporary cessations of body movement or ventilation, often co-occurring with background matching and visual displays to evade predation by both visual and non-visual predators(Bedore et al., 2015; Messenger, 1968; Moura et al., 2019). Freezing has been noted in a diversity of taxa, including cephalopods. As an example, the longfin squid *Loligo pealeii* reduces its movement and settles on the seafloor to minimize its presence to predatory teleosts, thus reducing the risk of attracting their attention (Bedore et al., 2015). Cephalopods, and in particular *S. officinalis*, represent an ideal system for studying non-visual crypsis as they fall prey to a diverse group of predators, many of which employ acute, non-visual sensory modalities while foraging (Palumbo et al., 2000).

This study aimed to explore the innate chemical recognition abilities of newly hatched cuttlefish in both normal and severe hypoxia conditions. This was accomplished by exposing the cuttlefish to odors from both predator (*Scyliorhinus canicula*) and non-predator (conspecific *S. officinalis*) sources. The research aimed to shed light on the effect of hypoxia on the chemical recognition abilities of cuttlefish and its potential implications for their survival and behaviour in their natural habitats. The results of this study would provide insights into the impact of hypoxia on the sensory abilities of marine organisms and how it affects their interactions with their environment.

Materials and methods

Collections

Egg masses of the common cuttlefish *S. officinalis* were collected in October 2022 from Caldeira de Tróia, a shallow water habitat near the mouth of the Sado estuary in Portugal. The eggs were immediately transferred to aquaculture facilities in Laboratório Marítimo da Guia, Cascais, and placed in two life support systems, with 60 egg capsules per system (30 L tanks) and replenished daily with fresh seawater. The water contained in semi-closed systems was filtered (0.35 μm) and UV-irradiated, with a 14-hour light and 10-hour dark cycle. Water quality was kept sufficient through wet-dry filters (bioballs matured with nitrifying bacteria), protein skimmers (Schuran, Jülich, Germany), and UV sterilizers (30W TMC, Chorleywood, UK).

New hatched cuttlefishes were randomly divided into 6 small boxes (16 cm L × 9.5 cm W) for a total of twelve animals in each one (Figure 2).

Experimental system

Six tanks were used, of which three were used for the hypoxia treatment replicates and three as control replicates., Ten animals were kept in culture in each tank for the duration of the experimental period (Table 1). During the behavioural tests, the animals from each tank were divided equally and randomly exposed to either predator or conspecific odour stimuli (according to Mezrai et al., 2020), resulting in five animals being tested with predator odour and five animals being tested with conspecific odour for each replicate. The hypoxia treatment simulated acute exposures of severe hypoxia (2 mg L -1 O_2) according to Terova et al (2008) for 12h.

conditions	**Number of rep. tanks**	**Animals in each rep.**	**tested odours**
Control	3	10 ind	Predator (5 ind) Conspecific (5 ind)

Hypoxia	3	10 ind	Predator (5 ind) Conspecific (5 ind)

Table 1. Experimental plan. Six tanks corresponded to the hypoxia treatment and its control, resulting in three replicate each condition. In each tank, ten animals were taken in culture for all the duration of the experimental period. During the behavioural tests, individuals coming from each tank where equally divided to be randomly exposed to two different odour stimuli.

These systems were supplied with natural seawater that was pumped from the sea, filtered with a 0.35 µ net and UV radiation, and kept under a semi-closed system to minimize bacterial activity. The systems were also equipped with biological filtration using bioballs matured with nitrifying bacteria. The temperature was controlled using extra tanks, a bain-marie system, and water chillers (Hailea, Guangdong, China) to maintain an average temperature of 18°C. Room illumination was provided through overhead fluorescent lighting (MASTER TL-D Super 80, 4000K, 3350 lumen) under a photoperiod of 12:12 day night. The experimental O_2 levels were adjusted automatically using solenoid valves controlled by an Arduino system (ATmega8, Arduino, Italy) connected to individual oxygen sensors (Strathkelvin 929, Mainz, Germany) immersed in each treatment sump, and to a power bar that sends the order from the Arduino to the solenoid valves, activating or deactivating them. The O_2 of natural seawater was reduced by injecting a certified nitrogen (N_2) gas mixture (Air Liquide, Miraflores, Algés, Portugal) via air stones (Figure 2). The hypoxia exposure in accordance with Terova et al (2008) lasted 12h.

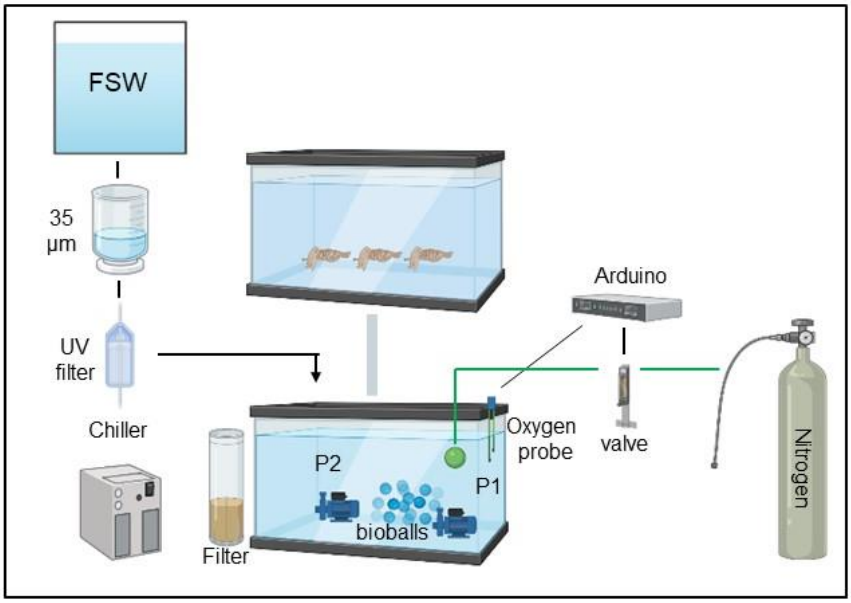

Figure 2. Experimental set up for one of the tanks for hypoxia treatment. Each replicate had an individual seawater supply with filtration of 0.35 µm net and UV filter and an individual sump with bioballs, oxygen sensor linked to the Arduino controller and one air stone to nitrogen gas mixture (only in hypoxia treatment), a pump to ensure mixing/circulation of water (P1) and another to elevate the water from the sump to the treatment tank (P2). Besides that, each sump had a sand filter and a skimmer.

To evaluate the innate chemical recognition capabilities of new hatched cuttlefishes in normal and hypoxia treatment, animals were exposed to predator and non-predatory odour. To obtain predatory odour, we used water coming from a *S. canicula* tank while for the non-predatory odour we used water coming from conspecific tank. According to previous test, the dilution factor used was 1:25.

The behaviour was evaluated in two different ways:

1) ventilation rate (VR), which is the number of ventilations per minute, rather than mantle contractions. VR was chosen as a measure because it can be used to monitor more

subtle responses to low-intensity stimuli, as suggested by Boal and Ni (1996). Additionally, decreased ventilation and bradycardia can be observed in cuttlefish after sudden visual or chemical stimulation, as reported by King and Adamo (2006). Unlike heart rate, VR is easily and directly observable in cuttlefish, either by noting the rhythmic motion of the collar flaps circulating oxygenated water to the gills, or by the movement of the funnel in response to pressure changes resulting from respiratory movements (inhalation and exhalation).

2) Behavioural response choices to the stimulus divided in: freeze, escape (sudden, erratic movement made as an attempt to flee the immediate area) and inking.

Behavioural tests

After the 12h exposure to the treatment, the new-borns were gently singularly transferred to the 50mL beaker where the behavioural tests were performed. Every individual was used in only one experimental session. The cuttlefish were placed in the beaker and acclimated for about 10 minutes, until they rested quietly on the glass bottom. The beaker was dimmed on lateral sides and on top to prevent visual inputs that could disturb the cuttlefish during the tests. Only the bottom of the beaker was left uncovered to permit the inspection by the still camera (Vixia HF R800 HD, Canon, Tokio, Japan; Figure 3). During the test, the camera was not moved to avoid adding any additional visual inputs to the animal. After 10 minutes of acclimation, the water from the tank containing predators or conspecifics (depending on the test) was gently added to the centre of the beaker using a transparent glass dropper. Previous tests were conducted to verify that this method of adding water did not cause any disturbance or reaction in the animals. Data collection was carried out by evaluating the response choices to the stimulus and manually counting the VR from videos. VR was recorded before the stimulation (VRbf), after 30

seconds (VR0.30), after one minute (VR1), after 3 minute (VR3) and after 10 minutes (VR10). Response choices to the stimulus were divided in: freeze, escape and inking.

Figure 3. Behavioural tests. Animals were recorded from the bottom with a camera. All the data were evaluated using the records.

Statistical analyses

Data were tested for normality and the homogeneity of variances by the D'Agostino and Pearson normality test. Further, the significance of differences among replicates was evaluated using two-way ANOVA. A value of $p < 0.05$ was chosen as a threshold for significance using GraphPad Prism 8.0.0 (GraphPad Software, San Diego, USA).

Results

Ventilation rate

To evaluate the innate chemical recognition capabilities of new hatched cuttlefishes, previously cultured in the experimental set up in control tanks and in hypoxia treatment

tanks for 12h, were exposed to predator and non-predatory odour. Each subject was used in a single experimental session and data was collected by observing their behaviour choices and manually counting their ventilation rate (VR) from videos. VR was recorded at several intervals: before the stimulation (VRbf), 30 seconds after stimulation (VR0.30), one minute after stimulation (VR1), 3 minutes after stimulation (VR3), and 10 minutes after stimulation (VR10). The data collected from the videos showed that there was no significant difference among all the cases. The data collected from the videos showed that there was no significant difference between animals exposed to hypoxia treatment and control animals, nor between animals exposed to the predator odour and non-specific odour. The VR remained always within the range from a maximum of 36 VR to a minimum of 31 VR (Figure 4).

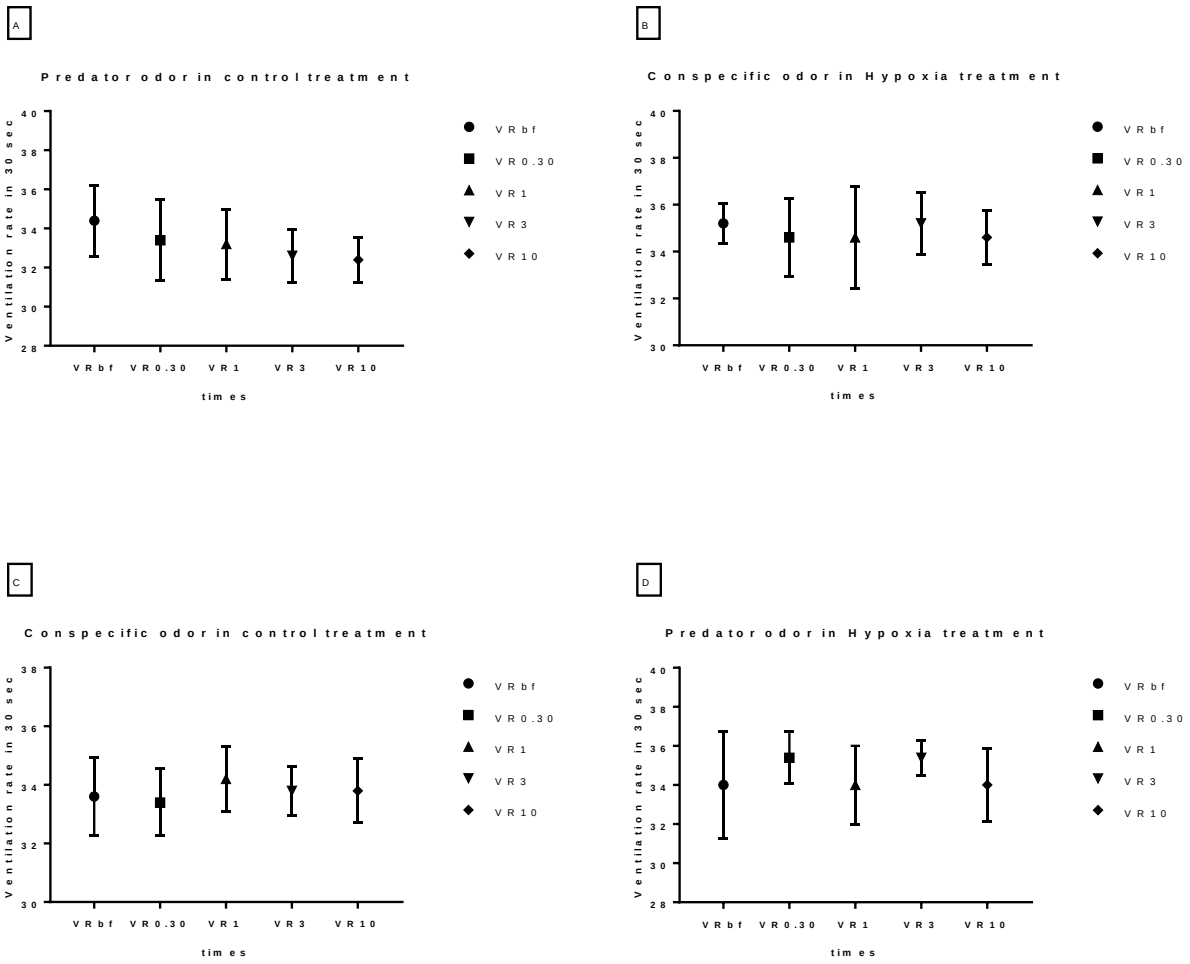

Figure 4. VR recorded at different intervals: before the stimulation (VRbf), 30 seconds after stimulation (VR0.30), one minute after stimulation (VR1), 3 minutes after stimulation (VR3), and 10 minutes after stimulation (VR10). In graph A) there are the results for animals from the control tanks exposed to predator ordour; B) there are the results for animals from the hypoxia treatment tanks exposed to conspecific ordour; C) there are the results for animals from the control tanks exposed to conspecific odour; D) there are the results for animals from the hypoxia treatment tanks exposed to predator ordour.

Behavioural response choices to the stimulus

As for the VR data, response choices to the stimulus were evaluated for each trial from the recorded videos. Response choices were divided in freeze, escape (sudden, erratic movement made as an attempt to flee the immediate area) and inking (Figure 5-6).

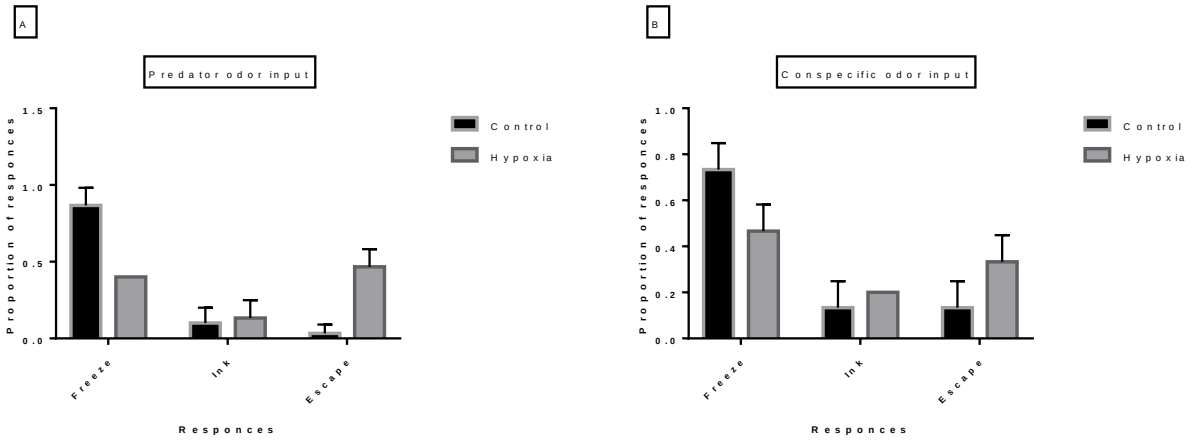

Figure 5. These graphs shows in proportion the different behavioural responses in animals coming from the control tanks and hypoxia tanks exposed to predator ordor (A) and conspecific odour (B).

Comparing the response choices coming from cuttlefish reared in the control tanks and hypoxia tanks, both exposed to predator odour stimuli, we can find a significant difference (p=0 0,0022; Figure 5A) with 90% of animals from the control that chose the freezing response against the 40% of animals which did the same choice from the hypoxia treatment. 50% of animals from hypoxia preferred the escape choice. Similarly, comparing the responses coming from cuttlefish from the control tanks and hypoxia tanks, but exposed to conspecific odour stimuli, we found again a significant difference (p=0,0474; Figure 5B) with 70% of animals from the control that chose the freezing

response against the 50% of animals which did the same choice from the hypoxia treatment.

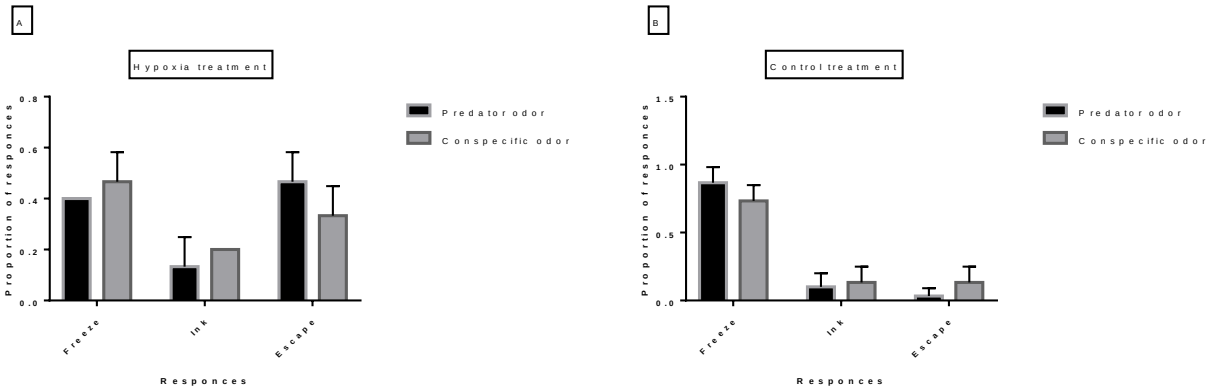

Figure 6. These graphs show in proportion the different behavioural responses in animals coming from hypoxia tanks exposed to predator and conspecific odour (A) and animals from the control tanks exposed to predator and conspecific odour (B).

On the other side, considering only cuttlefish reared in the hypoxia tanks and comparing the choices to predator vs conspecific odour stimuli, we found there was no significant difference (Figure 6A). Animals exposed to predator odour that chose to freeze where 40% versus 45% for animals exposed to conspecific odour. Similarly, considering only cuttlefish reared in the control tanks and comparing the choices to predator vs conspecific odour stimuli, we found again that there was no significant difference (Figure 6B). Animals exposed to predator odour that chose to freeze where 80% versus 75% for animals exposed to conspecific odour.

Discussion

Several studies have been conducted on various model organisms to evaluate the effects of future climate scenarios (Breitburg et al., 2018; Chan et al., 2008; Levin et al., 2009; Vaquer-Sunyer & Duarte, 2008). These studies aim to understand how changes in temperature, pH, and other environmental factors may impact the survival and reproduction of different species. Climate change is expected to have a significant impact on the world ecosystems and biodiversity, and it is important to understand how different organisms will be affected in order to develop conservation and management strategies.

Some studies have focused on the effects of ocean acidification, which is a direct result of increasing carbon dioxide (CO_2) levels in the atmosphere. As CO_2 dissolves in seawater, it creates carbonic acid, which leads to a decrease in pH. This can have a variety of negative effects on marine organisms, such as reducing growth and reproduction, altering behaviour, and impacting the development of their shells and skeletons (Diaz & Rosenberg, 2008; Fabry et al., 2008; Grantham et al., 2004; Kroeker et al., 2010; Seibel & Walsh, 2001). Other studies have investigated the effects of warming on different species, such as fish, amphibians, and reptiles. Warmer temperatures can lead to changes in the timing of reproduction, altered growth rates, and changes in the distribution of species(Helmuth et al., 2006). Some studies have also looked at the combined effects of different climate change-related stressors, such as increased temperature and ocean acidification. These studies aim at understanding how a range of stressors may interact and compound one another to affect the organisms.

Only recently, there have been new investigations focused on evaluating the effects of marine hypoxia. Hypoxia refers to low oxygen levels in the water, which can occur due to a variety of factors such as nutrient pollution and climate change (Levin et al., 2009; Vaquer-Sunyer & Duarte, 2008). Marine hypoxia can have serious consequences for marine organisms and ecosystems, as many species require oxygen to survive and thrive.

These studies aim to understand how different species and ecosystems may be affected by marine hypoxia, and how this stressor may interact with other environmental changes such as ocean acidification and warming. They investigate the changes in physiology and behaviour of the organisms exposed to low oxygen levels.

The aim of this study was to investigate how exposure to severe hypoxia could alter a fundamental behaviour such as predator response in model organism such as the cuttlefish. To evaluate the behavioural response, two different factors were considered: VR and response choices to the odour stimulus. In the case of comparing the ventilation rate obtained from all trials and with different stimuli, no significant difference was ever found. The choice of evaluating the VR was probably not good because it is a parameter that is difficult to evaluate as it had to be manually counted later on the video recorded during the trial. Since the operator must avoid any movement and any extra intervention during the test to avoid altering the behaviour response, in the case the animal moves even a little during the recording, there is a risk of losing the right focus of the camera, making it extremely complicated to accurately calculate the VR in a second moment. Instead, by taking into account the different behavioural responses such as freezing, ink or escape, the evaluation through video recording was much simpler. In this case, there was a more interesting result. Although there was no significant difference comparing the responses obtained from animals exposed to hypoxia treatment and tested with predator and conspecific odour stimuli as well as comparing responses from animals from control tanks tested with predator and conspecific odour stimuli, there were significant differences when comparing the responses between animals coming from the control tanks and hypoxia tanks exposed to predator odour and as well conspecific odour. These results show that the variable that cause the difference is not given by the stimulus of predator and conspecific odour, but rather by the exposure to hypoxia that made the animals more

prone to escape rather than settling on the bottom of the beaker. In fact, during the collection of data, the animals exposed to the hypoxia treatment were found to be much more stressed, difficult to handle and less inclined to stay still (as happened for the animals from the control group). This aspect will require further future tests and the search for a different evaluative parameter or further support for the choice of evaluating the ventilation rates.

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Section 4

General conclusions

Scientific research is aimed at discovering new information, understanding phenomena, and developing theories through the use of specific methods. It is often conducted by scientists in universities, government agencies, and private companies with the goal of expanding human knowledge, produce new ideas and understanding the natural world (Edquist, 2004; Hubbell et al., 2018). The scientific approach, while considered reliable, can have limitations such as researcher bias, lack of generalizability, and replicability issues. Additionally, scientific research can be highly time-consuming and expensive. However, the scientific approaches are continuously evolving and improving, and scientists are constantly working to address these limitations and improve the quality of research (Pinto, 2019; Schiffer, 2005).Innovation in scientific research can play a key role by leading to new discoveries and a deeper understanding of the studied subject. New and innovative approaches, such as multidisciplinary approach, can open up new areas of investigation and lead to new and unexpected discoveries that can have a significant impact on our understanding of the world (Milojevic, 2014; Schiffer, 2005).

According to Maul et al. (2017) and Payne et al. (2006), traditional protocols can have limitations in terms of “questions that can be asked”, “methods that can be used”, and the flexibility of the research design. This can lead to missed opportunities for important discoveries and potential biases in the research. To overcome these limitations, new approaches are intended to involve collaboration between researchers from different fields and the integration of different perspectives, methods, and techniques. This can lead to a more comprehensive understanding of research questions and the development of new and innovative methods.

In this thesis, I focused on marine research that is a complex and multi-disciplinary field that plays a crucial role in understanding the ocean, cover more than 70% of the Earth's

surface and play a vital role in regulating the Earth's climate, providing food and resources for human populations (Penesyan et al., 2010). Innovative methods and technology improvements played a vital role in advancing our understanding of marine ecosystems, allowing for greater access to previously difficult to study areas and organisms. Advancements in underwater imaging, remote sensing, sensor technology and automated culture systems have already provided researchers with new tools to monitor marine environment, to study marine populations, oceanographic processes, and the distribution and abundance of marine organisms (Lacroix et al., 2016; Muller-Karger et al., 2018). These technologies are also part of the intent to reform the way aquatic model organisms are cultured, allowing for precise control of environmental conditions and high-throughput experimentation, which could lead to a deeper understanding of biological processes that are common to other species, including humans.

According to these preconditions, the main objective of my thesis was to explore how innovative methods and automated culture techniques can be used to further improve the efficiency and reproducibility of marine research. The specific goals in detail were to investigate the potential of incorporating new techniques and a multi-disciplinary approach in my research, as well as to develop innovative automatic culture techniques for aquatic model organisms.

Highlighting the limitation of traditional methods and opportunities associated with innovative approaches, in Section 2, I presented studies that focused on previously approached research questions that have the potential for the introduction of new, innovative approaches. I worked mainly with the sea urchin, *Paracentrotus lividus,* as a model organism in these studies. *P. lividus* is already used to study a wide range of biological processes, including developmental biology, response to stressors, and genetics and molecular biology (Boudouresque & Verlaque, 2001, 2020; Roccheri &

Matranga, 2010). For this reason, I selected it as a valuable model organism, and I used it to approach different questions and limitations.

Conventional ecotoxicity tests are useful for identifying the harmful effects of a single pollutant on a model species but, as main limit, they do not consider the impact of a mixture of pollutants, which are commonly found in the environment (Hellou, 2011). To fully understand the impacts of complex combinations of contaminants, I proposed the introduction of mesocosm experiments as a new method of investigation, to test the physiological responses of individuals and communities in a more realistic environment. In my study, an experimental mesocosm set-up was used to investigate the effects of organic pollution on the health and reproduction on *P. lividus*. The results we obtained showed that the mesocosm (Recirculating Aquaculture System - RAS) tanks, which had limited water volume and no water changes, experienced a gradual deterioration of water quality, leading to stress responses and mortality events in the sea urchins. We found that the progressive increase of organic pollution affected the reproduction success of *P. lividus*, leading to morphological malformations, detected also by alterations in expression of genes in key pathways, in their offspring. These findings demonstrated the real potential of using realistic mesocosm experiments and molecular analyses to understand the impacts of complex combinations of contaminants on marine ecosystems and their need to lead for effective management strategies to protect them.

It is important to highlight the potential of the integration of the molecular approach in studies of various fields. The molecular approach in fact can improve them in several ways:

i) Selectivity: Molecular methods can also be used to specifically target and measure the presence or expression of genes associated with a particular physiological or biochemical

response, providing more detailed information on the specific effects of a pollutant or chemical on an organism.

ii) Sensitivity and early detection: By measuring changes at the molecular level, ecotoxicity tests can detect the effects of pollutants or chemicals before they cause visible damage, allowing for early intervention and management.

iii) Non-invasive: Molecular methods can be used to detect a response also without harm to the marine organisms (in our case had we sacrificed only a small part of the larvae obtained from the tested adults).

Additionally, *P. lividus* larvae are an important resource for scientific research as well as for aquaculture, as they are easy to maintain in the laboratory and have a consistent developmental program that is easy to follow (Castilla-Gavilán et al., 2018; Zupo et al., 2018). According to the potential results obtained with the introduction of the approach from different fields, I chose to investigate, for the first time through a molecular approach, the impact of maternal influences and culture conditions on the development and growth of this sea urchin offspring. In my study I found that both the maternal effects and the size of the culture tanks are critical to determine the reproductive success of this species, but the influence of the latter overwhelms that of the former.

Additionally, the study found that molecular analysis of the gene expression could be used as an indicator of stressful conditions for sea urchins and other marine invertebrates. We strongly believe that these findings will be useful for developing successful culture protocols for *P. lividus* for both research and commercial aquaculture purposes, but still further research is needed to improve yields and understand all the causes and effects on the urchins' development and survival.

Previous research indicated *P. lividus* as a suitable model organism for biotechnological studies such as investigating the potential of algal metabolites (Ruocco et al., 2018). The

activity of these compounds is typically tested on target model organisms using biological assays, and bioassays-guided fractionation are used to isolate, characterize, and study them to proceed for further investigate their potential applications.

Indeed, traditional approach involves observing the behaviour of target animals forced to graze on them, but this method may reveal limitations, particularly in terms of how the compounds must be administered through suitable feeds and supplements and how to preserve their characteristics María et al., 2014 (María et al., 2014).

To this end, we tested a new approach for administering algal extracts to *P. lividus* as a model organism. Encapsulation techniques were proposed as an innovative method to preserve the active compounds and administer them to an aquatic model organism. Furthermore, this technique was tested with two types of benthic diatoms, and I investigated their effects when included in alginate matrices and fed to the sea urchins. The results confirm activity already preciously noticed, demonstrating that this inclusion in alginate beads was able to perfectly preserve diatoms characteristics. This means that this method may be a useful technique for isolating diatom-derived bioactive compounds.

Ultimately, we concentrated on exploring the potential of incorporating a molecular approach in other areas of research. For this reason, we aimed to investigate the potential of using a molecular approach to better understand the mechanisms involved in the correlation between the early sexual shift in the shrimp *Hippolyte inermis* and the ingestion of *Cocconeis* spp. diatom, with the ultimate goal of optimizing the protocol for earlier identification of the molecular structure of the active compound. Traditional bioassays to identify this compound are a long and complex process that require significant manual labour, and also present difficulties such as the difficulty in finding enough ovigerous females and the risk of stress and altered biological response in the shrimp during the long culture period (Zupo & Messina, 2007). To address these

limitations, we proposed a molecular approach to early track the presence of the active compound in shrimp post larvae (five days after the settlement), which could significantly optimize the time and number of bioassays needed to identify the molecular structure of the apoptogenic compound. This could also lead to medical applications, such as developing new natural drugs for human anticancer therapies (Nappo et al., 2012).

To validate our approach we aimed at identifying key genes and molecular pathways involved in sexual differentiation by detecting the expression levels of these genes and comparing them to results obtained from the recently published transcriptome (Levy et al., 2021). Our results confirmed the expected patterns of expression, supporting our hypothesis that this new approach can support the traditional bioassay and lead to improvement in the investigations on these species and their peculiar correlation. According to the specific goals of my thesis, I also focused on the development of innovative automatic culture techniques for aquatic model organisms. In fact, the use of smart monitoring and automation in marine environments and model organism culture is already a main part in the intent to revolutionize the way we manage and exploit these resources, but further research are still needed to fully realize their potential.

For this reason, in Section 3, I delved deeper into the implementation of automatic culture techniques for aquatic model organisms. These organisms, both animal and plant, are widely used in biological research to understand the functions of life forms and in aquaculture as live foods or as targets of production. Therefore, it is important to develop flexible, programmable, and modular culture systems that facilitate the automatic production of demanding species, both for scientific and aquaculture purposes (Mutalipassi et al., 2018). In fact, the use of these devices can greatly reduce production costs and the need for personnel and fixed setups by consistently and cost-effectively repeating standard operations with high precision.

In the system outlined in chapter 7, an existing and patented system was employed and focused on its optimization to make it applicable to innovative cultures. The aim was to obtain a culture system that meets the needs of the species while minimizing the involvement of experienced biologists for daily maintenance. Data was collected on the growth and health conditions of *Botryllus schlosseri* juveniles and adult colonies to compare the results from our automatic system versus traditional system consisting of simple tanks relying on operator maintenance. Results showed significant improvements in growth rates and health status of the specimens in both life stages when using the automatic system compared to the traditional system. The automatic system also resulted in higher survival rates and better water quality. This suggests that the automatic system may be a solution to automate and simplify the rearing of small invertebrates in different life stages in the laboratory.

According to this evidence, in chapter 8, I aimed at devising a quite new system with a technologically advanced central control unit (CR1000X data logger). As previously argued, a multidisciplinary approach and collaboration from multiple fields of study can revolutionize the way to face a research demand and can also lead to the development of new and innovative techniques. In line with this goal, we collaborated with a team of engineers to make it possible. The Datalogger CR1000X is a useful tool for monitoring and controlling the culture of aquatic model organisms, with its real-time data collection and analysis capabilities and its ability to detect and alert to potential problems. Our preliminary results also confirm that CR1000X is a potential valuable tool for automating and improving the culture of aquatic model organisms. Our tests also highlighted that while commercial sensors were used due to their lower cost, they did require additional programming steps to increase accuracy. This suggests that researchers should be aware of the trade-offs between cost and accuracy when choosing sensors for their projects.

Moreover, the system described in this study, although currently set up for small tanks, can be considered a potential valuable tool for aquaculture as well. The system is flexible and can be used to monitor fish farming tanks of different sizes, and can also be supplemented with other parameters such as conductivity, salinity, and chlorophyll which are important for the healthy development of the animals and relevant for the market. Future studies will be needed to fully explore the potential of this technology and to identify best practices for using the CR1000X in aquatic culture systems. We are currently working on testing the efficiency of this automatic with the culture of *Idotea baltica* as a model organism.

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