

PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

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

Name of PhD student: Melania Siracusa

Title of PhD research: Emerging Biotoxins in marine organisms: chemical methods of analysis, bioaccumulation and eco-toxicological studies.

Name of PhD supervisor: Stefania Gorbi

Research lab name: Laboratory of Ecotoxicology and Environmental Chemistry- UNIVPM /Laboratorio Contaminanti Organici, Metalli Pesanti e Biotossine Algali - Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", sede di Ancona.

Cycle:

XXXVI

XXXVII

PhD Curriculum::

Marine biology and ecology

Biomolecular Sciences

Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:

Actea Mobile Laboratory

Advanced Instrumentation lab

Aquarium

MassSpec lab

MaSBiC

Simulation/informatics lab

Other. Please, indicate: Laboratory of Ecotoxicology and Environmental Chemistry.

ABSTRACT (1000 characters, including spaces):

Emerging Marine Biotoxins (EMBs) are microalgal and/or bacterial origin toxins for which distribution and toxicity data are limited. In the last years, they were detected in the Mediterranean Sea causing great concerns for the environment, human health and fishing; in this context it's important to investigate EMBs presence in the aquatic environment, their trophic transfer in seafood, and to identify biomarkers of exposure.

In this project chemical methods will be implemented to evaluate toxin profiles in field samples collected in the North-central Adriatic Sea, and the potential trophic transfer in organisms (fish) exposed to toxic microalgae through contaminated mussels (feeding fish), in mesocosm experiments. In exposed organisms the biological responses (biomarkers) will be studied. The research will be extended to the study of cyanobacteria and cyanotoxins, considered environmental Contaminants of Emerging Concerns, a threat of inland waters, that recently involved also coastal areas.

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

- BACKGROUND

Information on EMBs is still limited, in fact also EFSA (European Food Safety Agency) stressed on the need to investigate on this topic. PLTXs, especially ovatoxins (OVTXs) produced by the *Ostreopsis cf. ovata*, occurred, in the last decade, along the whole Mediterranean coasts, including Conero Riviera (North-central Adriatic Sea) [1]. CIs have been identified in shellfish from Europe including Italy [2]. A new toxic *Azadinium* species (*A. dexteroporum*) has been isolated in the Tyrrhenian Sea and traces of AZAs have been detected for the first time in mussels from the Adriatic Sea [3]. TTX is a toxin produced by bacteria (*Vibrio* sp, etc.), responsible for the fatal pufferfish poisoning, recently detected even in shellfish from Adriatic Sea [4]. Despite the presence of shellfish contaminated by EMBs, the primary producers, both toxic microalgae and bacteria, are rarely found in seawater. Exposure experiments to evaluate biological effects (biomarkers) of *Ostreopsis cf. ovata* and *A. dexteroporum* on mussels *Mytilus galloprovincialis* were performed, revealing an important biological impact of these EMBs [5,6]. Microcystins (MCs) are cyanotoxins with potential toxic effects in humans, although they are regulated only in drinking water. *Planktothrix rubescens* blooms, one of the most detected MCs producers, occurred frequently in Castreccioni Lake, the biggest drinking water reservoir in the Marche region (Italy). The lake was monitored over six years revealing intense cyanobacterial blooms and MCs levels were occasionally, quite above the legal limit [7].

- SCIENTIFIC AIMS

The project aims to elucidate toxicological impact of EMBs through a multidisciplinary approach, including:

- Implementation and validation of LC-MS/MS methods for the determination of the complex toxin profiles in shellfish (field samples), fish (exposed organisms) and toxic microalgal species.
- Evaluation by LC-MS/MS of the cyanobacteria ability to survive in different environmental conditions.
- Microbiological analysis characterizing potential TTX-producer bacteria.
- Study of the algae and/or toxins trophic transfer in marine organisms through exposure experiments.
- Eco-toxicological studies of molecular and cellular effects (biomarkers) induced by algae and/or toxins in sentinel organisms.

- WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Objective.

Toxic profiles, assessment of EMBs contamination levels in mussels analysed by LC-MS/MS, study of annual trends.

Methods.

In the first research year, implemented LC-MS/MS methods were used to analyse mussels collected during 2021. The EMBs contamination was evaluated using calibration curves built with certified materials, assuming a linear response.

Obtained Results.

CIs and AZAs

AZAs were found in mussels at low levels (10-13 µg AZA1 eq./Kg), with the predominance of AZA2 (65-100%). The maximum period of contamination was October-December in the Marche North coast. CIs also were found at low levels, the most abundant were Spirolides (SPXs) with a maximum contamination of 12-14 µg/Kg in the first part of the year (January-April). GYM A was found at LOQ levels (1-3 µg/Kg), then it was difficult to carry out geographical and temporal trends. AZAs and CIs still seem not to represent a risk for human health; in fact, levels are very far from legal limits (160 µg AZA1 eq./kg, Reg. EC 853/2004) for AZAs, and guidance level (400 µg sum of SPXs/Kg, EFSA opinion) for CIs.

TTXs

Results showed that mussels from Pesaro and Ancona natural banks were contaminated by TTX. Some samples (5%), collected from Ancona wild sites during June-July 2021, exceed the EFSA guidance level (44 µg TTX eq./kg). Mussels sampled at a specific point of Molo Portonovo (An, Italy) reached significant high levels of about 300 µg TTX eq./kg in early June 2021. In order to further investigate on this evidence, the mussel sampling campaign was repeated at the same site in June-August 2022. Moreover, a screening method for Paralytic Shellfish Toxins (PSTs) was implemented and used as tool for molluscs' official control. Monitoring PSTs is useful to better understand the phenomenon, since this class of toxins show chemical properties similar to TTX, with the same biological producers.

PLTXs

PLTXs were measured at low contamination levels (about 30 µg/kg) in mussels collected in September 2021 (EFSA guidance level is set at 250 µg/kg).

Expected Results.

Compartmentalization studies in TTXs contaminated mussels to evaluate the toxin distribution in different tissues (digestive gland, mantle, gills). This is useful to better understand the TTX contamination in shellfish.

WP 2. Objective.

Microbiological and molecular analysis to characterize potential TTX-producer bacteria.

Methods.

Methods for isolation and enumeration of *Vibrio* spp., PCR protocols for *V. alginolyticus*, PKS and NRPS genes (identified as TTX biosynthesis genes), optimized in the first year of research, were used to analyse mussel samples collected in 2021. The same protocols were also tested and optimized for phytoplankton, seawater and sediment.

Obtained Results.

In the analysed samples only colonies of *V. alginolyticus* were isolated and confirmed by PCR. Biosynthesis genes were found in the about 50% of samples. The co-occurrence of TTX and biosynthesis genes was checked in a lot of samples, underlining the necessity of a deeper investigation. *V. alginolyticus* was found also in phytoplankton, seawater and sediment, with a high concentration in July 2021, as for mussels.

Expected Results.

PCR analysis for phytoplankton, seawater and sediment.

WP 3. Objective.

Eco-toxicological study of trophic transfer and biological effects (biomarkers) induced by *Ostreopsis cf. ovata* in sea breams fed with contaminated mussels.

Methods.

The exposure experiments with control and exposure concentrations, were set up with 110 *Mytilus galloprovincialis* specimens for each tank. Mussels were acclimated for a week and then exposed to toxic *O. cf. ovata* for a month. Mussels accumulated OVTXs, as confirmed by LC-MS/MS analysis, were then used as feeding fish. Trophic transfer was studied exposing sea breams in 4 tanks (2/3 specimens per tank): 2 tanks for the exposure, 2 for control (Authorization to animal testing n° 680/2018-PR). Fishes were acclimated for a week, then fed with contaminated mussels for a further week. At the end of the exposure, sea breams were dissected and the different tissues collected (liver, muscle, gills and gastro-intestinal tract).

LC-MS/MS method already implemented for PLTXs analysis in mussels, was applied also for fish tissues, obtaining good performances. Transcriptomic analysis based on RNA sequencing and Focal Plane Array (FPA)-Fourier Transform Infrared Spectroscopy Imaging (FTIRI) measurements were carried out in liver samples of control and exposed fish.

Obtained Results.

LC-MS/MS analysis confirmed that mussels accumulated OVTXs with a maximum concentration of 150-200 µg/kg. The toxic profile was similar to that of *O. cf. ovata*. Livers, muscle, gills and gastro-intestinal tracts of contaminated seabreams demonstrated an OVTXs level always below the limit of detection (LOD). The analyses in seabreams livers indicated that an OVTX-enriched diet induced alterations of genes involved in the lipid metabolism. This suggests biological effects in exposed organisms.

WP 4. Objective.

Monitoring of microcystins (MCs) in Castreccioni Lake and in the near marine coastal area.

Methods.

Study of MCs extraction protocols for their applicability to various matrices (water, algae, shellfish). Optimization of LC-MS/MS parameters and evaluation of performances.

Obtained Results.

Water samples from Castreccioni Lake and shellfish (mussels and clams) were collected during 2022 from breeding sites of near coastal area.

Expected Results.

- Implementation of a LC-MS/MS method to investigate MCs in water, algae and shellfish.
- Evaluation of the ability of cyanobacteria to survive in different environmental conditions, such as brackish/marine waters.

- REFERENCES

- [1] Accoroni, S., Romagnoli, T., Colombo, F., Pennesi, C., Di Camillo, C.G., Marini, M., Battocchi, C., Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Tartaglione, L., Penna, A., Totti, C. Marine Pollution Bulletin, 62: 2512-2519 (2011).
- [2] Bacchiocchi, S., **Siracusa, M.**, Campacci, D., Ciriaci, M., Dubbini, A., Tavoloni, T., Stramenga, A., Gorbi S., Piersanti, A. Toxins, 12, 370 (2020).
- [3] Bacchiocchi, S., **Siracusa, M.**, Ruzzi, A., Gorbi, S., Ercolessi, M., Cosentino, M.A., Ammazalorso, P., Orletti, R. Toxicon, 108 :115-125 (2015).
- [4] Bacchiocchi S., Campacci, D., **Siracusa, M.**, Dubbini, A., Leoni, F., Tavoloni, T., Accoroni, S., Gorbi, S., Giuliani, M.E., Stramenga, A., Piersanti, A. Marine Drugs, 19, 304 (2021).
- [5] Gorbi, S., Avio, C. G., Benedetti, M., Totti, C., Accoroni, S., Pichierri, S., Bacchiocchi, S., Orletti, R., Graziosi, T., Regoli, F. Fish and Shellfish Immunology, 5:941-950 (2013).
- [6] Giuliani, M. E., Accoroni, S., Mezzelani, M., Lugarini, F., Bacchiocchi, S., **Siracusa, M.**, Tavoloni, T., Piersanti, A., Totti, C., Regoli, F., Rossi, R., Zingone, A., Gorbi, S. Marine Drugs, 17, 595 (2019).
- [7] Akyol C., Ozbayram E.G., Accoroni S., Radini S., Eusebi A.L., Gorbi S., Vignaroli C., Bacchiocchi S., Campacci D., Gigli F., Farina G., Albay M., Fatone F. Environmental Pollution, 286, 117535 (2021).

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

List of attended courses/seminars/schools

1. Elements of Marine Policy– DISVA
2. Microbial-mediated processes in aquatic ecosystems: from basic to applied research toward solving environmental problems- DISVA

List of conferences/workshops attended and of contributions eventually presented

1. “I PRINCIPI DELLE 3R NEGLI STUDI BIOMEDICI”- online 09 maggio 2022.

Part 3. PhD student information on publications

If not yet published, please indicate the publication status (submitted, accepted, in preparation...)

List of publications on international journals

- J1. **Siracusa, M.**, Bacchiocchi, S., Dubbini, A., Campacci, D., Tavoloni, T., Stramenga, A., Ciriaci, M., Dall’Ara, S., Piersanti, A. “A High Throughput Screening HPLC-FLD Method for Paralytic Shellfish Toxins (PSTs) Enabling Effective Official Control” *Molecules*, 27(15), 4702 (2022) <https://doi.org/10.3390/molecules27154702>.
- J2. Accoroni, S., Ubaldi, M., Bacchiocchi, S., Neri, F., **Siracusa, M.**, Buonomo, M.G., Campanelli, A., Totti, C. “Palytoxin-Analogues Accumulation in Natural Mussel Banks during an *Ostreopsis cf. ovata* Bloom” *Journal of Marine Science and Engineering*, 10 (10), 1402 (2022) <https://doi.org/10.3390/jmse10101402>.
- J3. Bacchiocchi, S., Campacci, D., **Siracusa, M.**, Accoroni, S., Gorbi, S., Piersanti, A. et al. “A TTX hot spot in the Adriatic Sea: study on the origin and causative factors of mussels contamination” (*in preparation*).
- J4. Giuliani, M. E., Bacchiocchi, S., Accoroni, S., **Siracusa, M.**, Campacci, D., Piersanti, A., Gorbi, S. et al. “Trophic transfer and subcellular effects of palytoxins in the gilthead seabream *Sparus aurata*: a focus on lipid alterations” (*in preparation*).

Ancona, 13/10/2022

Student signature

Melania Siracusa

Supervisor signature

Sepia G. B.