UNIVERSITÀ POLITECNICA DELLE MARCHE

Dipartimento di Scienze della Vita e dell'Ambiente DISVA

PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

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

Name of PhD student: Michela Panni

Title of PhD research:
Presence, behavior and risk assessment of pharmaceutical products in aquatic ecosystems

Name of PhD supervisor: Prof. Stefania Gorbi

Research lab name: Ecotoxicology and Environmental Chemistry

Cycle:
XXXVII

PhD Curriculum:
Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:
Actea Mobile Laboratory
Advanced Instrumentation lab
Aquarium

ABSTRACT (1000 characters, including spaces):

Active Pharmaceutical Ingredients (APIs) are considered contaminants of emerging concern, due to their ubiquitous presence in water systems and their potential adverse effects for non-target aquatic species. Although preliminary scientific studies highlighted the simultaneously occurrence of several APIs in natural environments, information on occurrence, bioaccumulation and adverse effects in coastal areas are still limited and fragmented. In this respect, the overall aim of my PhD study is to clarify the ecotoxicological impact of APIs, applying a multidisciplinary approach which integrates (1) field studies to characterize APIs occurrence in seawater, sediments and bioaccumulation in wild species, with (2) laboratory experiments to clarify the toxicity of single molecules and APIs mixtures to different bioindicator species. Overall results will be finally integrated to develop the first pharmaceuticals environmental risk assessment procedure in marine systems through a Weight of Evidence approach.

Part 1. Scientific case of the PhD Research

BACKGROUND

Active Pharmaceutical Ingredients (APIs) are well documented contaminants of emerging concern. Their ubiquitous occurrence in aquatic ecosystems is caused by the massive use in human and veterinary medicine and a limited removal by wastewater treatment plants (WWTPs) (Bayer et al., 2014; Mezzelani et al., 2018a). To date, several active principles from different therapeutic classes such as Non-Steroidal Anti- Inflammatory drugs (NSAIDs), lipid-lowering agents, cardiovascular and psychiatric drugs are ubiquitously detected in seawater and sediment worldwide, with variable levels ranging from ng/L to μg/L and ng/g, respectively (Moreno-González et al. 2015; Mezzelani et al. 2018a). Evidence of APIs uptake in microalgae, mollusks, crustaceans and fishes (Álvarez-Muñoz et al. 2015; Moreno-González et al. 2016; Ali et al. 2018; Mezzelani et al. 2020) along with APIs capability to interfere with physiological and biochemical processes in aquatic non-target organisms (Álvarez-Muñoz et al., 2015, Trombini et al., 2019, Cerveny et al., 2021, Cravo et al., 2022), determined an increased concern for potential long-term adverse effects to marine species. So far the majority of the studies aimed to clarify the environmental risk of pharmaceuticals focused on acute toxicity test, dosing concentrations usually orders of magnitude higher (mg/L) than those possibly found in aquatic ecosystems, thus revealing a low acute toxicity potential of these molecules in natural environment but also that the use of high concentrations in ecotoxicological bioassays may underestimate potential adverse outcomes in marine organisms exposed to low doses in chronic conditions (Mezzelani and Regoli, 2022).

An integrated ecotoxicological approach, which clarify APIs bioavailability, uptake and the onset of sublethal effects caused by environmentally realistic concentrations of APIs in non-target species, might represent a helpful tool to develop a more comprehensive environmental risk assessment of these emerging contaminants. Since pharmaceuticals typically co-occur as complex cocktail of several molecules with different chemical structures and modes of action, the ecotoxicological potential of single pharmaceuticals should be coupled to detailed studies on effects caused by APIs mixtures, in order to unravel potential additive or synergistic interactions. All these aspects have been so far overshadowed and limited investigated. In this respect, the aim of my PhD study is to clarify the ecotoxicological impact of APIs, both characterizing their occurrence in seawater, sediments and uptake in wild species and investigating under laboratory conditions the onset of adverse effects of both single molecules and mixtures, toward a final development of an environmental risk assessment procedure.

SCIENTIFIC AIMS

The overall aim of my PhD project is to study the ecotoxicological impact of APIs in marine ecosystems: (a) investigating the occurrence, spatial and temporal distribution of pharmaceuticals in seawater, sediments and wild species along the Adriatic Sea; (b) evaluating the bioaccumulation and the onset of sublethal adverse effects of both single molecules and mixtures, on a wide array of model species belonging to different trophic levels; (c) to finally develop the first comprehensive environmental risk assessment procedure for pharmaceuticals.

WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Field investigation: pharmaceuticals along the Adriatic Coasts

Objective: the aim of the field investigation is to study the APIs occurrence along the Adriatic Sea, characterizing temporal and spatial variation in water column, sediment and marine species.

Methods: in order to cover the entire basin of the Adriatic Sea, three areas characterized by different environmental and anthropogenic pressure were selected in the North, Central and South-Adriatic Sea. Evaluation of seasonal influence in APIs occurrence was considered, collecting samples both in winter 2021 and summer 2022. The first site namely Porto Garibaldi represents a coastal area close to the Po River Delta considered to be of national relevance as environmental source of land-based compounds to the Adriatic Sea. In Central Adriatic Sea, three main subareas were selected Ancona and Civitanova Marche and the area within the Ancona harbor which receive the outfall of the urban WWTP. The third area represents the pristine site close to the Marine Protected Area of the Tremiti Islands. Seawater samples were collected with pump by filling-up two bottles (500mL) in each sampling site, while sediments were sampled with the Van Veen grab (500g). All samples were stored at 4°C and -20°C. Species of commercial interest belonging to different trophic levels have been obtained from local fishermen. Liver and muscle tissues (n= 20) were dissected from the benthopelagic and benthic fishes including *Merluccius merluccius*, *Mullus barbatus* and *Solea solea*, while the entire soft tissues were collected from crustaceans *Squilla mantis.* A particular attention has been paid toward the Ancona harbor area, further collecting native mussels (*Mytilus galloprovincalis*) close to the WWTP outfall. All biological samples were stored at -20°C. All samples will be processed for the extraction, detection and quantification of APIs through liquid chromatography-mass spectrometry (LC/MS) techniques.

Expected/Obtained Results: the adaption and development of new analytical methods through liquid chromatography-mass spectrometry (LC/MS) techniques will allow the simultaneously extraction, detection and quantification of various APIs from both abiotic and biotic matrices. These activities are ongoing. Results will provide the first survey on spatial and temporal distribution on pharmaceuticals occurrence in seawater, sediments and selected species in the Adriatic Sea.

WP 2. Laboratory studies: bioaccumulation and sublethal effects of pharmaceuticals in marine species

Objective: the overall aim of the laboratory studies is to investigate, under controlled and ecologically relevant experimental conditions, short and long-term adverse effects caused by single pharmaceuticals and APIs mixtures in various marine species.

First experimental plan. Ecotoxicological potential of Ibuprofen, Paroxetine and their mixture in the Mediterranean mussels *M. galloprovincialis.*

Methods: mussels *M. galloprovincialis* were exposed during Winter 2021 to the antidepressant Paroxetine (PAR) and to the Non-Steroidal Anti-Inflammatory Drug Ibuprofen (IBU), both alone and in mixtures, to the environmentally realistic concentration of 1μg/L for 30 days, followed by 14 days in pharmaceuticals-free

seawater, intended as a depuration phase. Selected pharmaceuticals represent two of the most consumed drugs in Italy, also revealed to be accumulated in wild mussels from the Mediterranean Sea (Mezzelani et al., 2020). At the end of the exposure and depuration phases, tissues were collected and properly stored at -20°C and -80°C for the bioaccumulation analyses and measurement of a wide panel of biomarkers reflecting the perturbation of different cellular districts and biochemical pathways. Standardized protocols (Bocchetti et al., 2008) were applied to evaluate immunological responses (lysosomal membrane stability, phagocytosis rate and granulocytes/hyalinocytes ratio), genotoxic damage (loss of DNA integrity and micronuclei frequency), neurotoxic effects (acetylcholinesterase activity), peroxisomal proliferation (Acyl-CoA oxidase activity), single antioxidant enzymes (catalase, glutathione S-transferases, Se-dependent glutathione peroxidases, the sum of Se-dependent and Se-independent glutathione peroxidases and glutathione reductase), total glutathione, the total oxyradical scavenging capacity toward peroxyl (ROO•) and hydroxyl (HO•) radicals, accumulation of malondialdehyde, lipofuscin and neutral lipids.

Expected/Obtained Results: preliminary results on biomarkers parameters highlighted in all treatments (IBU, PAR, Mixture) an altered immunocompetence, a significant impairment of the oxidative metabolism, an increase of micronuclei frequency and DNA fragmentation, without remarkable differences between mussels exposed to pharmaceuticals alone compared to the Mixture. Modulation of such pathways, maintained even after the depuration phase highlighted a long-lasting effect of environmentally realistic levels of tested pharmaceuticals. These results will be integrated with the ongoing analyses on bioaccumulation. enabling new information on the ecological consequences of single APIs and mixtures for marine ecosystems to be provided.

Second experimental plan. Application of a battery of bioassays within the assessment procedure for the ecotoxicological impact of environmental pharmaceuticals.

Methods: a panel of 15 pharmaceuticals belonging to different therapeutic classes (Non-steroidal Antinflammatory drugs, Psychiatric drugs, Cardiovascular drugs and lipid lowering agents) were individually tested through a battery of bioassays which includes the algal growth inhibition of *Phaeodactylum tricornutum*, the embryotoxicity assay with *Paracentrotus lividus* and the bioluminescence test with *Aliivibrio fischeri*.
For some of these molecules their metabolites have also been tested. These selected APIs were also tested as mixtures, choosing six different combinations based on: (a) Mechanism of Action of single molecule, (b) combinations of pharmaceuticals typically measured in the environment and (c) mixtures used in human therapy. Pharmaceuticals were dosed at 1µg/L and 10µg/L, selected within a range of concentrations measure in marine/coastal environment. Obtained results were elaborated through the quantitative Weight of Evidence (WOE) model, which assign to each bioassay specific thresholds and weights based on the measured endpoint and sensitivity of the tested species, to finally provide a cumulative level of hazard of the entire bioassays battery.

Expected/Obtained Results: results highlighted different sensitivity of tested species toward selected pharmaceuticals and mixtures, with major effects on *P. tricornutum* and *P. lividus*. The overall elaboration with the WOE model revealed a higher hazard level for Gemfibrozil (Lipid lowering agent), Paroxetine,

Carbamazepine and its metabolite (Psychiatric drugs), compared to all the other tested APIs. Furthermore, higher level of hazard was measured for mixtures compared to single compounds. Quite interesting, in mixtures higher toxicity was not related to the exposure dose or to the number of combined drugs, revealing the need of future investigations to address mechanisms of interaction among these molecules.

WP3. Outreach activities

In this first year of PhD I participated to various dissemination events: SHARPER night (the European night of researchers, September 2021 and September 2022), SostenibilMente (event on sustainability organized by Ancona Municipality, November 2021), Sealogy (The European Blue Economy Show, November 2021) and laboratories for high schools. During these events, I had the opportunity to talk about aims and activities of my PhD project to citizen, students, public and private sector experts also through the preparation of poster, banner and brochures.

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Cravo A., Silva S., Rodrigues J., Cardoso V.V., Benoliel M.J., Correia C., Coelho M.R., Rosa M.J., Almeida C.M.M., 2022. **Understanding the bioaccumulation of pharmaceutical active compounds by clams Ruditapes decussatus exposed to a UWWTP discharge**. Environ. Res., 208, Article 112632, 10.1016/j.envres.2021.112632

Cerveny D., Fick J., Klaminder J., McCallum E.S., Bertram M.G., Castillo N.A., Brodin T., 2021. **Water temperature affects the biotransformation and accumulation of a psychoactive pharmaceutical and its metabolite in aquatic organisms**. Environ. Int., 155 (2021), Article 106705

Gonzalez-Rey M., Tapie N., Le Menach K., Dévier M.-H., Budzinski H., Bebianno M.J., 2015. **Occurrence of pharmaceutical compounds and pesticides in aquatic systems**. Mar. Pollut. Bull., 96, pp. 384-400, 10.1016/j.marpolbul.2015.04.029"

Mezzelani M., Gorbi S., Fattorini D., d'Errico G., Consolandi G., Milan M., BargelloniL., Regoli F., 2018. **Long-term exposure of** ***Mytilus galloprovincialis*** **to Diclofenac, Ibuprofen and Ketoprofen: insights into bioavailability, biomarkers and transcriptomic changes**. Chemosphere, 198, pp. 238-248, 10.1016/j.chemosphere.2018.01.148

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Part 2. PhD student information on the overall year activity

List of attended courses/seminars/schools

1. "The resolution revolution in Cryo-electron-microscopy, in Structural Biology and in Life Sciences", Martino Bolognesi, Department of Biosciences, University of Milan, 7 June 2022.
2. "Current threats to research ethics and how to cope with them", Marco Seeber, Department of Political Science and Management, University of Agder, Norway, 9 June 2022.
3. "Cambiamenti climatici, comunità e Sindaci resilienti: contributi e riflessioni con il mondo universitario"**,** Università Politecnica Marche, Facoltà Di Ingegneria, 12 October 2022

List of conferences/workshops attended and of contributions eventually presented

1. **M. Panni**, M. Mezzelani, G. d'Errico, M. Benedetti, S. Gorbi, F. Regoli (2022). Application of a battery of bioassays within the assessment procedure for the ecotoxicological impact of environmental pharmaceuticals. SETAC Italia, Siena, 15 September 2022.
2. M. Mezzelani, L. Peruzza, I. Bernardini, F. Buttari, S. Gorbi, G. d'Errico, **M. Panni**, D. Fattorini, M. Milan, F. Regoli (2022). Interactive effects of pharmaceuticals' mixtures in *Mytilus galloprovincialis.* Symposium on Pollutant Responses In Marine Organisms (PRIMO21), Gothenburg, Sweden, 22-25 May 2022.

Part 3. PhD student information on publications

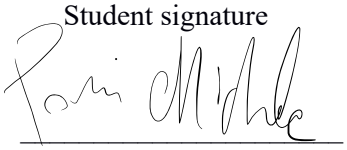
List of publications on international journals

1. M.E. Giuliani, M. Mezzelani, **M. Panni**, S. Gorbi. Evidence of similar hypolipidemic function of Fenofibrate and the natural bioactive compound Caulerpin in Precision Cut-Tissue Slices (PCTS) of *Mytilus galloprovincialis*
   In preparation for the Special issue in Marine drugs (MDPI)

2. **M. Panni**, M. Mezzelani, G. d'Errico, S. Gorbi, F. Regoli. Application of a battery of bioassays within the assessment procedure for the ecotoxicological impact of environmental pharmaceuticals. In preparation for Environmental Research

Ancona, 12/10/2022

Student signature



Supervisor signature