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

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

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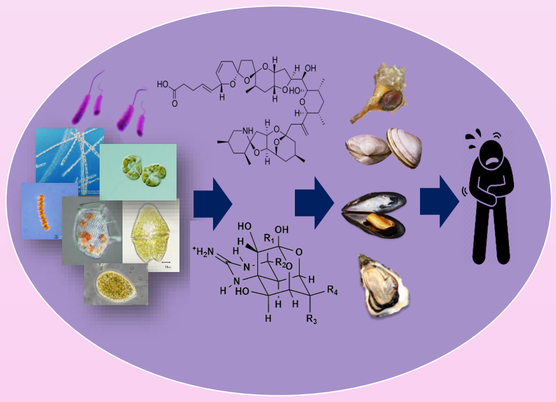
OVERVIEW

Emerging Marine Biotoxins (EMBs) are toxins from microalgal and/or bacterial origin including palytoxin-like compounds (PLTXs), cyclic imines (CIs), azaspiracids (AZAs) and tetrodotoxins (TTXs) for which toxicity data are limited. Algae and toxins are detected in seawater and seafood representing a threat for the environment and human health [1-3]. This PhD work aims to elucidate EMBs toxicological impact through a multidisciplinary approach including chemical, microbiological and ecotoxicological studies on EMB trophic transfer in the aquatic ecosystems.



METHODOLOGIES

SAMPLING PLAN ALONG THE MARCHE COAST

CIs/AZAs monitoring in mussels: biweekly frequency in breeding sites during 2021.

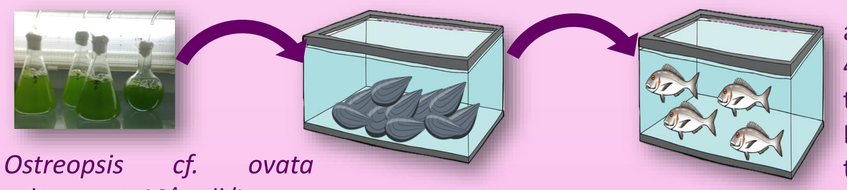
PLTXs monitoring in mussels: Ancona wild sites from July to October 2021, biweekly and weekly frequency during the alert period.

TTXs monitoring in mussels: Ancona and Pesaro wild sites from May to August 2021, biweekly frequency.

TTXs were also searched in mussels, phytoplankton, sediment and seawater collected in a specific wild sampling point (Molo Portonovo) from June to August 2021, with weekly frequency.

Sampling will be repeated in the next years

EXPOSURE EXPERIMENTS TO STUDY THE PLTXS TROPHIC TRANSFER

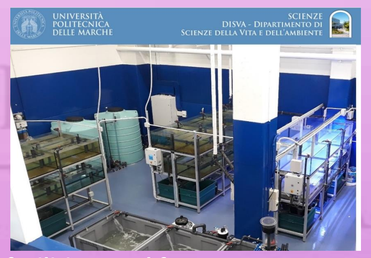


Ostreopsis cf. ovata cultures at 10^4 cell/L were prepared for mussels exposure.

A culture of *Skeletonema* sp. (10^6 cell/L) was also produced to feed control mussels.

Two 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.

Trophic transfer esperiments were accomplished exposing sea breams in 4 tanks (2/3 specimens per tank): 2 tanks for the exposure, 2 for control. 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 tissue collected (liver, intestine, muscle, brain, gills.)



UNIVPM-DISVA facilities used for mesocosm experiments.

MICROBIOLOGICAL AND MOLECULAR ANALYSIS TO IDENTIFY TTX PRODUCER

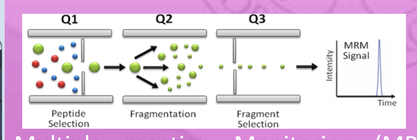
bacteria isolation according to ISO 21872-1:2017

PCR analysis

CHEMICAL METHODS IN LC-MS/MS

Extraction protocols and LC-MS/MS conditions were implemented [1-3].

ACQUITY I-Class XEVO TQ-S micro system (Waters)



Multiple reaction Monitoring (MRM) mode is used to detect EMBs.

BIOMARKERS

Biomarkers analyses including detoxification/metabolization pathways, oxidative stress responses, lysosomal alterations, peroxidation processes, genotoxic damages, physiological indices [2].

PRELIMINARY RESULTS

LC-MS/MS methods were implemented and validated for: **AZAs**, **CIs, PLTXs** and **TTXs.**

Validation data for the LC-MS/MS implemented methods.

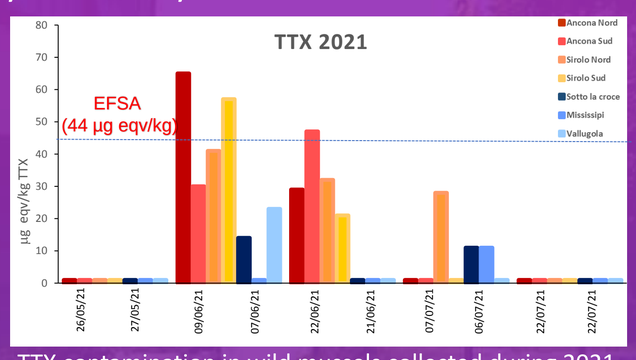
EMBs	*linearity (R^2)*	*Accuracy (R%)*	*LOQ (µg/kg)*	*precision (RSDr%)*
AZA1, AZA2, AZA3 (**AZAs**)	> 0.99	101 (AZA1), 99 (AZA2), 97 (AZA3) *	10	11 (AZA1, AZA2 , AZA3) *
GYM A, 13desMe SPX C (**CIs**)	> 0.99	94 (GYM A), 92 (13desMe SPX C) *	1	5 (GYM A), 9 (13desMe SPX C) *
PLTX (**PLTXs**)	> 0.99	74 (40 µg/kg), 87 (250 µg/kg)	40	9 (40 µg/kg), 7 (250 µg/kg)
TTX (**TTXs**)	> 0.99	99 (75 µg/kg), 97 (251 µg/kg)	10	7 (75 µg/kg), 8 (251 µg/kg)

*LOQ level

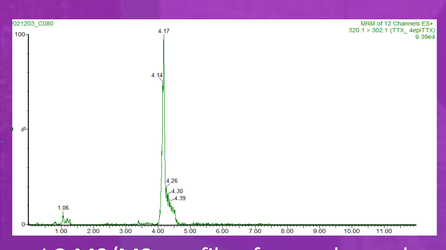
AZAs and **CIs** (about 10-15 µg/kg) were found in analysed mussels; due to the low levels, it can be excluded a risk for human health.

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). In the same period ARPAM reports on *O. cf. ovata* blooms in seawater.

TTXs were found in mussels from Ancona wild sites with concentrations comparable to EFSA guidance level of 44 µg TTX eqv/kg. Mussels sampled at Molo Portonovo reached significant higher levels of about 300 µg TTX eqv/kg in the early June 2021. The latter findings suggest a deeper investigation in the next years in May-June.

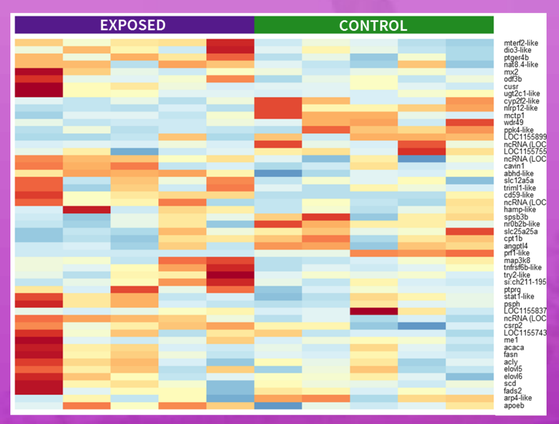


TTX contamination in wild mussels collected during 2021.



LC-MS/MS profile of mussel sample contaminated by TTX (June 2021).

Mussels exposed to *O. cf. ovata* accumulated PLTXs reaching levels of 175-200 µg/kg. Sea breams fed with contaminated mussels, seems not accumulated PLTXs in tissues, suggesting eventual fishes biotrasformation or elimination mechanisms. However, variations of lipidic profile and transcriptomic alterations of genes involved in lipid metabolism were observed in fish livers, suggesting biological effects in the exposed organisms.

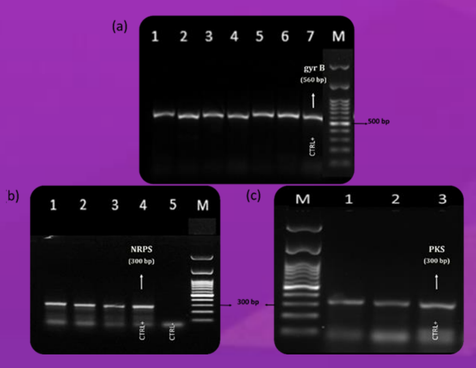


Transcriptomic changes were induced in fish liver by PLTXs-contaminated diet (e.g. alteration of genes involved in lipid metabolism).

PCR analytical protocols were set up [3] for :

- ✓ species-specific detection of *V. alginolyticus* and *V. parahaemolyticus*
- ✓ identification of PKS and NRPS genes (TTX biosynthesis genes)

Only *V. alginolyticus* was isolated from the mussel samples analysed by microbiological assay.



PCR for species-specific *V. alginoliticus* (a) and NRPS and PKS genes (b, c).

- ✓ LC-MS/MS analysis of TTXs in abiotic matrices (seawater, sediment, phytoplankton)
- ✓ compartmentalization studies in TTXs contaminated mussels
- ✓ PCR analysis to confirm the presence of *Vibrio* spp. and TTX biosynthesis genes
- ✓ eco-toxicological studies of molecular and cellular effects (biomarkers) induced by algae and/or toxins in exposed organisms.

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