MEMORIA CIENTÍFICO-TÉCNICA PROYECTOS COORDINADOS
Convocatoria 2022 - «Proyectos de Generación de Conocimiento»

AVISO IMPORTANTE - La memoria no podrá exceder de 35 páginas. Para rellenar correctamente esta memoria, lea detenidamente las instrucciones disponibles en la web de la convocatoria. Es obligatorio rellenarla en inglés si se solicita 100.000 € o más (en costes directos).

IMPORTANT – The research proposal cannot exceed 35 pages. Instructions to fill this document are available in the website. If the project cost is equal or greater than 100.000 €, this document must be filled in English.

1. DATOS DEL PROYECTO

TÍTULO DEL PROYECTO COORDINADO (ACRÓNIMO): *Impacto de la contaminación en la bioextracción de la ostra: Solución basada en la Naturaleza para la laguna del Mar Menor (MIToYSTER)*

TITLE OF THE COORDINATED PROJECT (ACRONYM): *Pollution impact on oyster bioextraction: Nature-based Solution for the Mar Menor Lagoon (MIToYSTER)*

DATOS DE LOS SUBPROYECTOS - ***SUBPROJECTS DATA***

SUBPROYECTO 1:
IP 1 COORDINADOR 1 (Nombre y apellidos. *Name and surname*)**:** Marina Albentosa Verdú
IP 2 COORDINADOR 2 (Nombre y apellidos. *Name and surname*)**:** Juan Bellas Bereijo
***TITLE*:** *Pollution impact on oyster bioextraction: Nature-based Solution for the Mar Menor Lagoon.*

SUBPROYECTO 2:
IP 1 (Nombre y apellidos. *Name and surname*)**:** Irrintzi Ibarrola Bellido
***TITLE*:** *Pollution impact on oyster bioextraction: Nature-based Solution for the Mar Menor Lagoon.*

SUBPROYECTO 3:
IP 1 (Nombre y apellidos. *Name and surname*)**:** Alatzne Carlosena Zubieta
***TITLE*:** *Pollution impact on oyster bioextraction: Nature-based Solution for the Mar Menor Lagoon.*

The Project described in this proposal was presented in the 2021 call for research projects of the Spanish National R+D+i Plan. The reviewers considered that the proposal was relevant and the topic was very important, being well justified in the context of the thematic priorities established in the call and of enormous relevance for the advancement of knowledge in the field of the transition of our society in accordance with the commitments of the European Green Pact. Also, the reviewers considered that the proposal can have a great scientific, social and economic impact due to the topic and the study area. Although the project achieved a good score, it was not enough to be funded. The main criticisms were related to the clarification and justification of methodological aspects related to the chemical analysis of contaminants and to the overlapping of objectives with projects of the research team, in progress at that time. For this proposal, we carried out a thorough review to make the project more viable based on feedback from reviewers. Regarding the first point, we propose converting the chemical analysis block, previously considered within subproject 1, as an independent subproject in this new proposal, led by the Applied Analytical Chemistry Group of the UDC (QANAP-UDC), reference laboratory of organic and metallic contaminants in marine matrices, especially in mass-limited samples such as those proposed in this project, and also a pioneering laboratory in the quantification of the proposed emerging contaminants. In addition, the chemical analysis potential of the Marine Pollution Research Group at the Spanish Oceanographic Institute (IEO-CSIC), which will be responsible for the analysis of traditional pollutants in the field experiment, has been highlighted. In this way, we intend to further strengthen the proposal, showing the analytical potential of the consortium. Likewise, we improved the description of the analytical methodologies to be used and the justification of the mixture of selected contaminants. Regarding the overlap with the RemediOS project (Biodiversity Foundation), in which only one of the IPs of subproject 1 participated, there was no activity, scientific objective, methodology, or any other coincident aspect between both proposals. RemediOS consisted of a proof of concept for the reproduction of the European oyster, *Ostrea edulis*, in the Mar Menor lagoon, and had no scientific objective. In addition, RemediOS ended in October 2022, so there would have been no time overlap. Finally, regarding the modelling activity, in this proposal we consider subcontracting this task, to better justify its viability.

2. *JUSTIFICATION AND CONTRIBUTION OF COORDINATION.*

The general objective of the coordinated project MIToYSTER is to contribute to effectively addressing the issue of the suitability of oysters for ecosystem restoration, in line with the goals of the United Nations Decade on Ecosystem Restoration (https://www.decadeonrestoration.org), that aims to "*halt the degradation of ecosystems that have been degraded or destroyed, as well as conserving the ecosystems that are still intact, to achieve global goals*", based on the premise that only "*healthy ecosystems can enhance people's livelihoods, counteract climate change, and stop the collapse of biodiversity*". Bivalves, in particular oysters, are species of great interest for ecosystem restoration, and the main objective of this proposal is to evaluate the effect of pollution on the biological status of the flat oyster (*Ostrea edulis*) and on its capacity to restore a eutrophic ecosystem. To this end, this proposal is based on a multidisciplinary, cross-sectional and integrated study to obtain a more complete understanding of the effects of pollution on the capacity of oysters to restore eutrophic ecosystems, using the Mar Menor lagoon as a case study.

The project originates from the collaborative work, since several years, between the IEO-CSIC, the UPV/EHU (Group of Ecophysiology and Ecotoxicology of Aquatic Organisms at the University of the Basque Country) and the QANAP-UDC, research groups that have coincided in other different projects. The confluence of the three research groups has made it possible to elaborate a proposal that will address the scientific challenge of studying the interaction between pollution and the nutrient extractive capacity of the oyster and its consequences for the restoration of degraded ecosystems. In order to achieve the project's objectives, the interdisciplinary and complementary work of the three research teams, which combines knowledge and expertise in the assessment of pollution on marine ecosystems (IEO group), in the physiological components of the energy balance of bivalves (UPV/EHU group) and in the chemical analyses of pollutants (QANAP-UDC) is essential. The IEO research group includes researchers specializing in the study of the biological effects of pollution through bioassays and biomarkers at different levels of biological organization, in the chemical analysis of pollutants in marine matrices, in the integrated assessment of pollution through the combination of chemical analysis and biological tools, and in the establishment of environmental quality criteria and standards. The UPV/EHU research group includes experts in the study of the different physiological components of the energy balance of bivalves, in the analysis of the impact of environmental and endogenous (genetic or epigenetic) parameters on the growth and reproduction capacity of bivalve populations and in aquatic toxicology of invertebrates. The QANAP-UDC group has a vast experience on the development of sensitive, selective and environmentally sustainable analytical strategies for the determination of metals and organic pollutants (priority and emerging contaminants and degradation products) in the marine environment and their implementation on monitoring studies. In addition, the consortium has a network of collaborators from other organizations including: Ramón Filgueira (University of Dalhousie, Canada) with expertise in nutrient bioextraction modelling; Eve Galimany (ICM-CSIC) with expertise in bioextraction techniques with bivalves; Luc Comeau (Fisheries and Oceans, Canada) expert in valvometry responses in bivalves; ANSE, a naturalist organization which will support field experiments; and the Native Oyster Restoration Alliance NORA, that will give support and advice on the ecosystem services provided by oysters. Staff and institutions involved in MIToYSTER are shown in Figure 1.

MIToYSTER is a multidisciplinary proposal covering: (i) a wide range of biological responses at different levels of biological organization (biochemical, cellular, physiological, behavioral, reproductive, individual), (ii) different phases of the bivalve life cycle (larvae, spat, juveniles, adults), (iii) the assessment of both pollution effects and the nutrient extraction capacity of the oyster, (iv) the chemical analysis of pollutants, and (v) laboratory and field approaches, that requires the application of a variety of specific methodologies, analytical techniques and expertise that justify the collaboration of the three groups to address the scientific challenges of the proposal.

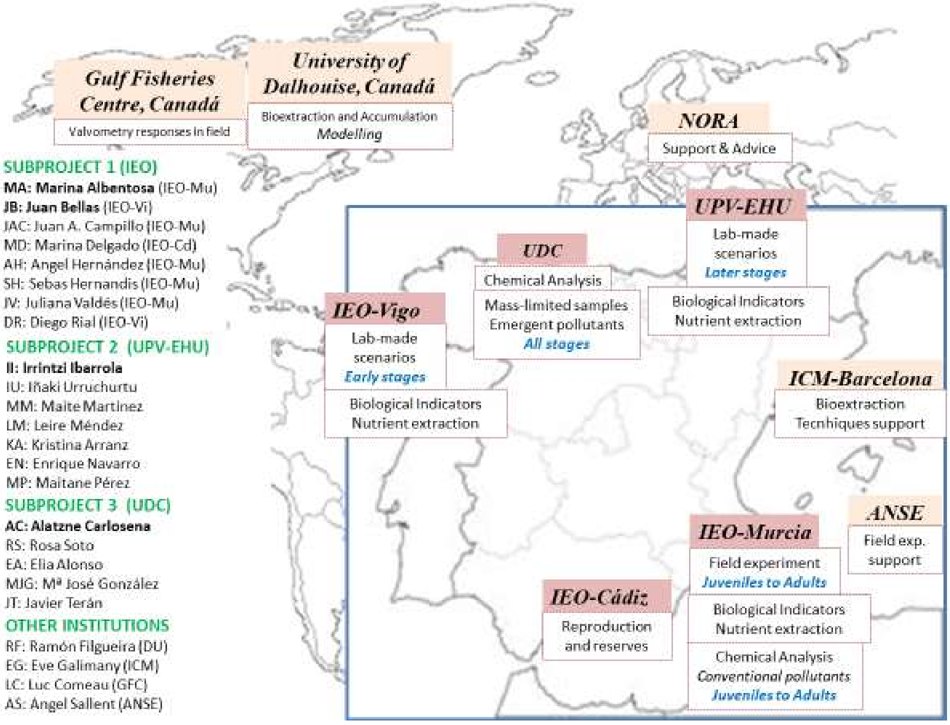

Figure 1. *Staff and institutions involved in MIToYSTER*

Interaction between the different objectives, tasks and subprojects

The project has not been conceived as a mere collection of separated scientific interests. Since the beginning, the project has been conceived as a whole, from a conceptual idea and from an integrative perspective, which would be impossible to reach by a single research team. The original ideas have been developed with the timing and responsibilities being assigned accordingly with the expertise and skills of every group. The research team of this proposal has been specifically built up to encompass the range of skills necessary to accomplish the overall aims of the project (Figure 2). It should be noted that the consortium is integrated by representative institutions in Marine Science of Spain (IEO, UPV/EHU and UDC), whose individual components have highly complementary profiles with high potential to positively interact. The project has been designed to efficiently combine the skills of the three teams, and to carry out complementary and retroactive research work, in order to fulfill the established objectives. With this aim, the project has been organized in 4 blocks structured in 7 work packages (WP): WP1, to evaluate the effect of pollution on the biological status of flat oyster throughout their life cycle; WP2, to assess the nutrient extraction capacity of the flat oyster under laboratory-simulated eutrophic conditions; WP3, to evaluate the interactive effect of pollution and eutrophication on the nutrient extraction capacity of the flat oyster in laboratory conditions; WP4, to evaluate the long-term interactive effects of pollution and eutrophication on the general health status of the flat oyster under natural conditions in the lagoon and WP5, on its nutrient extraction capacity, including its modelling; WP6, to analyze the pollutant bioaccumulation in the four oyster life stages under laboratory and field conditions; and WP7, to transfer, communicate and disseminate the project results to public authorities at national and European level, to the scientific community, and to the general public, and to develop collaborative work with NORA community. The three research groups, in addition to a high level of specialization and the necessary experience in the different research areas related to this proposal, have the complementary equipment and facilities to carry out the proposed research (see section 4). This coordinated project will be developed optimizing the synergy of the work carried out by each of the participating research groups. The information generated jointly by this coordinated project will be essential to provide advanced knowledge for the restoration of eutrophic ecosystems using Nature-based Solutions with the flat oyster *O. edulis*.

Necessity of all sub-projects

As mentioned before, the project has been conceived as a whole, so that coordination and responsibilities of tasks have been distributed following the conceptual diagram (Figure 2) and according to the experience of each team (Figure 3) as follows (more details of tasks in Section 4). Subproject 1 (SP-1, IEO) will assume the project coordination, and is responsible of WP1 (JB, IEO-Vi), WP3 (JB, IEO-Vi), WP4 (MA, IEO-Mu), WP5 (MA, IEO-Mu in collaboration with II, UPV/EHU) and WP7 (JB and MA, IEO-Vi and IEO-Mu). SP-1 will carry out the experiments in the laboratory with early life stages (larvae and spat, JB, IEO-Vi), the field experiments (from juveniles to adults, MA, IEO-Mu) and some of the chemical analysis of the field samples (JAC, IEO-Mu). SP-1 will coordinate the responses at the biochemical, cellular and behavioural level, and the assessment of pollution effects. Subproject 2 (SP-2, UPV-EHU) will coordinate the responses at the physiological, reproductive and individual level, and the assessment of the nutrient extraction capacity of the oyster. SP-2 is responsible for the WP2 (II, UPV/EHU) and WP5 (II, UPV-EHU in collaboration with MA, IEO-Mu), and leads the work done in the laboratory with late stages (juveniles and adults, II, UPV/EHU). Subproject 3 (SP-3, UDC) will carry out the chemical analysis of pollutants of all samples from the laboratory experiments and also analysis of emerging pollutants in field samples. SP-3 is responsible for the overall bioaccumulation studies included in WP6 (AC, UDC). The three subprojects, however, contribute equally to the experimental objectives and tasks. Therefore, although each subproject has its own specificities, the degree of interaction and integration between the three is very high at the team, objective and task level, which will facilitate synergies, communication and coordination, and hence all are necessary.

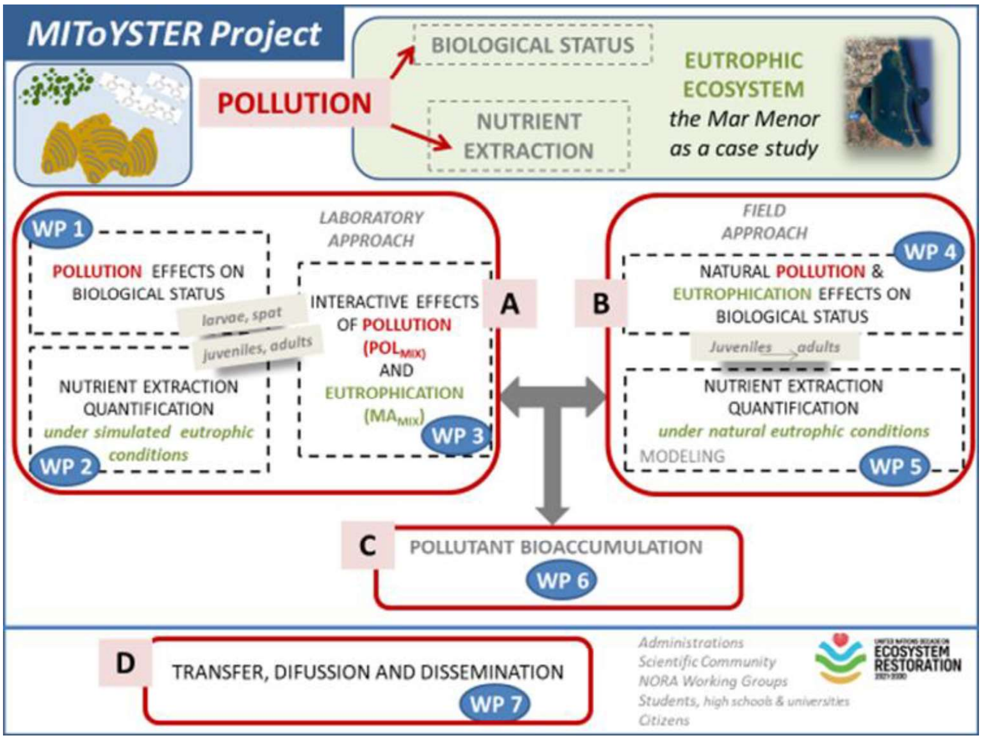

Figure 2. Conceptual approach of the Project organized in 4 blocks (A-D) structured in 7 working packages (WP)

Coordination mechanisms envisaged for the effective execution of the project.

The general coordination of the proposal will be carried out by the principal investigators of SP-1. To guarantee the effective execution of the project, close collaboration between all the components of the consortium will be needed. Therefore, the following mechanisms are foreseen: continuous supervision of the project through weekly communication between the IPs of the three sub-projects (e-mail, telephone or videoconference, as required); bimonthly on-line meetings, to ensure the degree of fulfillment of the project's objectives and the use of templates to check the progress of each task; creation of a working group on the project's intranet including the most relevant aspects and evolution of each line of work, which will allow all participants to be monitored; creation of a Dropbox platform to facilitate the exchange of documents and information between all the members of the consortium; organization of video conferences quarterly (and more frequently if necessary); and annual face-to-face plenary meetings of the whole consortium, including: a Kick-Off meeting at the beginning of the project for general planning and to define working strategies; two annual follow-up meetings, before submitting the annual report of the project, in which the different groups will present their progress, within which the results obtained so far will be evaluated; and a plenary meeting, in the last year, for a global discussion and the preparation of a synthesis paper. Each WP leader will hold specific meetings, including meetings for the planning of each task, follow-up meetings, and specific meetings prior to the plenary coordination meetings. Collaborators, interested institutions and stakeholders (e.g. regional and national government representatives, NGOs, universities), will be invited to plenary coordination meetings, and to specific WP meetings, as required.

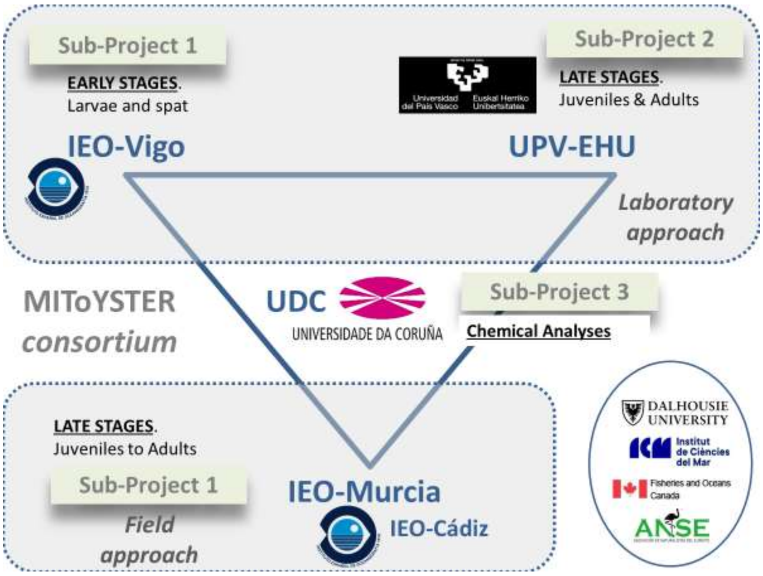

Figure 3. Distribution of project tasks among the partnership.

Added value expected to be achieved with coordination

In summary, the main added values of the coordinated project are:

- An interdisciplinary consortium formed by complementary groups that allow increasing knowledge and specific experience, equipment, techniques and infrastructures necessary to carry out a cross-sectional study. The only viable option to achieve the objectives of the proposal presented here is a coordinated project.
- The project has been designed and structured to favour the interaction between experts from different fields, promoting the transfer of knowledge and conducting interdisciplinary work of greater relevance and scientific impact.
- The coordinated project would also favour the visibility of the work and the results of the project by involving scientists of different competences.

As summarized in Figures 1 and 2, the three sub-projects are integrated globally organized into 4 blocks and 7 WP, with the participation of researchers from the three groups in all of them, considering the complementarity of the tasks to be carried out.

3. *JUSTIFICATION AND NOVELTY OF THE PROPOSAL.*

3.1. *Adequacy of the proposal to the characteristics and purpose of the selected modality.*

MIToYSTER is presented as an oriented research project, that is, it aims to solve defined problems; specifically, it aims to provide solutions to one of the most dramatic environmental crises of recent years in our country: the eutrophication of the Mar Menor. MIToYSTER is one of the research proposals generated within the framework of the *Mar Menor Oyster Initiative*-MMOI-, which is part of NORA since 2020. NORA (https://noraeurope.es/) is a European network founded in 2017 aiming at reinforcement and restoration of the native European flat oyster (*Ostrea edulis*) (https://noraeurope.eu/spain-the-mar-menor-oyster-initiative/). NORA supports the protection and ecological restoration of the native European oyster and its habitat in areas of its current or historical distribution. NORA provides a platform for the NORA community in order to collaborate and participate in knowledge exchange.

MMOI began to take shape in the summer of 2016 when the first serious episode of 'green soup' took place in the Mar Menor. At that time, one of the scientists behind this proposal, Eve Galimany, was working at the Smithsonian Marine Station (Florida, USA) on nutrient extraction with bivalves in the Indian River Lagoon, which had suffered, as in the Mar Menor, a serious episode of phytoplankton superbloom. The Florida experience was communicated to the regional authorities (CARM, Autonomous Community of the Region of Murcia) and in 2018 the BIVAREC workshop[1] was co-organized by the CARM and the IEO and attended by leading experts in bivalve energetic physiology

[1] Albentosa M., Galimany E. 2018. IEO Report. pp. 35.

(most of them included in the research groups of this proposal from the IEO and the UPV-EHU). The objective of the BIVAREC meeting was the estimation of the order of magnitude of the number of oysters that would need to be introduced to observe an effect on water quality. Based on oyster clearance data (volume of water filtered per unit time) available in the literature, and data on lagoon dynamics, it was estimated that a population in excess of 50 million oysters would be required. At the end of 2019, the Governing Council of the CARM approved an agreement on urgent measures to preserve the Mar Menor, reverse its current state and act in the event of emergencies[2]. A total of eight specific actions were contemplated, one of which consists of the introduction and testing of a bed of filter feeders from the Mar Menor. In collaboration with the CARM, the applicant group initiated contacts with US experts in bioextraction and in the recovery of coastal ecosystems degraded by eutrophication. Among them, Holly Greening, who was both Executive Director and Senior Scientist of the Tampa Bay estuary Program (Florida, US) and Rich Batiuk from the USEPA and responsible of the Chesapeake Bay Program, have been involved in the origin of MMOI.

MMOI is supported by 17 scientists belonging to different research institutions and led by the IEO (SP-1), which promotes the recovery of the flat oyster population that was in the lagoon in the 1980s as a bioremediation measure of the eutrophication of the lagoon. The UPV-EHU team (SP-2) has been collaborating with the IEO in the development of this initiative since the BIVAREC meeting in 2018. Some preliminary studies have been carried out during the last year, such as the genetic identification of the Mar Menor oyster and their physiological response to increased particulate matter[3]. In addition, the IEO has spent more than a decade studying the presence of pollutants in the waters of the Mar Menor[4] and their accumulation in the tissues of the organisms that inhabit it[5], as well as the effects that these pollutants trigger at different levels of biological organisation[6]. Given the great spatial and temporal variability of pollutant levels in the waters of the Mar Menor, their accumulation in sediments was also studied, in addition to their accumulation in organisms. The group responsible of the SP-3 (QANAP-UdC) has participated in most of these studies, particularly in those focussed on emerging pollutants detected in Mar Menor samples.

Within the framework of the MMOI, the Biodiversity Foundation (Spanish Ministry of Ecological Transition) has granted two projects, one of which has already been finished, the RemediOS project, which has been a proof of concept to reproduce the oyster of the Mar Menor. The other proposal is the RESALAR project, led by the NGO ANSE, whose objective is the environmental and salt recovery of the Marchamalo salt flats located in the Southern Basin of the Mar Menor. The IEO participates in RESALAR with an action about reproduction and fattening of oyster seed using the salt channels.

3.2. *Justification and expected contribution of the project to the generation of knowledge on the theme of the proposal. Starting hypothesis.*

Background and State of the Art

Marine environmental threats. Coastal marine ecosystems are subjected to different sources of stress caused by human activity, including changes in their physico-chemical variables (nutrients, oxygen, pH) or exposure to a mixture of chemical pollutants which constrain the functioning and structure of these ecosystems[7]. According to Nixon[8], *eutrophication* is the increase of the input of organic matter into an ecosystem due, mainly, to the increase of its primary production as a consequence of an excess of nutrients. The development of intensive agricultural practices with extensive use of fertilisers, the proliferation of industries, together with urban and touristic development in coastal areas are responsible for the input of nutrients, especially nitrogen and phosphorus, into coastal ecosystems directly and via rivers and atmosphere[9]. This abundance of nutrients determines the uncontrolled development of

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[2] http://www.carm.es/web/pagina?IDCONTENIDO=50610&IDTIPO=11&__PLANT_PERSONALIZADA=/JSP/CARM/plantillasPortal/contenidosSimples/consejosGobierno/plantillaConsejoGobierno.jsp&RASTRO=c$m22640,132#seccion2

[3] Albentosa M., et al. 2023. Aquatic Conservation.

[4] León V.M., Moreno-González R., Campillo J.A. 2014. In: Mar Menor: una laguna singular y sensible (V.M. León, J.M. Bellido, eds.) pp. 291-313.

[5] Campillo J.A. et al. 2014. In: Mar Menor: una laguna singular y sensible (V.M. León, J.M. Bellido, eds.) pp 315-339.

[6] Campillo J.A. et al. 2013. Aquat Toxicol 142–143, 365–379.

[7] Cajaraville M.P. et al. 2016. Estuar Coast 179,124-134.

[8] Nixon S.W. 1995. Ophelia 41,199-219.

[9] Andersen J.H. et al. 2006. J Plankton Res 28, 621-8.

phytoplankton blooms (called 'ecosystem disruptive algal bloom', EDABs)[10]. EDABs increase the turbidity of the water column, hindering the functioning of macrophyte populations whose losses further contribute to the organic matter generated by the EDABs. This increase in organic matter can lead to hypoxia[11], causing the death of marine fauna.

Coastal marine *pollution* is one of the main environmental issues of the last decades, and its prevention and control has become a priority objective. Marine pollution refers to the presence of toxic substances derived from human activities which causes deleterious effects either to the environment or directly to people[12]. The marine environment is threatened by mixtures of different types of chemical compounds ranging from conventional pollutants such as aromatic hydrocarbons, metals, pesticides, persistent organic pollutants, to pollutants of emerging concern such as pharmaceuticals, personal care products, flame retardants or micro- and nanoplastics[13].

Coastal lagoons are highly productive marine ecosystems that provide multiple services and resources to humans[14]. They have a high ecological, recreational and commercial value. They provide diverse habitats that promote biodiversity being special areas for nursery, feeding and refuge of numerous marine organisms. Due to all these values, coastal lagoons are highly populated and consequently, have become the marine ecosystems most impacted by human activity. The low water exchange with the adjacent sea makes them susceptible to pollution by nutrients and chemical compounds that end up in their waters from bordering watersheds. Moreover, coastal lagoons are highly vulnerable to climate change and extreme weather events.

The Mar Menor and the "green soup". This lagoon is located in the southeast of Spain and is the largest saline coastal lagoon in Europe. It is semi-circular in shape and is separated from the Mediterranean Sea by a strip of sand (La Manga). Seawater temperatures in summer can exceed 30ºC and drop to 10ºC in winter. The salinity of the lagoon currently ranges between 42 and 48 psu. Communication with the Mediterranean Sea is through 5 exchange areas called "golas". Water renewal is about 1 year[15]. The Mar Menor basin is made up of the plain of Campo de Cartagena dedicated to intensive agriculture and a network of dry wadis, except for the Albujón wadi, through which the irrigation surpluses of the entire cultivated area flow. Rainfall is very variable with few intensive events, mainly in spring and autumn.

The lagoon has always been an oligotrophic lagoon with transparent waters, with high salinity, around 50-53 psu in the middle of the last century, and plenty of singular biodiversity. However, most of the impacts that humans can have on coastal marine ecosystems have been suffered by the Mar Menor for decades. The intense mining activity carried out during the first half of the 20th Century in the Cartagena-La Unión mountain range caused heavy metals to be released to the lagoon with runoff water. During the 1960s, there was an important tourist development in all the riverside municipalities, especially in La Manga. In 1973, the Estacio channel was dredged and widened to make it navigable, which caused a "Mediterraneanisation" of the Mar Menor, lowering the salinity to 42-48 psu, which led to the greatest oceanographic transformation of the lagoon. In 1979, the Tajo-Segura water transfer began, which led to the transformation of the traditional rain-fed agriculture of the Campo de Cartagena into intensive irrigation. In 2015, and especially in the summer of 2016, a phytoplankton bloom was observed, transforming the lagoon water into a "green soup" (EDAB events). The most severe response to the turbidity produced by the phytoplankton bloom was the death of all lagoon vegetation in the deeper areas. Heavy rains in September 2019 (DANA) caused flooding that dumped large volumes of freshwater into the lagoon, triggering a series of events that resulted in the mass death of marine organisms living due to anoxia[16]. The second episode of mass mortality took place at the end of August

[10] Mercado J. et al. 2021. Mar Pollut Bull 164,111989

[11] Steckbauer A. et al. 2011. Environ Res Lett 6, 025003.

[12] Beiras R. 2018. Marine Pollution: Sources, Fate and Effects of pollutants in coastal ecosystems. pp 3-20, Elsevier Inc.

[13] Celander M.C. 2011. Aquat Tox 105S,72-77.

[14] Kennish M.J., Paerl H.W. 2010. In: Coastal Lagoons (M.J. Kennish, H.W. Paerl, eds.), pp 1-17, CRC Press.

[15] Pérez-Ruzafa A. et al. 2005. In: Coastal Lagoons: Ecosystem Processes and Modelling for sustainable use and development (I.E. Gönenc, J.P. Wolfin, eds.), pp 392-422, CRC Press.

[16] Ruíz J.M. et al. 2020. Informe IEO 166pp. https://www.miteco.gob.es/es/prensa/informe-ieo-mar-menor.aspx

2021 as a result of anoxia and the production of toxic compounds generated by the excess of nutrients reaching the lagoon[17]. To all these impacts must be added the pollution of the aquifer by nitrates as a result of intensive agricultural activity, which has also led to an increase in the water table of the aquifer, which in some places reaches the surface and ends up discharging into the Mar Menor. The multiple protection measures that have been granted to the Mar Menor over the years have been useless in protecting the lagoon. Thus, the lagoon is in a state of eutrophication that is difficult to predict and has altered its ecological stability and resilience[10].

Pollution in the Mar Menor. The lagoon is subject to the entry of pollutants of various types associated with the anthropogenic activities that take place in its surrounding (mining, agriculture, tourism, urban development, navigation). Although mining activity stopped several decades ago in the Mar Menor, mining wastes still enter the lagoon, especially during torrential rains, through seasonal rivers ("ramblas"), which can adversely affect the marine ecosystems. These wastes were initially deposited in the Southern basin of the lagoon, although hydrology, wind, salinity, temperature have redistributed them[18]. The loss of vegetation in the lagoon since the eutrophication crisis has led to the mobilisation of *metals and metalloids* that were retained in the sediments[19]. In addition to metals, the entry of *organic pollutants* is also very relevant. It has been described the entry of organochlorinated pesticides (OCs) and current-use pesticides (CUPs), polycyclic aromatic hydrocarbons (PAHs), pharmaceuticals and surfactants into the Mar Menor, mainly through the Albujón Wadi (the main catchment area of Campo de Cartagena). According to the review made by our group[4], the annual entry of organic compounds to the lagoon is of 18 kg of pesticides and 11 kg of pharmaceuticals, among which are the insecticide chlorpyrifos (5.6 kg) and the antibiotic azithromycin (4.2 kg). The presence of these pollutants in the water determines their accumulation in the tissues of the organisms living in the lagoon. Campillo and co-workers[5] studied the pollutants (PAHs, POCs, polychlorinated biphenyls –PCBs- and pharmaceuticals) accumulated in some bivalve species of the lagoon (oysters, cockles, clams and pen shells) showing a higher bioaccumulation of organochlorine compounds in oyster tissues.

Pollution and Eutrophication Interactions. Coastal zones are among the most endangered areas of the planet, since they are continuously subjected to multiple anthropogenic stressors. Large amounts of toxic chemicals, nutrients and organic matter are transported via rivers and waterways and are released into the coastal zone[20,21], altering coastal communities. In particular, rocky and soft bottom communities have been shown to be very sensitive to anthropogenic stressors such as turbidity, eutrophication and toxic substances, or their combination[21]. Since chemical pollutants, nutrients and organic matter share common pathways in the marine environment, they may act together, and their combined impact on coastal ecosystems is therefore a matter of concern[22,23].

The interaction between *eutrophication* and *chemical pollution* has been studied over the last years, but information is incomplete and fragmentary, dealing mainly with bioavailability and accumulation of pollutants rather than with their effects on organisms. Eutrophication (e.g. excessive N and P inputs) has been shown to facilitate the uptake of pollutants by phytoplankton and the subsequent transfer of pollutants into the food web. For instance, increases in metal concentrations in bivalves coinciding with increases in the concentrations of macronutrients in coastal waters, have been observed[22]. However, given its complexity and due to the vast number of chemicals and the large amounts of nutrients and organic matter that are released into the coastal environment, there is an urgent need to carry out in-depth studies to disentangle the underlying mechanisms and the environmental changes caused by the combined impact of these pollution and eutrophication factors, in order to detect and predict the potential impact on different functions of the ecosystem. This is especially relevant in the case of coastal

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[17] Ruíz J.M. et al. 2021. Informe IEO 24 pp. http://www.ieo.es/documents/10640/38594/informe+IEO_MarMenor_60921.pdf/36c4e2fc-7ab3-420d-b73d-8cf33478885e

[18] Muñoz-Vera A., García G. 2014. In: Mar Menor: una laguna singular y sensible (V.M. León, J.M. Bellido, eds.) pp 271-290.

[19] Alvarez-Rogel J. et al. 2019. https://canalmarmenor.carm.es/wp-content/uploads/2020/07/Informe-final-proyecto-BIOFOM-01-08-2019.pdf

[20] Lindeboom H. 2002. In: Oceans 2020: Science, Trends and the Challenge of Sustainability, 49-84.

[21] Kautsky L. 1998. Pure Appl Chem 70(12), 2313-2318.

[22] Zhao L. et al. 2021. Ecotoxicol Environ Saf 224, 112671.

[23] Nikulina A., Dullo, W. C. 2009. Mar Poll Bull 58(6), 905-915.

ecosystems with restricted water exchange such as coastal lagoons, where the prevailing muddy sediments constitute a depositional area favouring the concentration of pollutants[23].

Nature-based-Solutions. Different approaches are available to improve the quality and sustainability of ecosystems affected by eutrophication. The first nutrient management action should be done on land by reducing nutrient inputs to the system, for example, by implementing and improving treatment plants, installing or improving wastewater systems, or establishing better agricultural management practices[24]. But these actions are not enough because surplus nutrients discharged from land accumulate in sediments and marine muds over time, usually decades. Thus, these sediments act as a reservoir that constantly supplies nutrients to the water column[25]. For this reason, to carry out correct nutrient management in eutrophic coastal zones, both terrestrial and aquatic actions (carried out in the water column) are required[26]. According to the International Union for the Conservation of Nature[27], Nature-based Solutions (NbS) are actions aimed to protect, manage, and restore ecosystems impacted by human action, with the double objective of promoting human well-being and protecting and increasing biodiversity. Bivalves are key organisms in coastal areas due to the ecosystem services they provide, among others, water quality improvement through filter feeding[28]. Bivalve bioremediation is one of the greenest solutions to restore highly degraded aquatic ecosystems. The excess of nutrients of eutrophic ecosystems provokes the proliferation of phytoplanktonic organisms that are filtered and ingested by bivalves with high efficiency, transforming these nutrients into body tissues. The subsequent extraction of the bivalves supposes a withdrawal of nutrients from the system called *bioextraction*[29]. Different species of bivalves are used in bioextraction actions depending on the existence of autochthonous populations or the environmental conditions of each geographic area. On the east coast of the United States, the native oyster *Crassotrea virginica* is used[30] whose consumption is highly appreciated, so there is a perfectly developed cultivation and marketing. In one of the ecosystems most affected by eutrophication in Europe, the Baltic Sea, different species of mussels (*Mytilus edulis*, *Mytilus trossulus* and their hybrids) are being used in bioremediation projects[31]. Other species are of no commercial interest, but are interesting for their high filtration capacities and tolerance to a variety of environmental conditions. This is the case of the mussel, *Geukensia demissa*, which has been used in bioextraction actions in estuaries impacted by large cities[32]. In Australia, the oyster species *Saccostrea glomerata* and *Ostrea angasi* are used to restore disturbed ecosystems[33].

Oysters Restoration and Ecosystem Services provided. Marine restoration focuses particularly on habitat-forming species whose distribution and abundance has been reduced due to human activity and/or mass mortality events (usually associated with the human impact on marine ecosystems). These species, such as oysters, are structural components of ecosystems from which biodiversity and ecosystem services are generated[34]. The restoration of oyster populations is a widespread activity, especially in the United States and, for the last few years, in European countries around the North

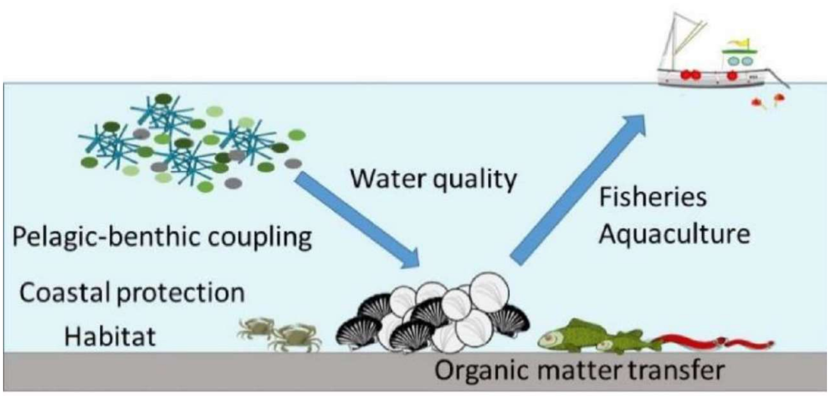

Figure 4. *Ecosystem services provided by bivalves*

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Sea[35]. Oysters live in aggregations forming reefs or beds which are among the most threatened marine habitats in the world[36]. The oyster reefs are three-dimensional structures which multiply their filtering potential compared to other bivalves.

Oysters provide important ecosystem services (Figure 4) since their biogenic reef building structures increase biodiversity and provide coastal protection. Oyster reefs/bed enhance denitrification, serve as nurseries for the early stages of many species and are of high cultural value. Their high filtering activity[37] can improve water quality on local scales, because oysters remove organic particles from the water and transform them into new tissues (growth). By removing particles from the water column, oysters can also increase light penetration to the sediment, and promote the recovery of seagrasses, another threatened and valuable coastal habitat. The drawdown of sediments together with the stabilizing effect of the reef, can also result in reefs acting as carbon sinks. However, substantial knowledge gaps remain regarding the provision of these ecosystem services, which can only be tackled through the combination laboratory and field studies to identify the degree to which these services are provided, and how factors such as pollution can interfere with the benefit of these services on the restoration of altered ecosystems. Though some processes occurring in bivalve populations that result in ecosystem services are well understood theoretically, quantitative evidence of the delivery of these services in the field is a relevant knowledge gap as it is the modulation of these processes by stress factors such as pollution.

The Mar Menor Oyster Bed. The progressive decrease in the salinity of the Mar Menor as a consequence of the dredging of the Estacio channel allowed the entry and settlement of new organisms from the Mediterranean, and, in 1972, the first flat oysters (*Ostrea edulis*) were documented in the Mar Menor[38]. During the 1980s and 1990s, a large flat oyster population developed, reaching 135 million individuals, giving rise to a small oyster fishery[38]. However, the harvesting of commercial-sized oysters was abandoned because of their unattractive appearance to the consumer (shell overgrowth), and the difficulty of their extraction. The last partial assessment of the oyster bed, carried out in 2006, indicated a population decrease of almost 10 times with respect to 1992. Currently, the population has practically disappeared, with only a few isolated specimens remaining.

The reasons for selecting the flat oyster for bioremediation actions in the Mar Menor have been: i) it was a well-developed species in the lagoon in the past, ii) its populations can develop three-dimensionally, which multiplies its filtering potential compared to other bivalves, iii) its development both horizontally and on reefs generates habitat, thereby increasing the biodiversity of the ecosystems they constitute, (iv) oysters are able to grow on soft and hard substrates compared to other bivalves in the Mar Menor which can only grow on soft substrates, and (v) the existence of the European network for the recovery of oyster populations (NORA, see below).

The Life Cycle of *Ostrea edulis*. The flat oyster *O. edulis* is a protandrous hermaphrodite, reaching sexual maturity first as a male (within 10-12 months postmetamorphosis), changing to female after the first spawning, and then to male again, in a cycle that persists throughout adult life (Figure 5). *O. edulis* presents a complex life cycle which includes external sperm release and internal egg fertilization in the pallial cavity of the females[39] (7-10 days), the *veliger larvae* (size of 150-190 µm in length) is released into the surrounding water (swarming)[40]. The larva has two valves, a complete digestive system and a ciliated organ, the *velum*, that is peculiar to bivalve larvae. The cilia of the velum allow larvae to swim and to stay in the water column and feed. The mature larvae, *pediveliger stage*, (240-330 µm) develop a functional foot and eye spot, and gill rudiments become evident. These larvae, between periods of swimming activity, use the protruded foot that functions as a sensory tactile organ to crawl and find a suitable substrate[41,42] to attach, metamorphose and settle, beginning its benthic existence. Metamorphosis is a critical period in the development of bivalves, during which the animal changes

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from a pelagic life to a sedentary benthic existence. The duration of the larval stage is dependent on environmental factors, such as temperature, and usually last for 8-12 days[39,42]. During the rest of the life cycle (postlarvae, spat, juvenile and adult stages), feeding takes place by means of filtration of the material in suspension (seston), at the level of the *gills*.

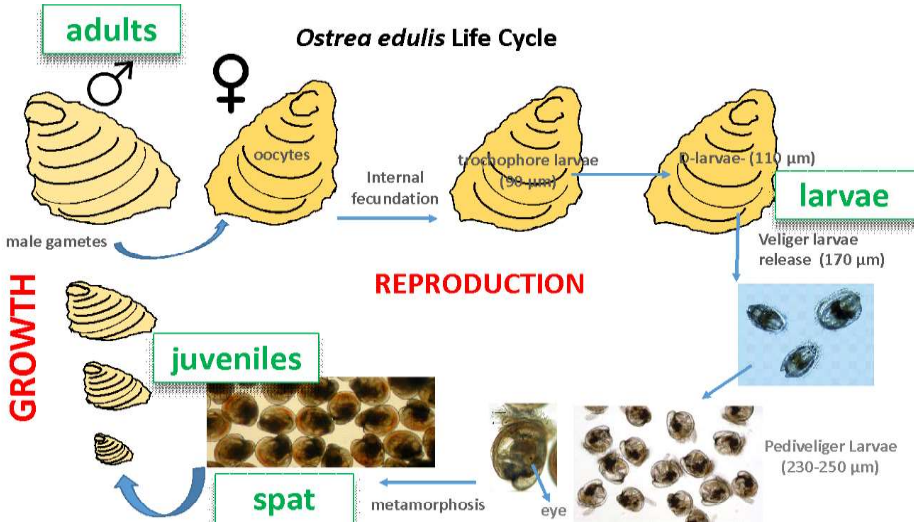

Figure 5*. Life Cycle in* Ostrea edulis *showing the life stages selected in this Project.*

Pollutant effects on the oyster biological status through their developmental stages. Because of their high sensitivity, early life stages of marine invertebrates are particularly suitable for the assessment of marine pollution. His and co-workers[43] looked over more than 100 years of research in bivalve aquaculture, and proposed the early stages of these invertebrates as the most sensitive organisms to pollution. Therefore, if the success of a species depends on its performance as it passes through successive stages of development, it is logical to use the most sensitive stages to assess environmental quality. Although the increase in weight has been suggested to account for the increase in resistance at later stages, the mechanisms that determine the differences in sensitivity between life stages are not clear. Earlier stages of development are less able to metabolize xenobiotics than adults and differences in morphology and functioning throughout the life cycle can also affect the uptake, accumulation and effects of pollutants[43]. The success of a future restoration project depends on guaranteeing the viability of each of the life stages of the species to be restored. It is therefore needed to assess the effects of the pollutants present in the Mar Menor on the viability and development of the oyster early stages. Thus, obtaining ecotoxicological data of all vital stages of *O. edulis* (larvae, spat, juvenile and adults) is crucial.

Pollution effects can be manifested at the cellular and molecular level altering a series of cytological and molecular parameters. A good pollution biomarker must present a quantitative response, proportional to the degree of pollution and must be sensitive. The release of uncoupled high-energy electrons from the oxygen molecule, due to the exposure to chemical pollutants, may cause serious injury to cells and tissues through the formation of reactive oxygen species (ROS). Under basal conditions, the adverse effects of ROS produced during cellular aerobic respiration are prevented by the *antioxidant defense system*, consisting of an array of low molecular weight scavengers and antioxidant enzymes[44]. Antioxidant enzymes such as peroxidases and catalases protect cells against the deleterious effects of oxyradical generation by maintaining endogenous ROS at relatively low levels and attenuating the damages related to their high reactivity. Among the enzymatic activities most used as biomarkers are glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD) or glutathione reductase (GR)[6]. The extent to which oxyradicals produce biological damage is dependent on the effectiveness of these antioxidant defenses. Lipid peroxidation (LPO) indicates the damage to cellular membrane lipids caused by ROS and is useful for assessing exposure to and effects of pollutants in bivalves[6].

Acetylcholinesterase (AChE) is an enzyme involved in the transmission of the nervous signal across cholinergic synapses. Once the depolarization is triggered in the postsynaptic neurone, the AChE hydrolyses the acetate group of the neurotransmitter to produce the inactive choline, interrupting the

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nervous signal. Synthetic chemicals such as organophosphate and carbamate insecticides selectively bind to AChE and inactivate the enzyme[45].

Micronuclei (MN) are nuclear abnormalities produced from fragments or entire chromosomes that lag in cell division because of a lacking or damaged centromere or a defect in cytokinesis. Their frequency in marine organisms is considered the most popular and promising marker of cytogenetic damage. Analysis of MN is increasingly being used for in situ genotoxicity assessment in aquatic media[46].

Valvometry. Bivalve shells provide the animal with the capability to isolate its internal tissues from the environment, becoming an effective behavioural mechanism to quickly respond to external stressors such as harmful algal blooms[47], hypoxia[48] or pollutants[49]. Rapid technological advancements based on the Hall Effect allow real-time monitoring of the movement of bivalve shells under natural conditions. Recently, initiatives around the world have been developed with the ultimate goal of implementing this behavioural response as an early-warning system of poor environmental conditions in terms of ocean water quality, e.g. MolluSCAN[50]. Therefore, the degree of valve opening (VvO) has implications for the biological status of the oysters and also as a tool to inform about the status of the ecosystem.

Nutrient extraction and oyster growth. For any bioremediation action with bivalves to be effective, the nutrient extraction capacity of the bivalves to be introduced into the eutrophicated ecosystem must be quantified. The evaluation of the extractive capacity is based on studying their feeding process, from quantifying the water filtered through their gills to assessing the amount of ingested organic matter used for energy and growth of the organism.

Oysters pump considerable volumes of water through their large gills and filter out particulate food items from the water column. *Clearance rate* is the volume of water filtered free of particles per unit of time. Captured particles are transported from the gills to the labial palps where the organic particles are selected (pre-ingestive sorting) and *ingested*[51]. The rejected particles are ejected as pseudofeces. Ingested particles are subjected to the action of extracellular enzymes in the stomach and the mechanical action of the crystalline style. Fine and digested particles are selected at digestive diverticula (post-ingestive sorting) where the intracellular digestion takes place. Undigested material is discharged through feces which, together with pseudofeces, make up the oyster biodeposits. *Absorption* efficiency is the proportion of ingested food that is absorbed. The *oxygen* needed for aerobic metabolism is extracted from the water flowing through the mantle cavity where the CO_2 is discharged. Gas exchange takes place in the gills where the hemolymph flows. As a consequence of protein catabolism, nitrogen molecules are produced, primarily NH_3/NH_4^+, that have to be *excreted*. The measurements of all these physiological rates (clearance, ingestion, absorption, respiration and excretion rates) can be integrated into the classical energy balance equation by Winberg[52] (C=F+R+U+P) and used to estimate *P* (P=C-(F+R+U) that is the energy available for growth (somatic, shell and reproduction) known as SFG –*scope for growth*-, where *C* is the energy consumed, *F* the energy lost in the feces, *R* and *U* are the energy losses in respiration and excretion, respectively. Oyster growth measurements (both direct and estimated through SFG) will allow us to quantify the nutrient extraction capacity of the oysters under different environmental scenarios simulated in the laboratory or under natural conditions in the lagoon. With these data we will parameterize a state-of-the-art Dynamic Energy Budget (DEB[53,54]) mechanistic model, which will be able to model the bioenergetics of *O. edulis* under different environments. Using DEB, we will estimate the extractive potential of the oyster under the environmental conditions of the Mar Menor, as well as hypothetical future conditions -climate change-, and thus develop the most appropriate bioremediation strategy.

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Pollutant effects on oyster nutrient extraction. Although the effect of pollution on the nutrient extraction capacity of organisms used in bioremediation has not been evaluated in-depth in the context of a eutrophication scenario as proposed in this project, information is available on the effect of pollutants on the growth and physiology of bivalves. In fact, growth (the consequence of the nutrient extraction) is one of the most sensitive measures of stress in an organism as it integrates the main physiological responses of energy gain and loss. In this sense, alterations of SFG measured under standardized laboratory conditions has been proposed[55] as a method of assessing the sublethal stress induced by pollutants in pollution monitoring programmes[56]. Moreover, the clearance rate is one of the most sensitive measures of the presence of pollutants in water and has been recommended as a non-destructive biomarker of aquatic pollution[57]. Further, valve opening is emerging as a potential non-invasive behavioural indicator of hypoxia[48] and contaminants[49]. It is therefore of great interest to assess the effect of pollutants in the Mar Menor on the extractive capacity of *O. edulis* prior to its use in a bioremediation action.

In this context, the MYToSTER Project will contribute to a better scientific knowledge on the effect of conventional and emerging pollutants on oyster growth, on their ability to restore eutrophic environments, and on the bioaccumulation of pollutants, which will serve and will be of help to better design strategies for the natural-based restoration of eutrophic ecosystems.

Starting Hypothesis

Bivalves, in particular oysters, are species of great interest for ecosystem restoration. For some species, especially where the habitat is at risk, a critical challenge is to restore sufficient habitat to allow for quantification of the ecosystem services associated with those species, considering stress factors (such as pollution) that may hamper their restoration capacity. To evaluate their restoration capacity, it is of utmost importance to gather a more complete understanding of the effects of stress factors on the ecosystem services of those species.

The following hypotheses have been formulated:

- Restorative actions based on oysters can improve the water quality of the Mar Menor and be used as a Nature-based Solution for the recovery of the environmental quality of the lagoon.
- The presence of pollutants negatively affects the general health status of oysters.
- The presence of pollutants negatively affects the capacity of oysters to extract nutrients (ecosystem service) in eutrophic ecosystems.
- The effect of pollutants on the general health status of oysters and on their capacity for nutrient bioextraction depends on their life stage.
- Eutrophication affects the resilience of populations to pollution.

In research-oriented projects

3.3. Justification and expected contribution of the project to solving specific problems linked to the selected thematic priority.

The improvements in the application of nature-based solutions to which this project is expected to contribute, are aligned to the thematic priority 6 of the Spanish National Plan for Scientific, Technical and Innovation Research 2021-2023: "Food, bioeconomy, natural resources, agriculture and environment", that addresses the strategic line: "Exploration, analysis and prospective of biodiversity", and to Horizon Europe cluster 6: "Food, bioeconomy, natural resources, agriculture and environment", that aims to reduce environmental degradation, halt and reverse the decline of biodiversity on land, inland waters and seas, and better manage natural resources through transformative economic and societal changes in urban and rural areas. Thus, MIToYSTER aims to generate knowledge and innovation in the field of marine biodiversity and enhance its value as a key factor for the future.

Also, the proposed research is aligned to the decade´s *UN Declaration* (https://www.decadeonrestoration.org/about-un-decade) aimed to restore ecosystems as a global movement (#GenerationRestoration) for the benefit of nature and humanity. Only healthy ecosystems

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[56] Albentosa M. et al. 2012. Sci Total Environ 435-436: 430-445.
[57] Toro B. et al. 2003. Rev Chi Hist Nat 76, 267-74.

can improve people's lives, counteract climate change and prevent biodiversity loss. The UN Decade Programme aims not only to influence government policies but also to involve citizens in a global movement to prevent the destruction of nature.

MIToYSTER's objectives are also linked with the *European Green Deal* (https://ec.europa.eu/clima/eu-action/european-green-deal_en) which aims to reduce greenhouse gas emissions (by 2050, Europe aims to become the world's first climate-neutral continent) and to preserve Europe's natural environment. MIToYSTER results will contribute to the *EU Blue Growth Strategy* (https://ec.europa.eu/oceans-and-fisheries/ocean/blue-economy/sustainable-blue-economy_es) whose goals are the support of economic activities in the marine and maritime sectors.

4. *OBJECTIVES, METHODOLOGY AND WORK PLAN.*

4.1. *General and specific objectives.*

General Objective:

The **main objective** of MIToYSTER is:

To evaluate the effect of pollution on the biological status of the flat oyster (*O. edulis*) throughout its development, and on its capacity to extract nutrients in eutrophic marine ecosystems, using the Mar Menor as a study case.

Specific objectives are:

- To evaluate the effect of pollution on the flat oyster in a eutrophic ecosystem throughout its life cycle.
- To assess the nutrient extraction capacity of the flat oyster under laboratory-simulated and under natural eutrophic conditions throughout an annual cycle.
- To evaluate the effect of pollution on the nutrient extraction capacity of the flat oyster.
- To evaluate the pollutant bioaccumulation in the four oyster life stages under laboratory and field conditions
- To establish the general health status of the flat oyster over an annual cycle in a polluted and eutrophic scenario such as the Mar Menor coastal lagoon.

4.2. *Description of the methodology.*

4.2.1. Oyster *life stages*

Since the effects of pollutants depend on the physiology and metabolism of each life stage, different stages of the life cycle of the oyster will be considered: larvae, spat, juveniles and adults. Four oyster life stages have been selected: newly released larvae to pediveliger (150-250 µm), spat (0.5-2 mm), juveniles (2-3 cm) and adults (>6 cm). Experimental organisms will be obtained from bivalves' growth-out facilities as the ones available at the two IEO centers (Murcia and Vigo) or from private companies as Ostreira –Spain-, Orkney Shellfish Hatchery –Scotland- or Mali Ston Bay –Croatia-, that usually produce flat oysters. The organisms required for laboratory exposure experiments can come from any of the sources described. However, the oysters needed for the field experiment in the Mar Menor must come from the lagoon itself for biosecurity reasons. For this purpose, oyster seed produced in the RemediOS hatchery (RemediOS Project) of the IEO-Mu is available for the MIToYSTER field experiments. Seed was produced in 2022 from broodstock collected at the Mar Menor and cultured with natural seawater from the lagoon that supplies the IEO hatchery. Currently, juveniles show an average size of 2-2.5 cm so they could have the required size (>6 cm) for the field experiment in 2024.

4.2.2. Eutrophication simulation (MA_{MIX})

A eutrophic ecosystem scenario will be simulated in the laboratory, reflecting the natural conditions observed in the Mar Menor lagoon during EDAB events[10] regarding total (TPM) and organic particulate matter (POM). For this purpose, microalgae culture facilities are available at the centers where the laboratory studies will be carried out (IEO-Vi and UPV-EHU). A mixture of several cultured microalgae (MA_{MIX}) will be used as a food source for acclimatization and exposition, so that cultures will be set up at different scales depending on demand. Selection of microalgae for the mixture will be done according to the characteristics (cell size and taxa) of the microalgae composition of EDABs described in the Mar Menor[10] and to their culture availability at the IEO and UPV-EHU facilities.