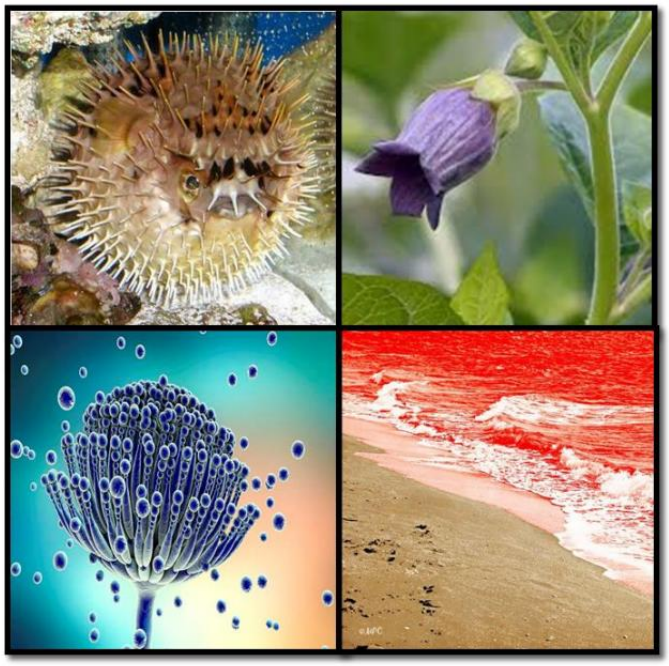
UNIVERSITÀ DI PARMA

SCHOOL OF ADVANCED STUDIES ON FOOD AND NUTRITION

SITOX

# Book of Abstracts

## 2nd MEETING ON "NATURAL TOXINS"



**September 18-19, 2019**
**AULA MAGNA, Via dell'Università 12**
**Parma, Italy**

The Italian Society of Toxicology (SITOX), together with the University of Parma and under the patronage of the European Food Safety Authority (EFSA), are organizing the 2nd Meeting on "Natural Toxins", in Parma, on 18-19 September 2019.

Natural toxins are toxic compounds that are naturally produced by living organisms. Some toxins are produced by plants, microorganisms such as pathogenic fungi, or microscopic algae and plankton as a natural defence mechanism against predators, insects or microorganisms, as biotic weapons in the plant-pathogen cross-talk or in response to climate stress.

These toxins are not harmful to the organisms themselves but they may be toxic to other creatures, including humans, when eaten. These chemical compounds share a large structural biodiversity, and differ in biological function and toxicity. Being most of them heat-stable, natural toxins can enter the food and feed production chain causing adverse effects in animals and humans, and representing a relevant cause of health and veterinary costs as well as economical losses.

After the success of the 1st Meeting on Natural Toxins, the workshop will move to Parma, in the middle of the Food Valley, for underlining the relevance of the topic in the agro-food sector. The workshop will be a multidisciplinary forum to showcase the most recent developments in chemical and toxicological methodologies, and face open challenges in the sector.

**SCIENTIFIC COMMITTEE**

Chiara Dall'Asta, Carmela Dell'Aversano, Corrado L. Galli, Carlo A. Locatelli, Antonella Penna, Ornella Rossetto, Silvio Sosa, Emanuela Testai, Aurelia Tubaro

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Luca Dellafiora, Martina Cirlini, Valentina Bernini, Francesco Martelli (University of Parma)

# Emerging risks due to natural toxins: link with climate change

A. Maggiore

European Food Safety Authority (EFSA), Parma

According to the EFSA's Founding Regulation (EC) No 178/2002 (Article 34), EFSA is required to establish procedures for the screening and analysis of information with a view of identifying emerging risks in the fields within its mission. The aim is to anticipate or even prevent future food safety challenges and risk assessment needs (data, knowledge, methodologies) thus contributing to preparedness. The achievement of this aim in the long-term may be based on the identification of drivers. They are natural or anthropogenic factors causing complex and interlinked changes that could put the European food system under severe stress. Because of them, food safety cannot be taken as granted in the future. Climate change is one of the most relevant drivers of emerging risks.

While a broad range of forward-looking studies and publications examine the impact of climate change on food security, future challenges for food and feed safety (including the emergence of natural toxins) as well as nutrition quality are often not specifically addressed. The CLEFSA project (Climate Change and Emerging Risks for Food Safety) aims at developing and testing new methodologies for emerging risks identification and to produce a characterised list of emerging issues/risks potentially affected by climate change. In particular, it explores the possibility of a) using a specific driver, climate change, for long term anticipation of emerging risks, using scenarios of climate change b) using horizon scanning and crowdsourcing to collect a broad range of signals from a variety of information sources, c) enlarging the knowledge network to experts for the specific driver from international EU and UN agencies and d) designing Multi-Criteria Decision Analysis tools for characterisation (including scoring) purposes through a participatory process.

A transparent and reproducible procedure has been designed including the following steps: 1) Building CLEFSA Network; 2) Designing identification criteria; 3) Identifying Emerging issues; 4) Designing characterisation criteria; 5) Building climate change Scenarios; 6) Developing a Tool for the characterisation; 7) Building the Characterisation group; 8) Scoring and characterisation; 9) Reporting.

This procedure will also bring together past and present EFSA initiatives in the area of climate change, thus, providing more transparency on how EFSA is addressing this global issue.

A survey has been launched to collect a broad range of issues, including weak signals, potentially affected by climate change. The scope of the survey has covered all EFSA's areas, including contaminants. More than 600 people responded, providing over 240 issues. The issues identified in the survey have been complemented by literature search, using on-line searching tools developed by other EU institutions, the EFSA Emerging Risks Networks (Emerging Risks Exchange Network – EREN and the Stakeholders Discussion group) and information stemming from EFSA's activity related to the subject.

A CLEFSA discussion group has been created constituted by experts from international EU and UN institutions and coordinators of large EU projects involved with climate change. The task of this group is mainly to support the design of a Multi-Criteria Decision Analysis tool for characterisation purposes and as focal points for the participatory characterisation exercise. The criteria to identify emerging issues potentially affected by climate change have been defined based on those used in the EFSA emerging risks identification process and adapted to the specific driver under analysis. A report will be produced and published at the end of the project (2020).

# EFSA's framework for the risk assessment of natural toxins in food

M. Binaglia

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The European Food Safety Authority (EFSA) is the reference body for risk assessment of food and feed in the European Union (EU). Its work covers the entire food chain – from field to fork. The assessments of risks for public health related to the dietary exposure to natural toxins in the food chain is a main area of activity under the responsibility of the EFSA Team and Scientific Panel on Contaminants in the Food Chain (CONTAM Panel). Natural toxins of relevance for food and feed contamination include primarily mycotoxins, plant toxins and marine biotoxins.

The assessment of natural toxins follows the standard four-pillar framework for chemical risk assessment, including hazard identification, hazard characterization, exposure assessment and risk characterization. For hazard identification and characterization of natural toxins, data from toxicological studies available in the public domain are collected and evaluated. Human and farm and companion animal dietary exposure assessment relies on the data on concentrations of natural toxins in food, submitted to EFSA by the EU Member States (EU MS) or other stakeholders, and food and feed consumption data. In view of the great diversity of adverse effects caused by various classes of natural toxins, the whole spectrum of approaches for the hazard characterization is considered, from the establishment of acute or chronic Health-based Guidance Values to the application of the Margin of Exposure approach for substances that are both genotoxic and carcinogenic. Alternative approaches, such as the use of the Toxicological Threshold of Concern (TTC) or read-across strategies are applied in case of insufficient data on specific toxins.

The scientific opinions of the CONTAM Panel are then used by risk managers, such as the European Commission or EU MS for possible revisions of the existing legislations on contaminants and/or other possible risk management actions. The presentation will give an overview on the process of how the EFSA CONTAM Panel performs risk assessments on natural toxins in food and feed.

# Immunomodulatory properties of *Alternaria* toxins

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Filamentous fungi of the Alternaria genus possess the ability to generate a spectrum of secondary metabolites with potential toxicological properties. Among these, the dibenzopyrone derivative alternariol (AOH) represents one of the major *Alternaria* toxins with respect to quantity, but was found to provide only marginally to the overall genotoxic potential of complex *Alternaria* toxin mixtures. However, recent studies argue for immunomodulatory activity of AOH, whereby the expression of several cell surface markers during the phorbol 12-myristate 13-acetate (PMA) induced differentiation of THP-1 monocytes to macrophages were modified. Moreover, in human primary macrophages, RAW 264.7 and differentiated THP-1 macrophages AOH was found to reduce the lipopolysaccharide (LPS) induced immune response [1,2]. Mechanistic studies revealed a suppression of lipopolysaccharide-induced NF-κB pathway activation by AOH. Subsequently, the secretion of the proinflammatory cytokines IL-8, IL-6, TNF-α was decreased whereas the protein levels of the anti-inflammatory cytokine IL-10 was enhanced. A distinct pattern of cytokine mRNA levels were monitored, varying between short- and long-term exposures. The impact of AOH on the immune response of macrophages was associated with modulation of crucial miRNAs. Actual studies demonstrate that the immunosuppressive activity of AOH is not limited to macrophages. In differentiated Caco-2 cells (human colon carcinoma) an immune response can be provoked by incubation with IL-1ß, resulting in enhanced expression of proinflammatory cytokines like e.g. IL-8. The presence of low micromolar concentrations of AOH was sufficient to suppress the immune response. Comparable to the observed effects in macrophages, AOH was found to modulate the expression of inflammation-related miRNAs also in differentiated colon cells [3]. Taken together, AOH itself doesn't induce a pronounced proinflammatory immune response, however, in an inflamed environment it possesses the ability to repress the appropriate immune response by targeting the NF-κB signalling pathway and regulatory miRNAs. Of note, ongoing studies indicate that the immunomodulatory activity is not limited to AOH, but might also be of relevance for other *Alternaria* toxins out of different structural classes.

Due to limited data with respect to hazard characterization and occurrence, *Alternaria* toxins are still rated as "emerging" mycotoxins. With respect to food safety, the presented results underline the necessity to consider potential immunomodulatory activity in future hazard characterization of Alternaria toxins.

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# Intestine, the forgotten target of mycotoxins

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As the most extensively exposed surface in the body, the intestinal mucosa has to face important chemical and biological challenges. The intestinal mucosa has three main physiological functions. It establishes a physical barrier between the internal milieu and the luminal content. The intestinal mucosa is also responsible for luminal nutrients digestion and their subsequent absorption. The mucosal epithelium is at the interface of immune system and luminal contents, including dietary antigens and microbial products. This implies a local defence mechanisms regulation that involves integrating all the signals that come from the external and internal world to preserve immune homeostasis steady-state conditions. Either of these intestinal physiological functions may be targeted by feed contaminants such as mycotoxins. Mycotoxins are produced on a wide variety of raw materials before, during and after harvest. Very resistant to technological treatments, mycotoxins can be present in food.. Among the "major" mycotoxins, Deoxynivalenol and Fumonisins have been studied especially for their toxicity on the intestine. They are not only locally toxic for this organ, but also dysregulate many intestinal functions and impair the immune response. This results in systemic toxicity leading to many symptoms and impairment of zootechnical parameters. Feed contamination with mycotoxins also increases translocation of bacteria across the intestine and thus intestinal and systemic infections. For Aflatoxins, Zearalenone and Ochratoxins, less data are available concerning their intestinal toxicity. The increased performance of analytical methods reveals new toxins, especially emerging ones, as well as "masked" or "modified" forms; it still needs to be determined if they represent a new for the intestine. Global surveys indicate that co-contamination occurs frequently but the health risk from exposure to a combination of mycotoxins is incompletely understood.

# New evidence in the risk assessment of ochratoxin A (OTA) in food

H. Steinkellner

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OTA is produced by various fungi of the genus *Aspergillus* and *Penicillium* and is one of the most important mycotoxins found as food contaminant.

In 2006, EFSA has published a risk assessment on OTA in food in which it concluded that OTA is nephro- immuno- and neurotoxic and a developmental toxicant at higher doses. OTA accumulates in the kidneys and upon chronic exposure to doses toxic to the kidney it also induces kidney and liver tumours in rodents. The results from genotoxicity tests are inconsistent but suggested that that DNA-damage is induced by oxidative stress. A tolerable weekly intake (TWI) of 120 ng OTA/kg bw was established based on a LOAEL of 8 µg/kg bw per day for renal toxicity in pigs and by applying an uncertainty factor (UF) of 450. Cereals and cereal products, wine, beer, grape juice, brewed coffee, cocoa and cocoa products and pork meat were identified as main contributors to OTA dietary exposure, which varied between 15 and 20 ng/kg bw per week and for high consumers between 40 and 60 ng/kg bw per week and thus were below the TWI of 120 ng/kg bw per week. It was noted that infants, children and certain high consumers could have higher exposures.

The European Commission (EC) has asked EFSA for an update of this previous risk assessment in which new toxicological studies (i.e. published since 2006) and more recent occurrence data of OTA in food need to be considered and which is currently being developed. As a first step a comprehensive targeted literature search and evaluation was carried out revealing that OTA is globally and intensely researched. Evaluation of new toxicokinetic data essentially confirm previous conclusions on OTA as a compound cumulating in the body and having marginal metabolism and that reactive metabolites binding to DNA *in vivo* have not been identified. While it could be confirmed that OTA is present in ruminant's milk only in traces it occurs in human breastmilk in relatively high amounts. A series of new biomarker studies with OTA have become available but many of these carried out with individuals exposed to OTA at levels not reflecting (much lower) exposures in European populations. There are no new data suggesting that OTA is acutely toxic but there is an abundance of new publications on repeated dose studies with OTA, many of these also attempting also to elucidate the mode of action for OTA toxicity. Essentially, these confirm the kidney as main target for OTA toxicity but in none of these new studies was an effect level lower than that previously established in pig studies obtained. As OTA is a strong kidney carcinogen in rats, the mode of action for tumour development is also a key aspect driving the selection of the risk assessment approach and will thus be considered extensively in the opinion. It will also be attempted to identify the main (and possible new) contributors to dietary OTA exposure and to discuss possible exposure of infants to OTA via breast milk.

# Pyrrolizidine alkaloids in tea and the revival of a healthy drink's good reputation

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Starting in 2013 a few hundred food products like honey and tea from retail market were analysed for pyrrolizidine alkaloids (PA) and revealed unexpectedly high PA amounts in teas. These findings disproved the previous assumption of honey being one of the main food sources of human PA exposure in Europe. Development of powerful LC-MS/MS methods for the determination of PA in those food products enabled a comprehensive data collection and thereby the assessment of the risk arising for human health. Due to the genotoxic carcinogenic effects of some PA, the margin of exposure (MoE) approach was applied for risk assessment. The outcome of the risk evaluation was that especially for adults who consume high amounts of (herbal) tea, for pregnant women, nursing mothers and for children the PA levels present in tea products could be of concern for their health. The derived MoE values far below 10.000 showed the need of risk management measures which were quickly initiated by food safety authorities and the producers of herbal tea and tea. Only a few years later, periodically conducted monitoring of (herbal) tea by the German and other European food control laboratories revealed a considerable decrease of PA levels in tea products. Currently, the establishment of maximum levels for PA in certain plant based foods including herbal tea and tea in the European Union is being discussed to further give legal certainty for producers and official control and to regain the focus on the numerous health benefits of herbal teas.

# Occurrence of tropane alkaloids in herbal teas, infusions and supplements

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Tropane alkaloids (TAs), secondary metabolites naturally produced by different species of infesting plants including Brassicaceae and Solanaceae (i.e. *Datura stramonium L.*), are potentially toxic compounds, those can indirectly contaminate crops. The contamination may occur when TAs plant producers are harvested together with the plants used for herbal teas preparation [1]. The most studied TAs are Scopolamine and Atropine, which is a racemic mixture of (+)-Hyoscyamine and (-)-Hyoscyamine, but at present more than 200 different TAs were identified in plant producers. In 2015 the European Commission drafted a recommendation about the monitoring of the presence of TAs in different food as cereal and cereal based products, gluten-free preparations, legume vegetable and also teas, herbal infusions and supplements [2].
For this reason, an UHPLC-ESI-MS/MS method was developed and applied for the determination of 4 main TAs (Scopolamine, Atropine, Anisodamine and Homatropine) in samples of herbal teas and infusions collected from the market.
The investigation regarded 60 samples of multi-component herbal teas, infusions and herbal supplements (extracts and tablets) normally used in herbal medicine and sold on the market. All the collected samples were submitted to the screening, evaluating the occurrence of the 4 selected analytes. Results highlighted the presence of TAs in 5 samples: in particular, 4 samples were resulted positive to atropine and among them one resulted also positive to scopolamine, while anisodamine was detected in one sample. Homatropine was not found.
In addition, the calculation of the daily intake related to the accidental ingestion of TAs due to the consumption of the teas containing these contaminants was also evaluated. The contaminations found in the samples resulted lower in respect to those observed by other authors and the intake measured was lower than the limit indicated by EFSA of 0.016 µg/Kg b.w, suggesting that the monitored situation does not represent a risk for the consumer health [1, 3, 4].

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# Study on potential *Clostridium botulinum* growth and toxin production in Parma ham

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The objective of this study was to investigate *Clostridium botulinum* growth and toxin production in industrially manufactured dry-cured ham, using typical Italian Parma-ham as prototype. The study focuses on the Parma-ham production phase identified at maximum risk to *C. botulinum* proliferation i.e. the transition from cold phase (salting and resting) to a phase carried out at temperature between 15 and 23°C (drying).

A preliminary *in vitro* test was carried out in order to verify the capability of 6 *C. botulinum* strains (1 type A, 4 type B and 1 type E strains) to growth in conditions of temperature, pH and NaCl concentration comparable to those of the beginning stage of ham drying. Five *C. botulinum* strains grew at 20°C and pH 6, four strains produced toxin when inoculated at a concentration equal to 103 cfu/ml at NaCl concentration of 4%, while when the inoculum concentration was 10 cfu/ml, NaCl concentration of 3% resulted the toxin-genesis limiting factor.

A challenge test with a mixture of the 5 *C. botulinum* strains selected by the preliminary *in vitro* test was performed on 9 thighs inoculated at the end of the resting phase. The study was designed to evaluate the potential growth and toxin production in extremely favourable conditions for the bacterium. Type B proteolytic *C. botulinum* toxin was produced after 14 days of incubation at 20°C in 2 thighs characterized by high weight, low number of days of resting and anomalous physiochemical characteristic (one for very low NaCl concentration (1.59%), the other for elevated pH (6.27) and both for high water activity values (>0,970)).

The results of this research confirm that the cold resting step is a critical phase in the production process of Parma ham for the investigated hazard. Based on the present study, the long resting phase adopted in the manufacturing of Parma hams is proven effective to prevent the growth of *C. botulinum*, an event which could not otherwise be excluded if the hams were processed under less stringent technological conditions.

# Detection of botulinum neurotoxins by Endopep-MS: a promising method for the replacement of the mouse bioassay

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The reference method for botulinum neurotoxin (BoNT) detection and identification, both in food and clinical samples, is the mouse bioassay. This method is sensitive, specific and is able to measure also the toxin activity but it is time consuming and requires the use of animals that represent an important ethical issue. Mass spectrometry has become an important analytical tool for many applications in microbiology, including bacteria identification. Recently a method for toxin detection and serotype differentiation of BoNTs based on LC-ESI-MS/MS and MALDI-TOF MS and called Endopep-MS, has been developed and successfully applied on field samples.

Endopep-MS method exploits the ability of BoNTs to cleave their substrate into toxin dependent specific positions. Therefore, this method consists on incubation of BoNTs with a peptide substrate that mimics the toxin's natural target. The reaction mixture is subsequently analyzed by a mass spectrometer, which detects any peptides within the mixture. Detection of the peptide cleavage products corresponding to their specific location indicates the presence of a particular BoNT serotype. If the peptide substrate either remains intact or is cleaved in a location different from the specific BoNT serotype, the sample is considered negative for the BoNTs presence. Moreover, the specificity of Endopep-MS method is enhanced through the addition of an initial purification and concentration step performed by means of magnetic protein G beads coated with antibodies against specific BoNT serotypes. Studies conducted with high performing MALDI-TOF instruments demonstrated that that this method can detect BoNT at levels comparable or lower than the mouse bioassay. We set up and validated an Endopep-MS protocol for the detection of BoNT/A, BoNT/B, BoNT/E and BoNT/F toxins in foods and BoNT/C and BoNT/D toxins in biological samples of animal origin by using the MALDI-biotyper: an instrument that results more and more diffuse in the microbiology laboratories for the routinely bacterial identification. The tests carried out showed that the EndoPep-MS is applicable to the MALDI-biotyper instrument with excellent results. The Endopep-MS method coupled with MALDI-biotyper instrument has proved to be able to detect the presence of all the toxins with an analytical sensitivity of ≤ 0.5 mLD50 for BoNT/A, BoNT/B, BoNT/D, BoNT/E and BoNT/F and 1 to 2 mLD50 for BoNT/C. The tests performed on both artificially and naturally contaminated samples showed a weak or absent matrix interference and suggested that the EndoPep is even more sensitive than the mouse test which is currently the gold test for the diagnosis of botulism.

# Toxin-antitoxin systems in bacteria isolated from food: a focus on genus *Lactobacillus*

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Toxin-antitoxin (TA) are two-component systems widely distributed in the genomes of bacteria and archea, involved in the management of adaptive response of microorganisms by regulating cell growth, death or persister formation as a result of stress conditions. All TAs currently known involve a stable toxin, that causes bacterial persistence or death by interfering with essential cellular processes, and a cognate antitoxin capable of repressing toxin activity. These systems are widely studied in pathogenic bacteria because persister cells avoid antibiotic-induced death by entering a physiologically dormant state and are considered a major cause of antibiotic treatment failure and recurring infections. *Lactobacillus* strains are associated with diverse ecosistems, including dairy products, plants and vertebrate gastrointestinal tract, and possess traits to adapt to the diverse niches, allowing them to survive to different stresses. Few studies have addressed TAs distribution in *Lactobacillus*, and their involvement in food ecosystems. For this purpose, we investigated the genome of 5 *Lactobacillus* isolates to assess the presence of TAs, and screened food isolates from genus *Lactobacillus* for their distribution and expression in response to food-related stresses. Sequencing results showed that each of the strain encodes peptides homologous to the type I Lpt peptide, as well as type II TAs. A screening on 17 *Lactobacillus* isolates allowed to study the distribution of a type II TAs, DinJ/YafQ, leading to the identification of sequence variants of the toxin YafQ. Their activity was verified by growth assays in *Escherichia coli*, exhibithing different toxic acitivity in this host. The strains harboring YafQ variants were exposed to various food-related stress conditions (low pH , high salt concentration, oxidative conditions, nutrient limitation and high temperature) and bacterial cultivability was measured, as well as the relative expression of DinJ/YafQ by RT-qPCR. The cultivability of the strains was only slightly affected in all conditions with the exception of thermal stress. The regulation of DinJ-YafQ TA system resulted strain-specific and the exposure to high temperature appeared to be the most significant stress condition able to modulate its expression. This study suggests that the presence of TAS in *Lactobacillus* strains genomes is ubiquitous, and their variety and distribution opens space for further investigations. Since these bacteria are widely used in industrial applications and are an important component of the human microbiota, TAs represent an interesting feature to understand the physiology of these bacteria in food and gut ecosystems.

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# Effects of Temperature, Growth Media, and Photoperiod on Growth and Toxin Production of *Azadinium spinosum*

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76% of all aquaculture production in Europe is conducted along the Western seaboard, and is an important contributor to job creation and the economy for these coastal communities. One of the limiting factors for the industry is the occurrence of biotoxin producing algae on which the shellfish feed. These algae can accumulate in shellfish to toxic levels (for human health) over a very short time period. The adverse impacts of harmful algal blooms (HABs) on farmed shellfish in Europe were estimated to cost €53.78 million per annum from 2001 to 2009. Quality assurance of the product, full compliance with regulations, and efficient product samplings are vital to ensure that any food safety risks are fully controlled and only product of the highest quality reaches the market.

Of particular concern to the Irish shellfish industry are the azaspiracids (AZAs). AZAs were discovered following a poisoning event in the Netherlands in 1995 after people consumed contaminated shellfish harvested in Ireland. The symptoms included nausea, vomiting, diarrhea, and stomach cramps. AZAs are cytotoxic, teratogenic to fish embryos and suspected lung tumour promoters. AZA1, -2 and -3 were identified as the source of the illnesses and have been regulated in shellfish since 2002.

To facilitate their study and subsequent biomonitoring, purification from shellfish and microalgae is performed to produce certified reference materials, required for accurate detection and quantitation in shellfish and water samples. Pure compounds are further used to assess toxicology, both *in vitro* and *in vivo*.

Being less complex, purification from microalgae rather than shellfish is preferable; however, challenges remain with respect to maximizing toxin yields. The impacts of temperature, growth media, and photoperiod on cell densities and toxin production in *Azadinium spinosum* were investigated. Final cell densities were similar at 10 and 18 °C, while toxin cell quotas were higher (~3.5-fold) at 10 °C. A comparison of culture media showed higher cell densities and AZA cell quotas (2.5–5-fold) in f10k compared to f/2 and L1 media. Photoperiod also showed differences, with lower cell densities in the 8:16 L:D treatment, while toxin cell quotas were similar for 12:12 and 8:16 L:D treatments, but slightly lower for the 16:8 L:D treatment. AZA1, -2 and -33 were detected during the exponential phase, while some known and new AZAs were only detected once the stationary phase was reached. These compounds were additionally detected in field water samples during an AZA event.

# *In vivo* and *in vitro* effects of azaspiracids

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Azaspiracids (AZAs) are lipophilic marine toxins produced by *Azadinium* and *Amphidoma* dinoflagellates and, after accumulation by edible shellfish, they can induce a foodborne poisoning in humans characterized by gastrointestinal symptoms. Among the different AZAs analogues, only AZA1, -2 and -3 are currently regulated in the European Union where their concentration in shellfish, expressed as AZA1 equivalents/kg, is determined using their toxic equivalency factors (TEFs), calculated from intraperitoneal lethality in mice (1.0, 1.8 and 1.4 for AZA1, -2 and -3, respectively). However, considering the potential oral exposure to these toxins, TEFs should be calculated by comparative oral toxicity data, as recommended by the European Food Safety Authority.

Thus, the acute oral toxicity of AZA1, -2 and -3 was investigated in female CD-1 mice. After 24 h from gavage administration, AZAs median lethal doses (LD50) were 443 μg/kg (95% CL: 350−561 μg/kg), 626 μg/kg (95% CL: 430−911 μg/kg) and 875 μg/kg (95% CL: 757−1010 μg/kg) for AZA1, -2 and -3, respectively. The derived TEFs, different from those previously obtained after intraperitoneal injection and suitable for regulatory purposes, were 1.0, 0.7 and 0.5.

Mice that died more than 5 h after the treatment or those sacrificed after 24 h (doses: ≥175 μg AZA1/kg, ≥500 μg AZA2/kg and ≥600 μg AZA3/kg) showed enlarged pale liver, while increased levels of glutamate dehydrogenase and/or transaminases as markers of potential liver damage, were recorded even at the lowest toxins dose. Blood chemistry revealed also increased serum levels of K+ ions at AZAs doses ≥500 μg/kg. Light microscopy showed tissue changes in the gastrointestinal tract, liver and spleen. No macroscopic, tissue or haematological changes were recorded two weeks post exposure, indicating reversible toxic effects.

These data suggest the liver as a target organ of these toxins. Hence, an *in vitro* study was carried out on immortalized human hepatocytes (IHH) to investigate the effects of AZA1, -2 and -3 on liver cells. AZAs induced an increased mitochondrial activity in IHH cells after 24 h exposure. The effect, not related to a proliferative stimulus, was dependent on the activation of mitochondrial complexes I and II, caused by a perturbation of ionic balance. In particular, the AZAs-dependent increase in mitochondrial activity was selectively reduced by the blockage of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels, which suggests their key role in modulating the effects of AZAs at the hepatic level.

# Emerging issues due to saxitoxins and tetrodotoxin in the mediterranean sea: an analytical and molecular combined approach

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Harmful algal blooms (HAB) cause serious impacts to human health, marine environment and economic maritime activities at many coastal sites. Therefore, there is an urgent need for validated analytical methods for determination of toxins in the food chain and of molecular methods for detection and count of HAB species, species-specific identification and reliable quantification of cell densities.

Saxitoxins (STXs) are a group of neurotoxins produced by marine dinoflagellate belonging to genus *Alexandrium*, *Gymnodinium* and *Pyrodinium* that may cause a fatal paralytic syndrome in humans following ingestion of contaminated seafood. Tetrodotoxin (TTX) is also a natural toxin produced by marine bacteria, well known in Japan to cause lethal food poisonings following ingestion of contaminated puffer fish (fugu). Despite presenting different structural features, STXs and TTX exert similar toxic effects and, most importantly in an analytical perspective, they are co-extracted under the same conditions; thus, availability of a methodological approach for their combined detection is desirable.

This study aimed to investigate STX-related risk in the Mediterranean area [1]. Genus- and species-specific primers and probes designed on rDNA ribosomal and STX genes allowed to develop and apply new identification and counting qPCR based assay, which proved to be more rapid, sensitive and specific when applied in the water column. In the recent aquaculture system investigated for the STXs producing species, the sxtA1 gene qPCR assay can support the analytical methods for STX determination in seawater and shellfish especially at early warning stage of toxic blooms. At the same time, three different instrumental platforms and 3 separate analytical methods were used to investigate the presence of a wide array of STXs and TTX in seawater and shellfish (mussels, clams) collected in spring/summer 2015 to 2017 in the Mediterranean Sea. A very high STX contamination in mussels emerged, unprecedentedly found in Italy, with maximum total concentration of 10850 □g STX eq/kg of shellfish tissue measured in 2016. The sxtA1 gene content was correlated with toxin presence in environmental samples to provide an indication of STX-related risk during a bloom. The amount of sxtA1 gene was in the range of 1.38x105–2.55x108 copies/L and the STX concentrations ranged from 41-201 nmol/L in seawater. In addition, for the first time TTX was detected in Italy in most of the analyzed samples in the range 0.8-6.4 µg TTX eq/kg.

The recurring blooms of STX-producing species over the 3-year period, the high STX levels and the first finding of TTX in mussels from the Syracuse Bay suggest that human health concerns exist and that monitoring programs of STXs and TTX in seafood should be activated in this geographical area.

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# Improved detection of marine neurotoxins through the creation of an ouabain and veratridine resistant (OV-R) neuroblastoma (neuro-2a) cell line

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Seafood products contaminated with marine biotoxins pose a risk to human health. These toxins are often potent in minute amounts and contained within complex matrices; requiring sensitive, reliable, and robust detection methods. The mouse neuroblastoma cytotoxicity assay (CBA-N2a assay) is a cell-based, sensitive (<1 ppb limit of quantification), high-throughput, *in vitro* method, capable of detecting sodium channel-specific marine biotoxins at concentrations below current human health guidance levels (e.g. 0.1 ppb for C-CTX-1 eq.). The N2a assay method can distinguish between voltage-gated sodium channel (VGSC) specific effects on cell membranes, such as toxins that activate (e.g., ciguatoxins (CTX), brevetoxins (PbTX)) or block (e.g., tetrodotoxins, saxitoxins) the target VGSCs. The sensitivity and specificity of the cells in this assay to compounds activating VGSCs is due to the addition of the pharmaceuticals ouabain (O) and veratridine (V). Assay sensitivity is dependent on the pre-treatment of cells with O to inhibit the sodium-potassium pump (which would otherwise permit cells to compensate for excessive sodium ion flux) and V (which increases sodium channel permeability through a blockage of the voltage-gated sodium channel into an open position). The presence of O\V increases the concentration of intracellular sodium and the addition of sodium channel activating toxins (i.e., PbTx and CTX) further exacerbating the intracellular sodium concentration, resulting in dose dependent cell mortality. This assay exceeds the sensitivity of the traditional mouse bioassay and other current analytical methods and presents a valuable tool for toxin analysis in food and environmental samples. However, this method has not yet been validated.

In this study we describe a method for creating an ouabain and veratridine resistant (OV-R) neuroblastoma (neuro-2a) cell line for the N2a assay. Because O\V themselves cause cellular death it can be difficult to distinguish the source of cellular toxicity; and without OV-R, mortality rates from O/V may exceed the range of utility for the assay. Therefore, creating an OV-R cell line from new Mouse (Mus musculus) N2a cells (Neuro-2a (ATCC® CCL131™)) purchased from the American Type Culture Collection will enable a more standardized practical use of this assay in laboratories. We demonstrate the toxicity of O/V to newly purchased cell lines and how to create an OV-R cell line for use in existing CTX and PbTx testing protocols. Here we demonstrate that without desensitizing new cells, the addition of O/V, at levels described in the existing literature, can result in 60-80% cell mortality; compared to OV-R cell mortality at 10-30%, well within the range of acceptable utility for this assay, and enhanced toxin detection using commercially available standards PbTx-3 by 30% and CTX3C by 40%. This study is the first step towards standardizing a method for routine analysis of CTX and PbTx. Here we present our results, with implications for future studies. Liquid Chromatography coupled to High Resolution Mass Spectrometry methods for the detection of tetrodotoxins, saxitoxins, and PbTx's have already been developed in our laboratories, and currently a method for CTXs is under development. This will allow the comparison of results obtained by analytical methods and the OV-R N2a assay.

# Insights in marine toxin recognition strategies after severe food poisoning events following consumption of fish

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Food poisoning events such as ciguatera or tetrodotoxin poisoning after consumption of fish can cause severe poisoning of consumers, which may include death. A good understanding of the poisoning event is crucial to better describe the different steps involved in the process, better tackle the problem and eventually develop management and preventive strategies.

For this purpose, it is important from the first stages to have in place toxin recognition strategies in order to identify and quantify the toxins involved in these events. From a clinical perspective, this includes identifying and gathering the symptoms conducting to the food poisoning and obtaining biological samples of patients in order to detect potential toxins. It also involves gathering the food that was responsible for the event, and be able to have reliable methods in order to unequivocally identify the toxins and be able to quantify them. This is not only important to better describe the event, but further measures to set maximum permitted levels of toxins in food will rely on these type of data.

Examples of the identification and quantification of toxins in several episodes conducting to ciguatera or tetrodotoxin poisoning will be presented.

# Kinetics differences among microcystins variants: any relationship with toxicity?

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Microcystins (MC) toxicity is congener-specific; however, the *in vitro* inhibition of PP1/PP2A (the key molecular event of MC toxicity) by single MC variants is comparable. Consequently, MC toxicokinetics, including both transporter-mediated uptake and biotransformation reaction, seems to be the critical point to explain the variant-specific toxic potential. Glutathione conjugation, either occurring spontaneously or catalyzed by GST, is the accepted main step in MC detoxification. We characterized the *in vitro* human conjugation of different variants (MC-LR, MC-RR, MC-YR, MC-LW and MC-LF), characterized by different structures and lipophilicity, the latter experimentally determined in the same conditions using the OECD guideline n° 117. The ranking from the most to the least lipophilic is : MC-LF>MC-LW>MC-LR>MC-YR>MC-RR.

Using single human hepatic recombinant isoforms (GSTA1, A2, A4, M1, T1 T2, P1, and O1) and human liver cytosol (HLC, pool of 200 donors) the kinetic parameters Vmax, Km and Cli were calculated for MC-LR, MC-RR, MC-YR, and MC-LW. All tested recombinant GSTs were active in conjugating MCs, with comparable catalytic efficiency although the ranking was different, as well as the kinetic behavior. The spontaneous reaction was generally predominant when compared to enzymatic reaction. The reaction between MC-LF and GSH (both spontaneous and enzymatic) was very limited and no kinetic parameter could be calculated. This variant is therefore poorly detoxified in the human liver and further investigations are recommended on its toxicity.

The variant which is most efficiently detoxified (Cli values about 2-3 folds higher) is MC-RR, which is the least acutely toxic. In addition, with MC-RR and only to a lesser extent for the other in the presence of GSH depletion, such as the one occurring being co-exposed to paracetamol or other GSH-depleting xenobiotics, the enzymatic reaction was by far predominant, giving relevance to the possible interindividual differences due to the activity of polymorphic GST isoforms.

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# Bacterial fermentation and Marine toxins: the case of tetrodotoxin and okadaic acid

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Recently, bacterial fermentation has been studied as a possible way to biodegrade, modify or bind toxins. In this work the effect on some marine toxins, tetrodotoxin (TTX) and okadaic acid (OA) has been tested. TTX is a bacterial neurotoxin associated to consumption of the typical pufferfish-derived delicacy fugu. This toxin is actually produced by several strains belonging to *Vibrio*, *Alteromonas* and *Pseudomonas* genus that can colonize the gastrointestinal tract of fishes and terrestrial animals. This potent neurotoxin targets the voltage gated sodium channels causing paralysis and several other symptoms. OA, an inhibitor of protein phosphatases, is the main algal toxin responsible for diarrhetic shellfish poisoning (DSP). This toxin is produced by *Dinophysis*, *Exuviaella* and *Prorocentrum*, three dinoflagellates genus that colonize the major part of seas and oceans. DSP manifests as intense diarrhoea and severe abdominal pains and is associated to consumption of molluscs. For this study several strains, isolated from food niches, belonging to different lactic acid bacteria species and bacteria isolated from marine environment were tested. A simplified model system was adopted in which live bacteria are directly contacted with the toxins TTX and OA in order to evaluate the interactions. Detection and quantification of TTX and OA was performed by UHPLC-MS/MS technique. In order to determine a possible interaction between toxins and bacteria, the percentage of the toxic compounds calculated following bacterial contact was compared to that of the control samples. This preliminary study on the effects of bacteria on marine toxins may represent the basis for the development of a method to support *in vivo* studies that can lead to the adoption of tools able to limit the algal blooms occurring in the seas. The next purpose to investigate the nature of the interactions among toxins and bacteria could open interesting perspectives.

# Effects of the marine toxin palytoxin on *Artemia franciscana*

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Palytoxin (PLTX) is a non-protein marine toxin, originally isolated in 1971. To date, PLTX has been found in several marine organisms including *Palythoa* and *Zoanthus* corals, *Ostreopsis* dinoflagellates and *Trichodesmium* cyanobacteria. Currently, PLTX and its analogues are distributed worldwide, although PLTX was initially identified in tropical waters. Despite its toxicity in humans, associated to the oral, inhalation, cutaneous and ocular exposure, toxic effects have also been described in marine animals such as death of *Patella caerulea* (Gastropoda) and *Monodonta turbinate* (Gastropoda), abnormal bysssus of *Mytilus galloprovincialis* (Bivalvia), loss of spines of *Paracentrotus lividus* (Echinoidea), abnormal arms of *Coscinasterias tenuispina* (Asteroidea). In addition, various bivalves (such as clams, scallops, oyster, and blue mussels) exhibit growth rate reduction, production of mucus, lesions in different tissues, immune responses and high mortality rates. Hence, considering the potential eco-toxicological impact of PLTX, the aim of this study was to evaluate the effects of PLTX on *Artemia fransciscana*, a micro-crustacean of the Artemiidae family frequently used in toxicological and ecotoxicological analysis.

On this model, PLTX, at environmental-relevant doses (0.1, 1, 10 nM), induced a time-dependent mortality of adult *Artemia franciscana* (21 days in culture) starting after 12 h exposure and reaching a mortality of 100% of animals after 24 hours at the highest dose (10 nM) . Hence, an exposure of 12 hours was chosen as the lowest exposure time at which PLTX-induced mortality significantly appeared. After 12 hours, PLTX did not significantly alter protein content in treated animals, while a slight increase of ROS production was recorded, suggesting a possible oxidative damage. Since locomotion alteration in *A. franciscana* adults was observed after treatment with PLTX, in particular at the higher concentration, cholinesterase activity was quantified in treated animals to investigate its possible role in PLTX toxic effects. In addition, to investigate if animals' death was due to a non-efficient detoxification capability, the activity of gluthatione S-transferase was measured.

In conclusions, these results demonstrate that environmental-relevant doses of PLTX are able to induce significant mortality of *A. franciscana* animals, as a model of eco-toxicology screening. Experiments are ongoing to elucidate the relevant mechanism(s) of toxicity.

# Developments on the presence of tetrodotoxins in Mediterranean shellfish from the Marano lagoon in the northern Adriatic sea - Italy

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Tetrodotoxins (TTXs) are highly potent neurotoxins responsible of *Tetraodontidae* puffer fish-poisoning widespread in Asiatic waters. In recent years TTXs have been detected also in bivalve mussels from the Mediterranean Sea i.e. Greece, and from European countries facing the Atlantic Ocean i.e. England, Netherlands, Spain. The bioaccumulation of these emerging toxins by edible shellfish could represent a potentially threat for food safety. In 2017 and 2018, at the end of May high levels of TTXs were detected in Italy in Mediterranean Mussels (*Mytilus galloprovincialis*) collected in the geographic area of "Ficariol San Piero" of the Marano Lagoon in the frame of the EU monitoring program for the regulated marine biotoxins. The amount of TTXs detected, 541 µg/kg in 2017 and 216 µg/kg in 2018, exceeded abundantly the alert level indicated by EFSA (44 µg/kg) [1]: The TTXs toxins were already detected in Italy [2], however, the occurrence of such a high levels in a restricted area, in the same period of the year over different years and in similar environmental conditions (e.g. water temperature ranged between 19-25°C) deserved further investigation. Therefore, in 2019, an explorative sampling in the same geographic area was implemented with the aim of investigating possible factors behind the TTXs production.

Samples were collected on weekly basis from April to July and included both autochthonous (*Mytilus galloprovincialis* and *Ostrea Edulis*) and seeded shellfishes (*Mytilus galloprovincialis and Crassostrea gigas*), marine sediment and sea water. Simultaneously, environmental parameters such as water temperature, salinity, oxygen content and pH were monitored across the whole period, using a multi-parameter data buoy; data regarding nutrients in the water were gathered as well. Once collected, samples underwent across different analysis: HILIC-MS/MS [3,4] for the detection of TTXs on mussels and oysters specimen; isolation of bacterial strains putatively involved in TTXs' production [5]; extraction of total DNA and RNA from both bivalves and marine sediment, to identify genetic determinants involved in the TTXs' biosynthetic pathway [6] and to investigate the composition of the resident microbial community over time, by means of Next Generation Sequencing approaches. Furthermore, to unravel the role of *Prorocentrum cordatum* in TTXs production [7], characterization of harmful algae in the water column was carried on.

Preliminary results revealed levels of TTXs above the suggested safety threshold for humans from both oysters and mussels, sampled from the end of May up to the end of June, with 501 µg/kg being the highest TTXs level, found in mussels.

These findings suggest the constant presence in that particular geographic area and time period, of biotic and/or abiotic factors promoting the production of TTXs, which are planned to be investigated in the near future.

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# Distribution of ovatoxins in the food chain: ten years of monitoring along the Campania coast

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Since the end of the last century, blooms of the benthic marine dinoflagellate *Ostreopsis*, initially described from tropical regions, have been increasingly reported from rocky shores of the Mediterranean coasts. This phenomenon has caught the attention of public health protection authorities because during some *O.* cf. *ovata* blooms, an unusual incidence of a respiratory syndrome has been observed in people exposed to marine aerosols during their recreational or working activities. A multidisciplinary network involving toxicological, ecological and chemical expertise revealed *O*. cf *ovata* as a toxic species able to produce congeners of palytoxin (PLTX), named ovatoxins (OVTXs). These compounds can inducie adverse effects in marine animals and humans. Although nowadays an in-depth risk evaluation posed by OVTXs is still lacking, the Italian Ministry of Health enacted guidelines for the management of *O.* cf. *ovata* blooms along the Italian coasts [1, 2] and the European Food Safety Authorithy [3] suggested a provisional tolerance limit of 30 μg of PLTX equivalents per kg of mussel tissue.

As an implementation of national directives, a Monitoring Plan on the distribution and the impacts of *O.* cf. *ovata* was established in the Campania Region by Regional Committee Decree n. 62 27/07/2007, including three activity phases: routine, alert and alarm. Routine activity takes place in periods and at places where *O. ovata* concentrations and the deriving risks are low. It includes an analytical component, with observation and sampling of coastal seawater, benthic macroalgae and seafood, along with an educational component and a syndromic surveillance and risk communication plan. The next phases take place when a risk due to possible toxic aerosol and seawater or to toxin accumulation in specific seafood is highlighted by the routine activity, with measures differing depending on the kind of anticipated risk. The alert corresponds to phases of possible risks due to increasing *O. ovata* concentrations, leading to bloom development. It implies more intense controls and sampling on the different matrices. The alarm is triggered by high *O. ovata* concentrations and/or high levels of toxins in the seafood.

Since 2007, every year from June through October, seawater and seafood samples have been collected from a variety of sampling stations along the Campania coast and analyzed to determine: i) *O.* cf. *ovata* distribution and cell numbers at up to 46 sampling sites; and ii) seafood contamination level. The Monitoring Plan has allowed to identify hot-spots along the Campania region, to assess the distribution of OVTXs in the food chain, and to enact restrictive measures which have so far been effective in protecting public health.

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# Presence of Cyanobacteria and *Ostreopsis ovata* in Italian bathing waters

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In the last years, an increase of algal blooms in bathing waters has been observed, due to various factors (climatic changes and human activities); in particular, some environmental elements (temperature, light intensity, ultraviolet radiation, wind, pH, nutrients, salinity ...) influence the growth rate of these algae and their toxicity. The Bathing Water Directive 2006/7/EC and the Italian law (Decree 116/2008; Decree 97/2010 and subsequent amendments), require monitoring controls on the presence of potentially toxic algal species, in order to preserve the health of bathers. For this purpose environmental monitoring is carried out by the National Environmental Protection System (SNPA), represented by the Institute for Environmental Protection and Research (ISPRA) and the Regional Environmental Agencies (ARPAs). The presence of *Ostreopsis ovata* along the Italian coasts is reported. *Ostreopsis ovata* is a benthic potentially toxic microalgae that can produce analogues of Palitoxin (PLTX) including ovatoxins (OVTXs). Moreover, is reported the presence of potentially toxic species of cyanobacteriae in bathing waters lakes.