enviromed

Next generation toolbox for greener pharmaceuticals design and manufacturing towards reduced environmental impact

D2.2 - Report on the design of ecotoxicity studies and monitoring campaigns

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August, 2023

Funded by the European Union

Deliverable	D2.2 - Report on the design of ecotoxicity studies & monitoring campaigns
Work Package(s)	WP2 – ENVIROMED specifications, requirements and preliminary work preparation
Task(s)	T2.3 – Ecotoxicity study & environmental monitoring campaigns design
Dissemination Level	Public (PU)
Due Date	30-09-2022
Actual Submission Date	07-08-2023
WP Leader	TUW
Task Leader	CNR
Deliverable Leader	CNR
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Document History				
Version	**Date**	**Description**	**Comments**	**Editor(s)**
v0.1	20-02-2022	Document initialization	Draft document	P. Italiani (CNR)
v0.2	16-11-2022	First internal version	Revised draft document	P. Italiani (CNR) D. Melillo (CNR) R. Marino (CNR) K. Kypriotis (EYDAP)
v0.3	20-07-2023	Second internal version	Restructured and updated document	P. Italiani (CNR)
v0.4	27-07-2023	Third internal version	Chapter on KPIs and revised document with comments	K. Chatzipanagiotou (IRES) G. Antonaropoulos (IRES)

Funded by the European Union

This project has received funding from the European Union's Horizon Europe research and innovation programme under grant agreement No 101057844

				A. Giakoumaki (RISA)
v0.5	01-08-2023	Version ready for peer review	Revised version based on received comments	P. Italiani (CNR) K. Kypriotis (EYDAP)
v0.6	07-08-2023	Reviewed document draft	Peer review	M. Dimitriadou (RISA)
v0.7	07-08-2023	Reviewed document draft	Peer review	M. Vasileiadis (ALPES)
v1.0	07-08-2023	Final version	Ready for submission	P. Italiani (CNR)

Executive Summary

The deliverable D2.2 is the output from Task 2.3, which aims at designing the ecotoxicity studies and planning the environmental monitoring campaigns. In particular, this deliverable encompasses: the design of the marine monitoring campaign, which includes the sampling of marine species and wastewater; the identification and the description of the ecotoxicological assays for performing the ecotoxicity study; the preliminary list of metrics that will be used to estimate the Key Performance Indicators throughout the project, associated with the full life cycle of pharmaceuticals.

Regarding the identification and the description of the ecotoxicological assays for performing the ecotoxicity study, it should be pointed out that the ecotoxicity study in the ENVIROMED project will be exclusively focused on the evaluation of immune-related parameters in the selected marine species. The choice to study the capacity of selected pharmaceuticals to affect the immune system of marine invertebrates is extremely important for the evaluation of animal health and wellness.

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List of Acronyms

Table 1: Acronyms and abbreviations

Term	Definition
DoA	Description of Action
E-factor	Environmental Factor
KPI	Key Performance Indicator
LCA	Life Cycle Assessment
LPS	Lipopolysaccharide
PMI	Process Mass Intensity
PCR	Polymerase Chain Reaction
PP	Pharmaceutical Product
RNA	Ribonucleic Acid
TGIP	TransGenerational Immune Priming
WP	Work Package
WWTP	Wastewater Treatment Plant

1. Introduction

This report is related to the design of the environmental monitoring campaign, the design of the immune-ecotoxicity studies, and the definition of metrics for the Key Performance Indicators (KPIs). The design proposed in the DoA is mainly confirmed without any particular changes, apart from the inclusion of a motoring campaign in Italy as explained throughout the document, and the elimination of two general parameters in the context of ecotoxicity study. Indeed, after an in-depth analysis, the investigation of parameters such as reproduction and development has been reconsidered and, then, excluded, unlike what is indicated in the description of Task 2.3 in the DoA. The reason lies in the huge number of animals that would be required and, in the decision to comply with the 3R principle of animal experimentation. Moreover, studying the capacity of selected pharmaceuticals to affect the immune system of marine invertebrates is extremely important for the evaluation of animal health and wellness as well as sufficient and exhaustive. Therefore, general parameters, such as survival, susceptibility to stress/infections, generation of innate memory or TGIP, encapsulation/melanization reactions, will be evaluated. No changes have been made for functional/molecular parameters.

The eco-immunotoxicity studies will be performed by taking into account all assays and technical considerations recently described and addressed in a review publication (authored by the ENVIROMED's partner, CNR) [1] on methodological approaches and *in vivo* and *in vitro* models which offer excellent possibilities for realistic and meaningful immunotoxicology assessments for every kind of toxicant, both chemical and particulate. The assays to be used in the ENVIROMED project are described in the deliverable.

One of the main aims of the ENVIROMED project is to improve the understanding of the environmental impacts of pharmaceutical products (PPs) throughout their lifecycle (i.e., from their production to the end of their lifetime). This includes, but is not limited to, the impact of solvents, chemicals and energy used during PP synthesis, the impact of cleaning agents, solvents and water used during the cleaning of manufacturing equipment between synthesis batches, and the impact of PPs to the environment and living organisms (i.e., ecotoxicity) upon their release, either via the wastewater treatment of PP-containing waste of production facilities, or via the release of PPs and their metabolites by the retailers or consumers (e.g., release after human consumption and metabolism to wastewater, release of unused or expired products to wastewater or solid waste streams). To that end, several metrics are listed in this report, which can be used to calculate KPIs related to PPs along their lifecycle throughout the ENVIROMED project. Metrics for KPIs designated in this deliverable are selected to measure the performance and environmental impacts of both the manufacture of PPs (i.e., inputs and outputs of the manufacturing process), and the ecotoxicity impacts of PPs upon release to the environment. KPIs and metrics are described in Chapter 2 of this deliverable. For KPIs related to ecotoxicity, a more detailed description of the approach to investigate throughout this project is given in the remainder of Chapter 1 and in Chapter 3 of this deliverable, including both the environmental monitoring campaign design, and the design of the ecotoxicity assessments.

The ecotoxicity of pharmaceuticals will be assessed with *in vitro* and *in vivo* immunotoxicity studies of PPs (as pure compounds) and of samples from monitoring campaigns (i.e., seawater samples acquired in the vicinity of municipal wastewater treatment plant discharge points). The former (i.e., pure compounds) can provide valuable insights into the ecotoxicity of PPs upon release from the manufacturing facilities, as well as disposal of unused PPs. The latter (i.e., seawater in the vicinity of wastewater treatment plants) will improve the understanding of ecotoxicity effects of PPs at the end of their lifecycle (i.e., after consumption, metabolism, and potentially partial transformation via wastewater treatment processes). Importantly, the

seawater around a wastewater treatment plant does not only contain (partially metabolized and/or transformed) PPs, but also a mixture of several other compounds excreted and disposed of in municipal sewage. Therefore, while these ecotoxicity studies will not reveal the isolated ecotoxicity effect of PPs, they will allow the realistic estimation of the combined (and potentially synergistic) ecotoxicity impact of PP-containing municipal sewage effluents.

In vivo testing will assess the impact of pharmaceuticals on filter feeding marine environmental species (tunicates, molluscs, and echinoderm) upon short-term exposure, measured as mortality and susceptibility to infections/stress. *Ex vivo*/*in vitro* assays will include the evaluation of changes in cell populations and stress parameters on immune cells.

The main points/considerations for the immune-ecotoxicity assessment are described below, while detailed protocols of the assays will be provided with the reports/deliverables planned in the project as soon as the research activities will be carried out. Some protocols' details need to be still set and implemented (i.e., immune cells' culturing condition, short-term exposure to ambient water pollution and chemical pollutants).

This task is strongly linked to the activities planned in Tasks 6.3 and 7.4 starting at M14 and M28 of the project, respectively. Specifically, the eco/immunotoxicity studies will start during the second year of the project (Task 6.3) and will continue throughout the third and fourth year in conjunction with the "Environmental monitoring campaign 3 – Marine Involvement" (Task 7.4).

Comments on "Ethical clearance and permissions" are also included into the document.

1.1 Structure

This document is structured as follows:

- Chapter 1: Introduction
- Chapter 2: Metrics for Key Performance Indicators of Pharmaceuticals
- Chapter 3: Ecotoxicity study and environmental monitoring campaigns design
- Chapter 4: Ethical clearance and permission
- Chapter 5: Conclusions
- Chapter 6: References

2. Metrics for Key Performance Indicators of Pharmaceuticals

Several scientific studies have been performed on the environmental impacts of PPs along their lifecycle, and have identified processes, materials and emissions that have a significant contribution to the environmental impacts of the pharmaceutical industry. For example, Pietrzykowski and co-workers [2] have investigated the production of monoclonal antibodies with Life Cycle Assessment (LCA) methodology, and concluded that a significant share of the environmental impact is due to the energy and water requirements of the cleaning process for the equipment between production batches. Lee and co-workers [3] also highlighted the significant impact of solvent use and subsequent solvent waste treatment related to cleaning between batches for the production of 4-d-Erythronolactone, using Life Cycle Assessment methodology, and recommended a switch from batch to continuous manufacturing, which could significantly improve the environmental performance of this process. Similarly, Osorio-Tejada and co-workers [4] used LCA methodology to investigate the synthesis of benzoxazole, and concluded that energy and solvent use have a significant contribution of up to 88% to the environmental impacts for batch production processes, which can be reduced by switching to continuous manufacturing. Besides the impacts that stem from the production process of PPs, other investigations have focused on the End-of-Life treatment of unused or expired medicines. For example, Cook and co-workers [5] compared different disposal options and concluded that disposal via wastewater (i.e., by throwing PPs in the toilet or sink) has a much higher environmental impact compared to disposal via solid waste or take-back schemes. While the majority of publications on the environmental impacts of PPs focus on upstream processes (i.e., manufacture and packaging), a significant share of the impacts may stem from the ecotoxicity as a result of PP release to the environment at the end of their lifecycle [6], and therefore the ecotoxicity of PPs to different organisms is a very important factor to consider when estimating the impacts along the entire lifecycle of PPs.

In addition to LCA methodology for quantifying the environmental impacts, the application of which can be limited due to lack of available inventory data for PP synthesis, simpler metrics are also often used, such as the Process Mass Intensity (PMI) and the Environmental Factor (E-factor), described in detail in Deliverable D2.1 of the ENVIROMED project. While the use of such simplified metrics allows a quick estimation of the environmental impacts when comparing different processes, they may not always be useful to determine hotspots within a process that have significant contribution to the overall impacts. For example, Ott and co-workers [7] investigated the manufacture of several PPs and reported that a Palladium-based catalyst, while only contributing to 0.1 kg/kg of total key input materials, can be responsible for over 90% of the environmental burdens of the process, primarily due to the supply of this scarce element. Nevertheless, simple performance metrics can be useful to provide a quick assessment of the hotspots within a production process, particularly when reported in combination with each other as well as with LCA to estimate the impacts and compare process alternatives for PP synthesis routes.

Based on the information provided in this chapter, related to the hotspots of environmental impacts along the lifecycle of PPs, the European targets for decrease of Greenhouse Gas emissions by 2030 and to become climate neutral by 2050 [8], as well as the different methodologies and metrics that can be used to estimate these impacts (i.e., LCA, PMI, E-factor, and their combination), the following metrics are proposed for the ENVIROMED project, which can be used to calculate KPIs throughout the duration of the project's planned activities and interventions to conventional manufacturing and monitoring processes:

a. Water used = Total water withdrawal (to be measured as mass or volume of water used per synthesis batch)

b. Energy used = total energy used (to be measured as units of energy used per synthesis batch)

c. Solvent used = Total solvent used (to be measured as mass or volume of water used per synthesis batch)

d. Waste generated: Total waste generated (to be measured separately for liquid and solid waste generated per synthesis batch)

e. Emission: Total Greenhouse Gases emitted (to be measured as kg of CO_2 equivalents emitted per synthesis batch)

f. Quality of the water: RQ = PEC/PNEC (wherein RQ = Risk Quotient, PEC = Predicted effect Concentration, PNEC = Predicted not effect concentration) (to be estimated for liquid waste generated per synthesis batch, assuming exposure of target organisms to the waste)

g. Process Mass Intensity = kg of material input per kg of pharmaceutical product (to be estimated per synthesis batch, considering the amount of PP produced after purification per batch)

h. E-factor = kg of waste per kg of pharmaceutical product (to be estimated per synthesis batch, considering the amount of PP produced after purification per batch)

3. Ecotoxicity study and environmental monitoring campaigns design

3.1 Environmental monitoring campaign

3.1.1 Circulation in the Inner Saronikos Gulf

Saronikos Gulf is located on the western edge of the central Aegean Plateau (Eastern Mediterranean), near the city of Athens (Figure 1). It communicates with the Aegean Sea at its southern end and is bounded by the coast of Peloponnese to the west and the coast of Attica to the north and east. The Inner Saronikos Gulf is delimited geographically by the coasts of Attica, Salamis and Aegina islands and is submitted directly to the effects of the wastewater discharge from the Psyttalia outfall.

Since the beginning of the 1970's, when the first marine environmental research studies started in the Saronikos Gulf, it was suggested that water circulation is determined by the prevailing winds. The circulation in the Inner Saronikos Gulf is in most cases cyclonic (counterclockwise direction) due to the prevailing northerly winds (N, NE, NW). Anticyclonic circulation is observed during southerly winds and is usually less intense. Water circulation in the water column is determined by the stratification conditions. During strong stratification conditions (i.e., in August when there are dominant North winds) in deeper layers, under the pycnocline, there is a tendency to reverse the cyclonic circulation of surface waters, while in homogenization periods there are no significant changes in circulation by depth.

Figure 1. The Saronikos Gulf

3.1.2 WWTP of Psyttalia and dispersion patterns of outfall

The wastewater treatment plant (WWTP) operated by EYDAP is located at the island of Psyttalia, an uninhabited island in the Saronikos Gulf between the harbor of Piraeus and Salamis Island, Greece (Figure 2). It covers an area of 0.375 square kilometres. The WWTP has been in operation since 1994 and includes pre-treatment, primary and secondary treatment with advanced biological nitrogen removal, sludge treatment and cogeneration of electrical and thermal energy. The average supply of incoming wastewater is in the range of 730,000 m^3 per day. The processed outflow of the WWTP is diffused through pipelines in the Saronikos Gulf with reduced organic load of wastewater at a rate of 93% and nitrogen by 80%. Treated wastewater is discharged southwards of Psyttalia by two diffusers at locations: (1) 37o55.519'N 23o34.722'E (depth 63m) and (2) 37o55.843'N 23o36.143'E (depth 57m), (Fig. 3). The salinity of Saronikos Gulf doesn't present intense seasonal or spatial differences and ranges between 38.5 and 39.5, with the minimum values near the area of the WWTP outfall, where there is a constant supply of freshwater.

Treated wastewater is mixed with freshwater, having always less density than seawater at the discharge locations and consequently has the tendency to upwell to the surface. During the stratification period (June to November) the wastewater field is trapped below the thermocline-pycnocline, at depths of 35 to 60 meters, and is dispersed by dominating currents at this depth. During the period of homogenization (January to April) or weak stratification, the wastewater field is upwelling to the surface with strong presence above 15 to 20 meters. Results from field studies of the Hellenic Centre for Marine Research (HCMR) [9] between 2000 and 2004 on the dispersion patterns of the wastewater field reveal that:

- In 60% of the cases the field was transported west-northwest along the southern shores of Salamis Island.
- In 20% of the cases the transport had an east-southeast direction, along the shores of Attica.
- In 5 % of the cases after an initial dispersion westwards or eastwards, the circulation transfers the field south to the central part of the Saronic Gulf.
- In 10 % of the cases the dispersion patterns were inconclusive.

In the same field studies, it was found that the influence of the outfall decreases considerably with the distance and does not seem to affect the Saronikos Gulf, as far as the surface nepheloid and the bottom nepheloid layers are concerned. In the Psyttalia area higher values of nutrients, particulate and dissolved organic carbon and chlorophyll (phytoplankton biomass) were detected than in the inner, the outer and the western areas of the Gulf. Therefore, the Psyttalia area could be considered as mesotrophic to eutrophic.

Figure 2. Psyttalia Wastewater Treatment Plant (WWTP) location

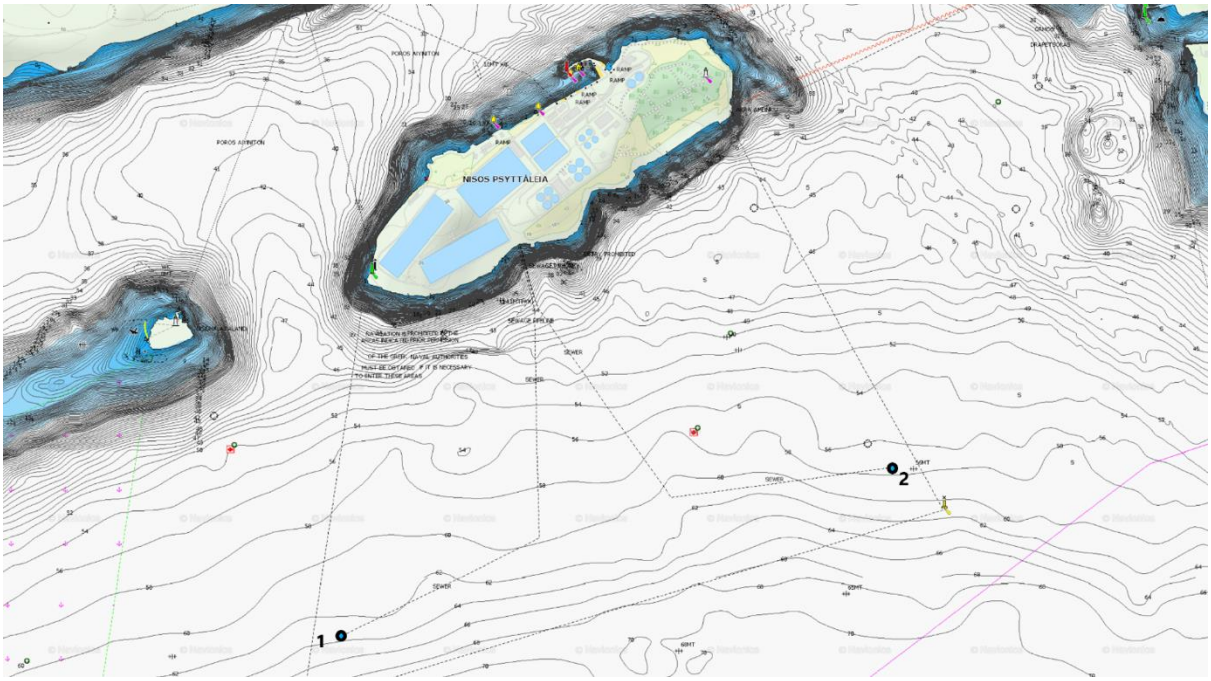


Figure 3. Sewage pipelines discharge to sea at locations: (1) 37°55.519'N 23°34.722'E (depth 63m), (2) 37°55.843'N 23°36.143'E (depth 57m)

3.1.3 Marine water and fauna sampling

The suggested points of sampling follow two different transects, one heading southeast of Psyttalia (transect A) and one heading west (transect B), as shown in Figure 4. The first points are near the coast of Psyttallia (points A1 and B1), with the following points at a distance of 500m and 1500m from the start (points A2, B2 and A3, B3 respectively). Sampling depth will be at maximum 40m, as there is no occurrence of hard substrate organisms below that depth (in depths over 30-40 meters the sea bottom is flat, and the substrate is mud or/and silt). Marine fauna will be collected around the sampling points, in areas with hard substrate, mainly points A1, B1, B2. Water samples will be collected by a Niskin type water sampler while marine fauna will be collected by scuba diving.

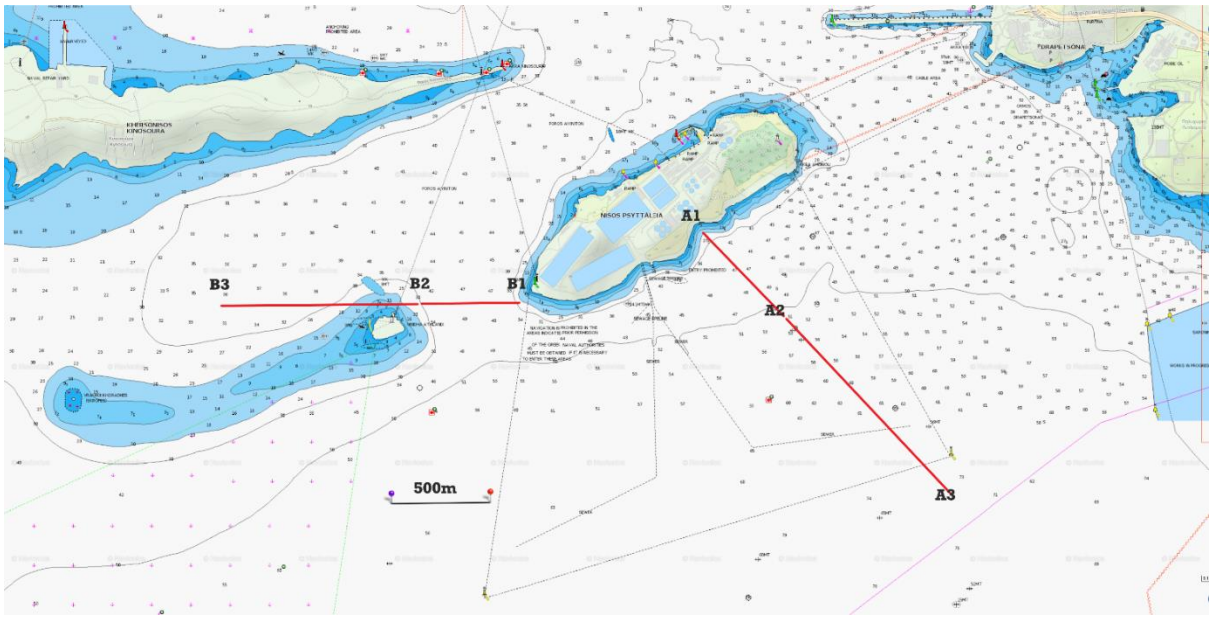


Figure 4. Suggested sampling points for marine water

The environmental monitoring campaign dedicated to the marine environment will mainly consider the presence of pharmaceuticals in the water in the selected site of the WWTP of Athens. Water samples will be collected in principle at least at two sites around WWTP, at three points of distance and at three different depths (surface, bottom, mid depth). The sampling procedure will be repeated four times, in winter, spring, summer and autumn. The volume of water samples is reported in the Annex. Salinity, temperature and depth will be measured on site for each sample taken. Water samples will be filtered to remove suspended material, aliquoted and shipped to an accredited laboratory in order to measure the concentration of pharmaceuticals. EYDAP will be responsible for these measurements by subcontracting the work to the Laboratory of Analytical Chemistry, Department of Chemistry of the University of Athens. These water samples will be tested on specimens from three marine species, the tunicate *Ciona robusta*, the mollusc *Mytilus galloprovincialis* and sea urchin *Paracentrotus lividus* collected respectively in the small sea of Taranto, in the bay of Naples, and Acciaroli, near Gulf of Salerno. The selected marine species will be also collected if present at the site of the environmental monitoring campaign at the time of water sampling, following the sampling procedures described in the Annex.

As concerns water sampling, three important parameters have to be taken into account, two related to the distance from the pollutant site and one related to the depth. Regarding the distance at which it would be appropriate to collect the water, it is necessary: a) to ensure that the salinity of the water is between 25 and 39 PSU (Practical Salinity Unit) since in order to

set up the *in vivo*/*vitro* test, it is extremely important to know the osmolarity of the seawater which the animals will be exposed to, and the salinity of the water should be specified on each water sample shipped; and b) to know more or less the concentration gradient of pharmaceuticals micropollutants in the water surrounding the sampling sites.

Regarding the depth, in addition to the salinity parameters, it is necessary to keep in mind which are the areas inhabited by the marine species selected for ENVIROMED. This aspect is particularly important for the validity of eco-immunotoxicology assessment.

Regarding sampling procedures of marine species, they should be organized considering the habitat and availability of the selected species. In detail, *Ciona robusta* adult lives attached to any substrates at a depth from 0 to 40m; *Mytilus galloprovincialis* lives attached on the rocks in the intertidal zone up to a depth of 40m; *Paracentrotus lividus* lives on the hard rocks and in *Posidonia oceanica* meadows at a depth from 0 to 30m. All these species are available throughout the year, except the tunicates that have a seasonal fluctuation due to their sensitivity to high and low temperatures. Thus, mussels and sea urchins can be found and collected throughout the year, while Ciona can be found mostly in spring and autumn.

Despite the seasonal fluctuation of the selected animal populations, which makes it less likely to encounter animals in the field at all sampling instances, water sampling in winter, spring, summer and autumn makes sense because the ecosystem certainly varies and these variations could change the status (e.g., in terms of solubility or chemical form) of the pharmaceutical pollutants in seawater and wastewater, or even their concentration. For example, there could be species of algae, protozoa etc. that absorb or modify the pharmaceuticals pollutants, which may be more abundant or metabolically active during certain seasons due to temperature changes or nutrient availability, as well as seasonal fluctuation of consumption of pharmaceuticals for the population of Athens, which would consequently affect the concentration of excreted pharmaceuticals in the wastewater.

3.1.4 Environmental monitoring campaign in Italy

A monitoring campaign in Italy has been designed and only recently annexed in the work plan activities that will be performed in Task 6.3. The only purpose of this campaign is to collect sample marine fauna from sites different from those chosen in Greece and previously described. The animals collected during the campaign in Italy should be used as control animals, as a contingency plan in case of failure to get naïve animals (born and bred in laboratory) for the immunological tests. The suggested points of sampling in Italy are selected based on the availability of the selected marine species present in the different sites, that is: Gulf of Napoli for *Mytilus galloprovincialis*, Gulf of Taranto for *Paracentrotus lividus* and Acciaroli, near Gulf of Salerno, for *Ciona robusta*, as indicated in Figure 5. The sampling procedure of marine species will be performed as described above. The only differences will be that a single sampling point (near the coast) and a single depth (mid depth) will be used for this sampling campaign. These animals will be kept in animal care facility of Stazione Zoologica Anton Dohrn of Naples. This facility uses the seawater from the Gulf of Naples.

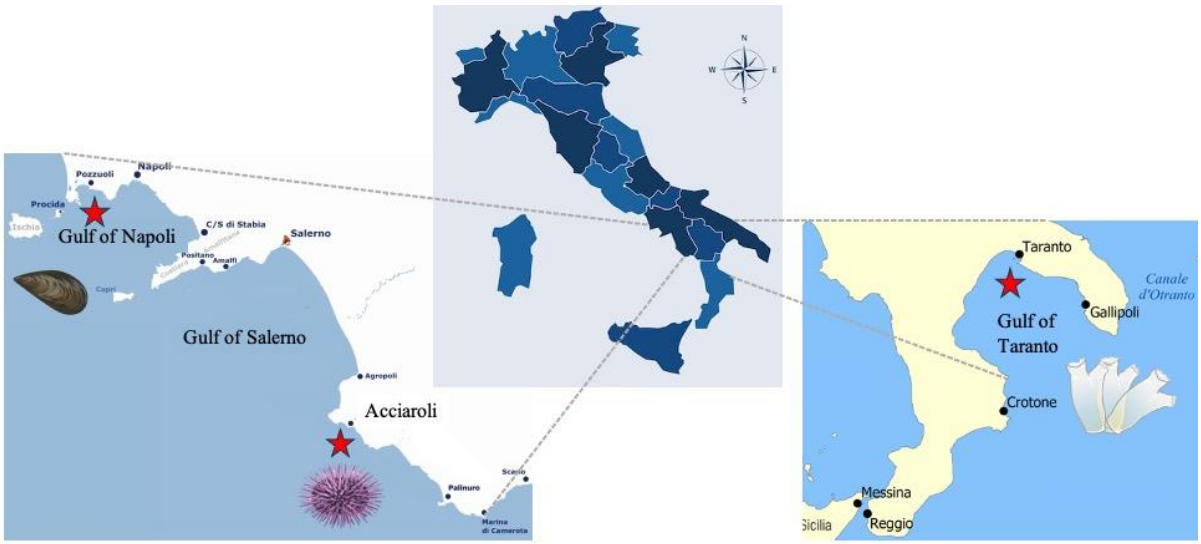


Figure 5. Suggested sampling points for marine invertebrates in Italy

3.2 Eco-Immunotoxicity studies

In order to perform eco-immunotoxicity studies, ENVIROMED will address three marine invertebrates with different characteristics, the tunicate *Ciona robusta* (sea squirt), the mollusc *Mytilus galloprovincialis* (Mediterranean mussel), and the echinoderm *Paracentrotus lividus* (Mediterranean Sea urchin) (Figure 6). These three invertebrates were chosen for some specific advantages in toxicological studies [1]. They all share a number of conserved innate immune functions with human beings, which make them suited as human proxies relative to those functions, in addition to representing models for environmental toxicology.

Seawater samples collected by the environmental monitoring campaign and containing pharmaceutical pollutants, and artificial seawater with selected pharmaceuticals identified by ENVIROMED partners (WP2 and WP6) will be tested on adult individuals from the 3 marine invertebrate species collected respectively in the small sea of Taranto, in the Gulf of Naples, and sea near Acciaroli in aquaculture and marine protected areas. In order to evaluate the concentration of pharmaceuticals in waters of animal collection sites, samples of seawater will be collected and sent to EYDAP which will be responsible for the water analysis. This will allow to set a baseline of pharmaceutical pollutants concentration to which our animals are exposed. In addition, a sample of seawater from the animal care facility of Stazione Zoologica Anton Dohrn should be also analyzed, as we expect pharmaceutical pollutants in the Gulf of Naples.

All the analysis described will be also performed on adult individuals and on tissue and cells isolated from of the 3 marine invertebrate species collected during the Environmental monitoring campaign in Greece, if animals are present at the site of water sampling.

In all species immune parameters will be analyzed to assess the possible toxicity of pollutants at tissue, cellular, and organism level.

Short-term experiments will be given greater consideration (range of exposure ca 2-3 hours). For long-term experiments there are two main limiting issues: a) the amount of water needed and collected from the environment is unmanageable, since around 400lt of seawater are required for each sampling season (see Annex), that are hard to collect, ship and store in the laboratory due to high cost, labor and storage room demands; and b) the animals exposed for a

long time, for example, to pharmaceuticals, would be subjected to a very high level of stress due to the living conditions, as they should be kept in a beaker rather than in a tank due to the limited water volume availability. This would make it very difficult to distinguish between the effects due to stress and those due to the presence of pharmaceutical pollutants. Indeed, the response to stress is generally very high and could overshadow the response to other threats, and a stressed animal is not a good model for toxicological studies.

All the experiments should be performed with collected seawater from Greece and with artificial/clean seawater where selected pharmaceuticals will be added. However, the experiments with collected seawater from Greece will be decided later based on the available water from Greece and based on the presence or not of the selected pharmaceuticals.

Figure 6. Selected marine invertebrates

3.2.1 C. robusta

3.2.1.1 Effect at tissue level

Transcription studies on gut and pharynx. To investigate the impact of pollutants at tissue level, gut and pharynx organs (the immunocompetent organs) will be collected from 20 individuals, exposed either to polluted seawaters from Greece or seawater with added pharmaceutical compounds at specific concentrations, to extract total RNA for transcriptomic analyses. Three different categories of genes will be analyzed: general immune-related genes (*C3-1, C3ar, Il17-1, Il17-2, Il17r, Tnf, Tgfb, Lbp, Tlr-2, Tlr13, Cd36*), organ-specific immune-related genes (hemocyte and gut-specific *Vcbp-B* and *Vcbp-C*) and oxidative stress-related genes (*Sod-A, Gst, Gr*). Control animals will be individuals not treated or exposed to pharmaceuticals, kept in clean seawater. This approach requires the animals' sacrifice, and therefore replicate groups will be set up for other analyses.

***Ex vivo* assays on haemocytes.** Another tool to investigate the effect of polluted waters at tissue level is the short-term toxicity assays carried out on haemocytes collected from treated animals. Briefly, haemolymph containing circulating cells will be collected and both functional and diagnostic assays will be performed. First, haemocyte subpopulations count will be run. Then, the evaluation of the haemocytes' viability as well as the quantization of apoptotic and necrotic cells will be established by using a kit (Annexin V-FITC kit) that allows the differentiation among early apoptotic cells (annexin V positive, propidium iodide negative), necrotic cells (annexin V positive, PI positive), and viable cells (annexin V negative, PI negative). In addition, lysosomal membrane stability will be evaluated by the neutral red retention time (NRRT), while dynamic changes and functions of the lysosomes will be

evaluated with a LysoSensor, a dye that accumulates inside acidic vesicles with a fluorescence intensity proportional to acidification. All above assays are quick and do not require cell fixation. Conversely, enzymatic assay for phenoloxidase is carried out on fixed cells. Lastly, the immune abilities of haemocytes will be measured through a phagocytic assay. About 20 animals will be enrolled per assay.

3.2.1.2 Effect at organism level

Immune memory establishment. The impact of pharmaceuticals and pollutants on the whole organism will be assessed in terms of immune memory establishment. Briefly, following exposure to polluted seawater from Greece or seawater with added pharmaceuticals (priming), animals will be maintained in tanks with non-polluted seawater, fed and properly oxygenated for one week (resting time). After resting they will be challenged with a microbial stimulus, the LPS. Then, the expression profile of selected immune and stress genes, listed above, from immunocompetent organs, namely gut and pharynx, will be evaluated by real time PCR. Moreover, the analysis of epigenetic changes will be also performed. These data will be compared with data from control group animals (not treated) to find out the development of immunological memory.

Metabolomic studies. The rapid changes in metabolite production induced by pharmaceuticals on individuals will be assessed through a metabolomic approach. This unbiased analysis allows the identification of biomarkers for toxicity and inflammation without previous knowledge of the specific metabolites or metabolic pathways involved in the studied phenomena. Specific kit will be used for the extraction of metabolites from hemolymph and immunocompetent organs and data will be comparatively profiled by a UPLC-MS (ultra-performance liquid chromatography-mass spectrometry) untargeted approach.

3.2.1.3 Effect at cellular (haemocyte) level

Pure pharmaceutical substances will be tested directly on haemocytes collected from healthy animals. Then, the possible effect of substances will be evaluated through the short-term toxicity assays, described above. In this way it is also possible to carry out dose-response studies. Moreover, the difference in terms of reactivity against prototypical stimuli (such as LPS) will be evaluated by comparing the cells isolated from sampling animals and those isolated from healthy animals.

3.2.2 M. galloprovincialis

The experimental procedures for *M. galloprovincialis* are the same as in *C. robusta* in terms of exposure time and amount of sea water. In Mytilus, as in Ciona, besides the haemolymph, there are two immunocompetent organs, namely the gills and digestive gland, available for quoting response to pollutants. However, as it is not possible to extract total RNA from haemocytes, even the transcriptomic studies will be carried out on immune-competent organs. Transcription studies include a set of selected genes involved in cell proliferation and apoptosis [proliferating cell nuclear antigen (PCNA) and tumor suppression protein 53 (p53), respectively] and in immune response [Extrapallial protein precursor (EPp), Lyso, Toll-like receptor i isoform (TLR-i), mytilin B (MytB), myticin B (MytC), and fibrinogen-related protein (FREP)] that will be evaluated by qPCR. Gills and digestive gland from treated animals could be collected to study/detect histologically an inflammatory response that in gills appears as a consistent infiltration of hemocytes and impairment of the ciliated epithelium, whilst in digestive gland it emerges as a loss of consistency in the tissues, vacuolization of digestive tubules, and massive

infiltration of white adipose tissue. Viability and immune ability assays will be run on haemocytes with the same procedures of *Ciona robusta* as well as metabolomic studies on all three immunocompetent tissues.

3.2.3 P. lividus

The main criticism of *P. lividus* model is the large amount of water needed per individual. For this reason, we could not run experiments with seawater sampled in Greece. Alternatively, animals will be submerged in sea water containing known amounts of pharmaceuticals and then their effects on coelomic cells will be tested. In the sea urchin, the immune response is brought about by the heterogeneous population of circulating cells within all coelomic spaces, whose composition is modulated by environment. In sea urchin the transcriptomic analyses will be done on coelomic cells as well. Examples of immune responsive genes are *Tlr, p38 Mapk, Nf-kb* and *Jun*. Lastly, an appraisal of the response at organism level, that is the establishment of an immune memory, is not possible, as no methods for assessing immune memory have been proposed yet.

4. Ethical clearance and permission

Since the use of invertebrates is not considered as animal experimentation, no ethical clearance and permission are needed. Indeed, studies on the three selected marine species do not need ethical review and approval, in accordance with the national and international legislation regulating animal experimentation. All activities will be performed according to the Italian DLgs 26/2014, the European Directive 2010/63/EU and the associated "A working document on Animal Welfare Bodies and National Committees to fulfil the requirements under the Directive".

All the invertebrate models involved in ENVIROMED have the advantage of complying with the 3R principle of reducing, refining, and replacing animal experimentation. However, in this perspective, adherence to the ARRIVE guidelines will be strongly followed in order to strengthen the quality and utility of immunotoxicology research. These guidelines have been developed to improve the quality of reporting in research using animals [10] and imply that all investigations on animals should describe essential information, including the number and specific characteristics of animals used, details of housing and husbandry, and the experimental, statistical, and analytical methods applied in the study.

5. Conclusions

The aim of this deliverable was to define metrics for the Key Performance Indicators, to design and plan the environmental monitoring campaigns and to describe the immune-related parameters and methodological approaches/assays to perform the ecotoxicity study.

The campaigns for environmental monitoring have been designed as described in Task 2.3, that is, by selecting the sampling sites, planning of timing for sampling and type/amount of samples, describing the sampling procedures and the organisation of the sampling activities.

The ecotoxicity studies have been designed considering in vivo and ex vivo/in vitro experimental procedures, and a series of toxicity evaluation criteria, prioritized based on the expected relevance/impact. In view of what is described in the DoA, the three marine species and the functional/molecular parameters (*i.e.*, haemocyte counts, haemocyte subpopulations, phagocytosis of bacteria, production of phenoloxidase, release of lysozyme, expression of immune and stress-related genes) have been confirmed and described. Conversely, general evaluation parameters, such as survival, susceptibility to stress/infections, generation of innate memory or TGIP, and encapsulation/ melanization reactions have been included, except development and reproduction.

All the methodological approaches for immune-ecotoxicity study have been selected considering all the technical issues.

References

1. Auguste M, Melillo D, Corteggio A, Marino R, Canesi L, Pinsino A, Italiani P and Boraschi D (2022). Methodological Approaches to Assess Innate Immunity and Innate Memory in Marine Invertebrates and Humans. Front. Toxicology 4:842469. doi: 10.3389/ftox.2022.842469
2. Pietrzykowski M et al. An environmental life cycle assessment comparison of single-use and conventional process technology for the production of monoclonal antibodies. J. Clean. Prod. 2013, 41, 150-162. doi: 10.1016/j.jclepro.2012.09.048
3. Lee CK, Khoo HH and Tan RBH. Life Cyle Assessment Based Environmental Performance Comparison of Batch and Continuous Processing: A Case of 4-d-Erythronolactone Synthesis. Org. Process Res. Dev. 2016, 20, 11, 1937–1948. doi: 10.1021/acs.oprd.6b00275
4. Osorio-Tejada J et al. Life cycle assessment of multistep benzoxazole synthesis: from batch to waste-minimised continuous flow systems. Green Chem., 2022, 24, 325-337. doi: 10.1039/D1GC03202J
5. Cook SM et al. Life Cycle Comparison of Environmental Emissions from Three Disposal Options for Unused Pharmaceuticals. Environ. Sci. Technol. 2012, 46, 10, 5535–5541. doi: 10.1021/es203987b
6. Emara Y et al. Modeling pharmaceutical emissions and their toxicity-related effects in life cycle assessment (LCA): A review. Integr. Environ. Assess. Manag. 2019, 15, 1, 6-18. doi: 10.1002/ieam.4100
7. Ott D et al. Life Cycle Analysis within Pharmaceutical Process Optimization and Intensification: Case Study of Active Pharmaceutical Ingredient Production. ChemSusChem, 2014, 7, 12, 3521-3533. doi: 10.1002/cssc.201402313
8. Document 52019DC0640, COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE EUROPEAN COUNCIL, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS The European Green Deal, COM/2019/640 final. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=COM%3A2019%3A640%3AFIN
9. Siokou-Frangou, I. (2004). Monitoring of the Saronikos Gulf ecosystem affected by the Psyttalia sea outfall (Final Report – in Greek). Athens: HCMR.
10. Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., and Altman, D. G. (2010). Improving Bioscience Research Reporting: the ARRIVE Guidelines for Reporting Animal Research. Plos Biol. 8, e1000412. doi: 10.1371/journal.pbio.1000412

ANNEX: Sampling procedure

- SEAWATER SAMPLING

Water samples will be collected in principle at least at two sites around WWTP, at three points of distance and at three different depths (surface, bottom, mid depth). The sampling procedure will be repeated 4 times, in winter, spring, summer and autumn. The amount of sea water required for ecotoxicity experiments for both model organisms (only tunicates and bivalve) is about 36 lt (around 18 lt per model) per sampling site and per season. Considering the different points and times of sampling, the total amount of sea water needed should be at least 300-400 Lt per season.

Concerning the amount of seawater needed to expose animals, approximatively 0.5 lt of "polluted" seawater is required for 3 animals to be exposed for 2-24hr. So at least 3,5 L of water is needed for 20 animals.

- MARINE INVERTEBRATE SAMPLING

Ascidians (*Ciona robusta*)

Adults of *Ciona robusta* live in clumps and usually all of them have similar size. Animals with size of at least of 7-10 cm should be collected.

Collecting:

1. In order to avoid harming animals, the animals should be kept attached to the soft substrates (*i.e.*, ropes) and collected by cutting substrates, or if it is not possible (*i.e.*, they are attached to hard substrates like rocks), the material connecting the posterior end of body with substrates should be cut.

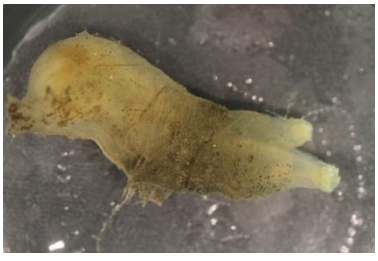
2. The animals have to be kept in large tanks of seawater in which they were living (100-200 ml sw/animal) until the shipment and kept at 15-18°C.

3. At least 100 animals/sampling site should be collected.

(**Note**: rate of survival during the shipment is more or less 30%)

Packaging and shipping:

10-20 animals should be kept in a 20-liter bag filled with 15-18°C seawater, then the bags should be put in a polystyrene box (do not overlap bags with animals) along with freezer packs.



ENVIROMED is interested to collect *Ciona robusta* (shown in the pictures on the right); however, if it should not be possible, other species of Ciona present in the site would be acceptable.

Mussels (*Mytilus galloprovincialis*)

Collecting:

1. Collect at least 100 animals/sampling site

(**Please note**: rate of survival during the shipment is more or less 30%)

2. Soon after the sampling, the mussels should be kept at 15 °C seawater

Packaging and shipping:

Mussels should be kept in bag nets tightly closed, then the bag nets should be put in a polystyrene box. The animals must be overlaid with ice or other cooling tools, on the condition that the temperature should be 4°C throughout the shipment.

Sea Urchin (*Paracentrotus lividus*)

Collecting:

Collect at least 10 animals/sampling site

Packaging and Shipping:

Put sea urchins in tanks (see picture on the right), 1 lt of seawater per animal; then put the tanks in a polystyrene box with whatever tools to maintain the temperature at 16-18°C throughout the shipment.