



Corso di Dottorato di Ricerca in Scienze della Vita e dell'Ambiente - Ciclo XL

Emerging Freshwater and Marine biotoxins: contamination levels, trophic transfer and biological effects

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OVERVIEW

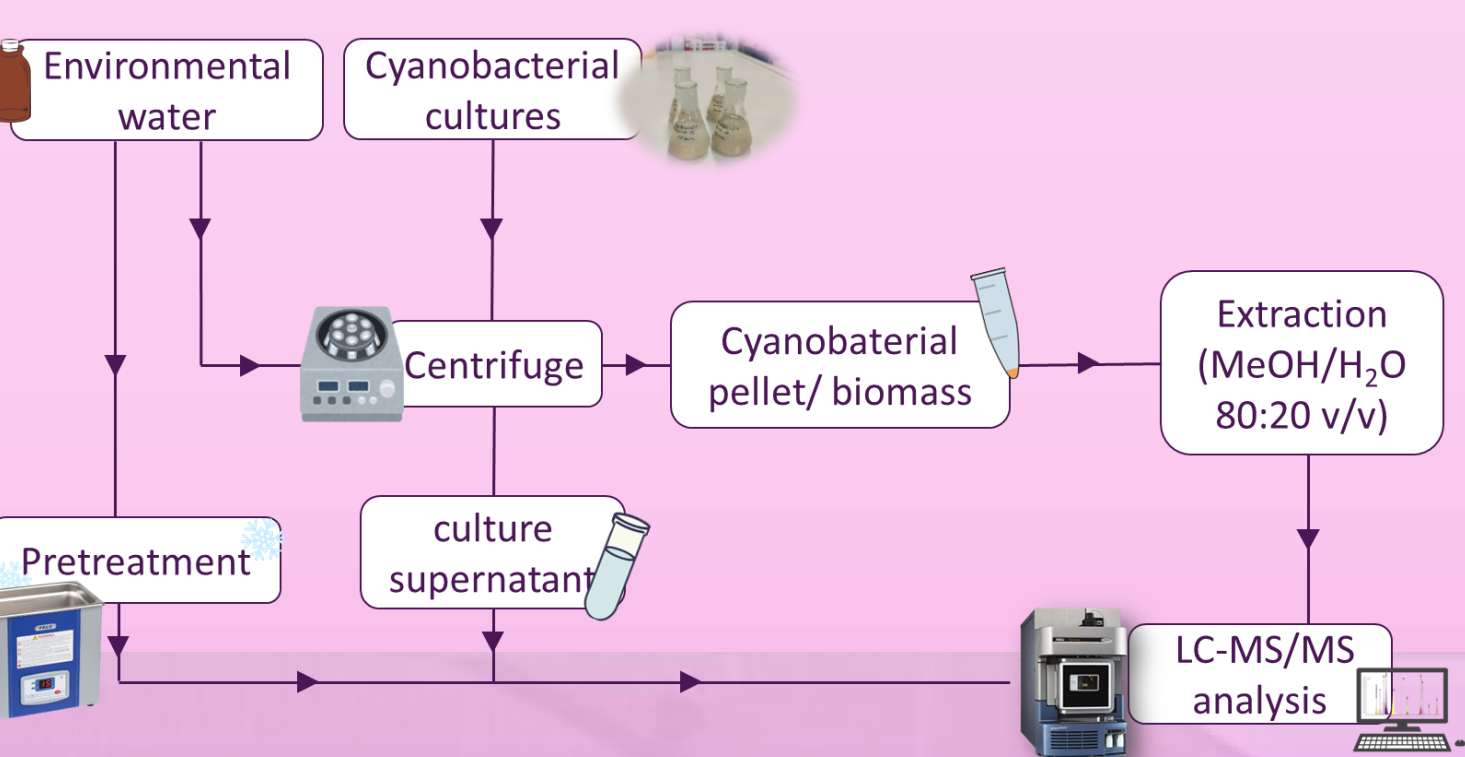
Emerging Biotoxins (EBs) are toxins produced by microalgae and/or bacteria from marine and freshwater habitats for which toxicity data are limited. Contaminations by Emerging Marine Biotoxins, including tetrodotoxins (TTXs), and freshwater EBs, such as cyanotoxins, are frequently reported in Italian basins, representing a threat for humans, animals and environments. This PhD work aims to **investigate levels, contamination trends and ecotoxicological impact of cyanotoxins and TTXs, in freshwater and marine environments, in the Marche region, through a multidisciplinary approach.**

METHODOLOGIES

A chemical method based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and an enzyme-linked immunosorbent assay (ELISA) have been developed in order to evaluate the presence of Microcystins, an harmful group of cyanotoxins, and Tetrodotoxins. TTXs are not yet regulated, but a guidance level of 44 µg TTXs kg⁻¹ shellfish meat has been established by European Food Safety Authority (EFSA). On the other hand, the World Health Organization (WHO) issued maximum limits for the analogue MC-LR of 1 and 24 µg L⁻¹ in drinking and recreational waters, respectively.

CYANOTOXINS

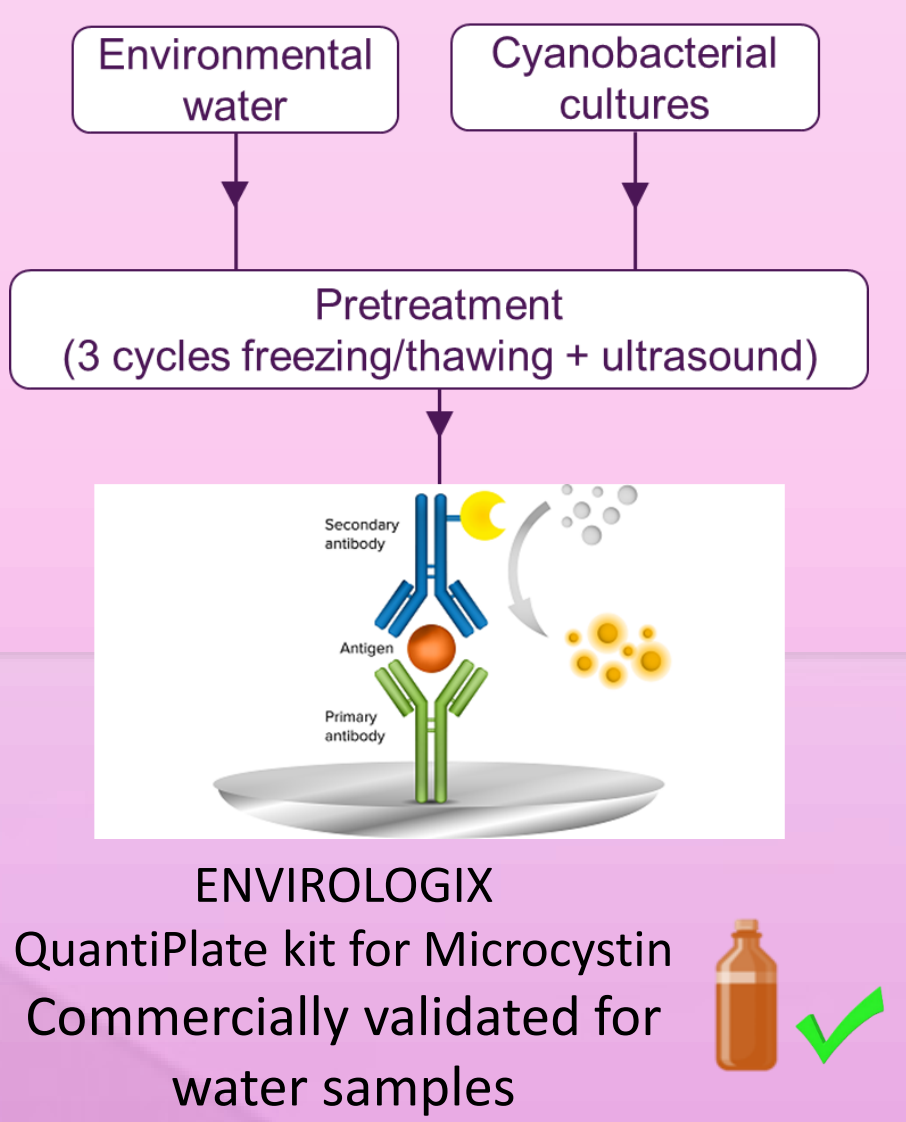
Determination of Microcystin by LC-MS/MS



LOD and LOQ ELISA vs LC-MS/MS

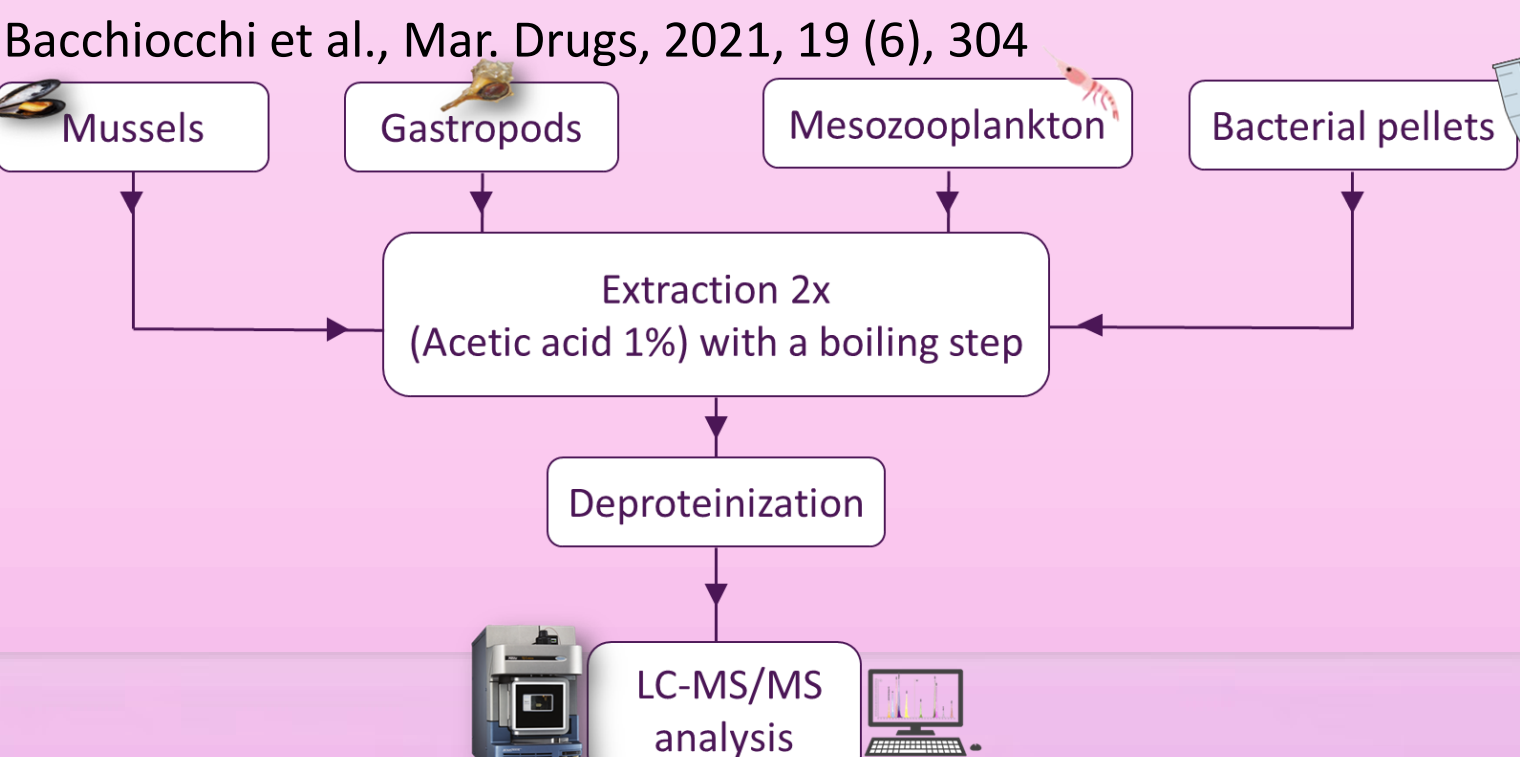
	LOD µg/L (water)	LOQ µg/L (water)
ELISA kit	0,1	0,2
LC-MS/MS	0,03	0,1

Enzyme-linked immunosorbent assay



TETRODOTOXINS

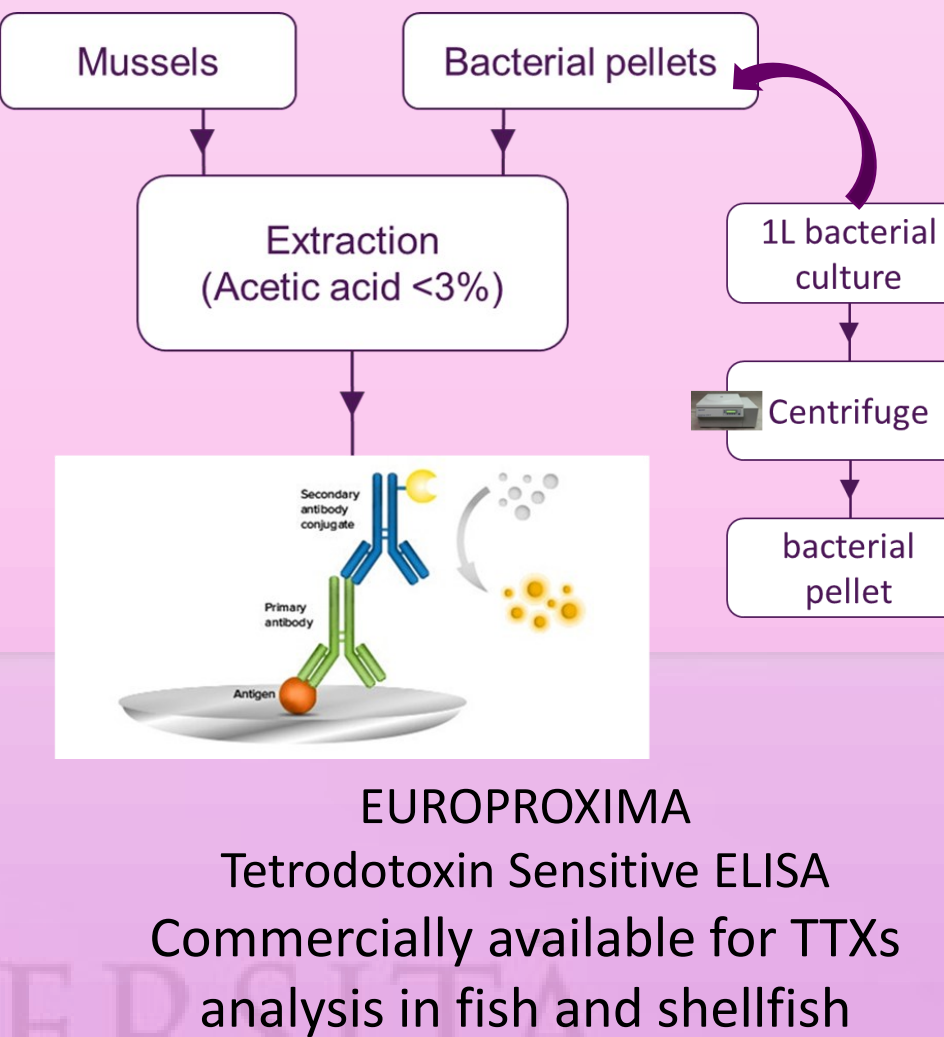
Determination of Tetrodotoxin by HILIC-MS/MS



LOD and LOQ ELISA vs LC-MS/MS

	LOD µg/kg (mussel)	LOQ µg/kg (mussel)
ELISA kit	1,4	4,0
LC-MS/MS	3,0	8,0

Enzyme-linked immunosorbent assay



PRELIMINARY RESULTS

CYANOTOXINS

Implementation of MC's analysis methods

TREATMENT	GLASS µg/L	GLASS %	PLASTIC µg/L	PLASTIC %
NO TREATMENT	1,70	2	1,60	3
ULTRASOUND TREATMENT (15 min, 180 W, 20°C)	1,50	2	1,90	4
1 CYCLE (freezing at -20 °C / thawing at 20°C)	62,4	83	2,30	5
2 CYCLES (freezing at -20 °C / thawing at 20°C)	50,8	68	9,90	20
3 CYCLES (freezing at -20 °C / thawing at 20°C)	64,2	85	5,80	17
3 CYCLES (freezing at -20 °C / thawing at 20°C) + ULTRASOUND TREATMENT (15 min, 180 W, 20°C)	75,1	100	48,4	100

Different sample preparation procedures, using both glass and plastic items, were assayed on a monoclonal culture of *Planktothrix rubescens*, a cyanobacterium producing MCs, in stationary phase. Pre-treatment of the sample with 3 cycles of freezing/thawing followed by sonication using glass containers was found to be the best approach, therefore it was chosen for the preparation of environmental water samples.

ELISA kit vs. LC-MS/MS

screening method for waters

preliminary tests on cyanobacteria cultures (*P. rubescens*) in stationary phase

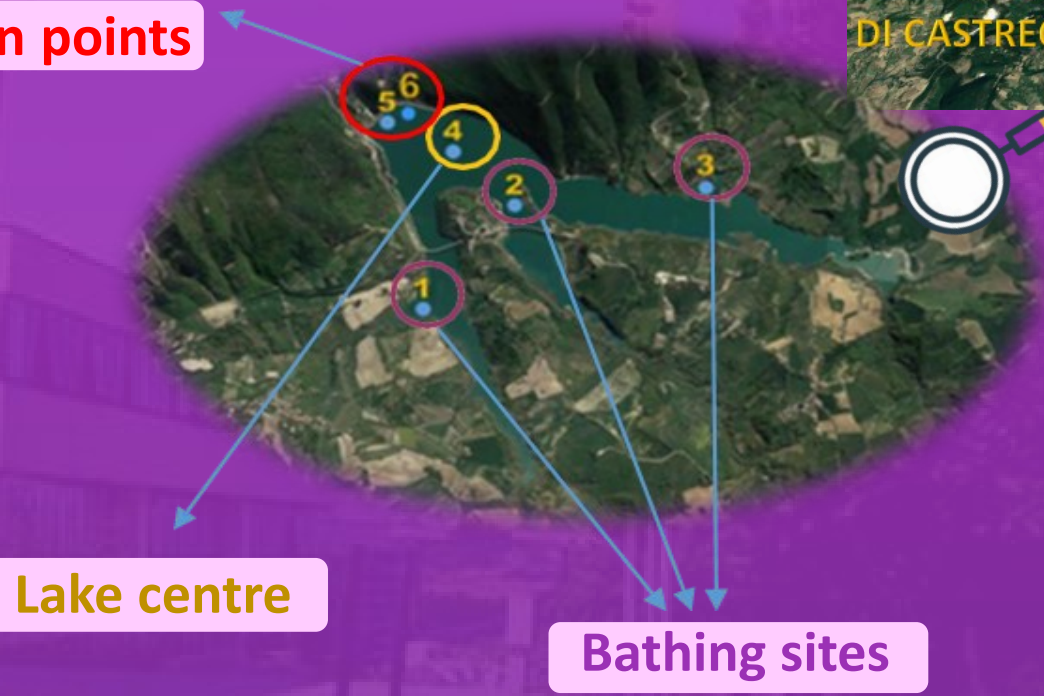
Obtained results: 100 µg/L (ELISA) vs 75 µg/L (LC-MS/MS) (overestimation of about 33%)

ELISA kit is quick, cheap but overestimates the contamination level of the samples, features that makes it suitable as screening method. Then, the contaminated samples can be confirmed using LC-MS/MS, achieving an accurate quantification of MCs level and information on the toxic profile.

Definition of the study area

The study area includes the Castreccioni Lake (MC) [1], the emissary Musone river and the marine coastal area close to the river mouth.

Potabilization points



Castreccioni Lake is periodically interested by *P. rubescens* blooms. Sampling points in the lake were identified to follow the contamination phenomenon.

- Work in Progress**
- ✓ Biweekly sampling of Castreccioni Lake for cyanobacterial blooms by ARPAM and analysis of MCs' concentration by IZSUM
 - ✓ Identification and characterization of different *P. rubescens* chemotypes able to produce and release MCs
 - ✓ Evaluation of MCs' bioaccumulation in bivalve mollusks bred in the coastal area near the river mouth

TETRODOTOXINS

ELISA kit applicability to bacterial cultures

ELISA kit matrix extension tests		
Sample ID	Conc. (µg/kg)	Recovery %
V. alginolyticus 26-2 TA MB spike 60 µg/kg	62,60	104
V. alginolyticus 26-2 TA APA spike 60 µg/kg	63,75	106

TTX is produced by different bacteria genera (e.g. *Vibrio*), so the ELISA kit was tested for its applicability to bacterial pellets by spiking at different TTXs concentration. At high TTX contamination levels, the response obtained is more than satisfactory, while at lower more tests are needed.

ELISA kit as screening method

TTXs			
Sample	Sampling site	Sampling date	average µg/kg N=3
Mussels	Passetto	31/05/2024	19,4
Mussels	Trave	31/05/2024	34,5
Mussels	Passetto	10/06/2024	43,2
Mussels	Trave	10/06/2024	119
Mussels	Passetto	26/06/2024	13,6
Mussels	Trave	26/06/2024	56,9
Mussels	Molo	26/06/2024	22,8
Mussels	Passetto	17/07/2024	2,73

Samples collected as part of an earlier oceanographic campaign were analyzed to assess the contamination of mussels with TTXs.

ELISA vs LC-MS/MS			
Methods	Mussel samples' TTX concentration (average µg/kg N=3)		
	Passetto 10/06/2024	Trave 10/06/2024	Passetto 17/07/2024
ELISA kit	68,1	149	5,16
HILIC-MS/MS	43,2	119	2,73
Δ%	45	22	62

Samples contaminated with different TTX concentrations were analyzed to compare the performances of the ELISA kit with the LC-MS/MS method. The results show that at high contamination levels the methods have an acceptable variability, while at lower contamination levels the ELISA kit overestimates. The ELISA assay can be used as a screening method, while the LC-MS/MS method can be used as confirmation method.

Definition of the study area

Studies conducted on mussels from the Marche coasts [2, 3] suggest that the Conero Riviera (Ancona) is a possible hotspot for TTX accumulation. In particular, 'Molo Portonovo', a sampling site not included in official controls, should be investigated.



- Work in Progress**
- ✓ Identify the Limit Of Detection (LOD) and Quantification (LOQ) of the ELISA kit for bacterial pellets
 - ✓ Monitoring of Molo Portonovo (mussels and other biotic and abiotic matrices) and classified natural beds during the period May-September

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- [1] Akyol, Ç., 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. *Environ. Pollut.* 2021, 286.
[2] Bacchiocchi, S.; Campacci, D.; Siracusa, M.; Dubbini, A.; Leoni, F.; Tavoloni, T.; Accoroni, S.; Gorbi, S.; Giuliani, M.E.; Stramenga, A.; et al. *Mar. Drugs* 2021, 19, 304.
[3] Bacchiocchi, S.; Campacci, D.; Siracusa, M.; Dubbini, A.; Accoroni, S.; Romagnoli, T.; Campanelli, A.; Griffoni, F.; Tavoloni, T.; Gorbi, S.; et al. *Mar. Drugs* 2023, 21, 8.