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Determination of microcystin-LR in clams (Tapes decussatus) of two Sardinian coastal ponds (Italy)

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ABSTRACT

The presence of microcystin-LR (MC-LR) was monitored in Tapes decussatus harvested in two Sardinian ponds (Cabras and Tortolì, Italy) in spring and summer. After solid phase extraction, samples were analyzed using a screening enzyme-linked immunosorbent assay (ELISA) followed by a liquid chromatographic coupled to tandem mass spectrometer (LC–MS/MS) analysis. Results obtained through the ELISA test showed the presence of microcystins with a maximum concentration in August for Cabras pond (0.55 ng/g) and in September for Tortolì pond (0.85 ng/g). The LC–MS/MS analysis did not confirm the presence of MC-LR suggesting that results obtained with the ELISA technique could be due to the presence of other microcystins. According to the tolerable daily intake suggested by the World Health Organization, these results hint that clams harvested in these ponds are safe for human health. These data can contribute to enrich the knowledge about the healthiness of Sardinian ponds and of their products.

Cyanobacteria, also known as Cyanophyceae, are photosynthetic organisms that can proliferate in marine and freshwater bodies with high nutrient loads as a result of anthropogenic enrichment. They produce many secondary metabolites; some of them are toxins that, when released into water, can cause environmental and health problems (Pereira et al., 2015). Toxic metabolites are classified into four categories: hepatotoxins, dermatoxins, neurotoxins and cytotoxins (Carmichael, 2001; Pearson et al., 2010; Schmidt et al., 2014). Among them, the most studied and widely distributed, are the hepatotoxic microcystins (MCs) mainly produced by *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc* (Ríos et al., 2013). They are monocyclic heptapeptides containing an unusual amino acid, the (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA), that is essential for the expression of their biological activity (Dawson, 1998). To date, nearly 90 variants of MCs have been identified (Welker and Von Dohren, 2006); they differ primarily in the two L-amino acids at positions 2 and 4, and in the demethylation of *d*-erythro-β-methylaspartic acid and/or *n*-methyldehydroalanine at positions 3 and 7, respectively (Gutierrez-Praena et al., 2013). The most frequent and studied variant is microcystin-LR (MC-LR) with the variable aminoacids leucine and arginine in position 2 and 4, respectively (Ghazali et al., 2010) (Fig. 1).

The primary mechanism of MC-LR toxicity consists on the irreversible inhibition of serine/threonine protein phosphatases 1 and 2 A (Freitas et al., 2015) leading to protein hiperphosphorylation, destruction of cytoskeleton, deregulation of cell division and tumor promoting activity (Martins and Vasconcelos, 2009). Health hazards of MCs lead the World Health Organization (WHO) to establish for MC-LR a provisional guidelines value of 1 μg/L in drinking water and a provisional tolerable daily intake of 0.04 μg/Kg body weight/day (Preece et al., 2015). To date, in Italy, there is only a national limit for cyanotoxins and cyanobacteria in bathing water, but there is not an established limit in drinking water (Mariani et al., 2015). Furthermore, many studies have also shown that MCs accumulate in a wide range of aquatic organisms including seafood consumed by humans (Chen and Xie, 2007; Preece et al., 2015), indicating a potential threat to human health. The presence of MCs was assessed in some Sardinian reservoirs (Mariani et al., 2015; Sulis et al., 2014) but, to our knowledge, at the moment, no information is available on the possible presence of MCs in clams of Sardinian aquatic environment. There is concern that people who rely on local seafood as a source of dietary protein may be particularly at risk of MCs exposure (Preece et al., 2015). In this work, clams (Tapes decussatus) samples were monitored in order to evaluate the possible accumulation of MC-LR. Clams, known to be filtrating organisms and bio-indicators of environmental quality, were collected in two Sardinian areas: Cabras and Tortolì ponds. In recent years, Cabras pond has been subject to a dystrophic crisis with the development of Cyanophyceae (Padedda et al., 2012). Cyanobacterial blooms were also found in the surrounding areas of Tortolì pond (Funari

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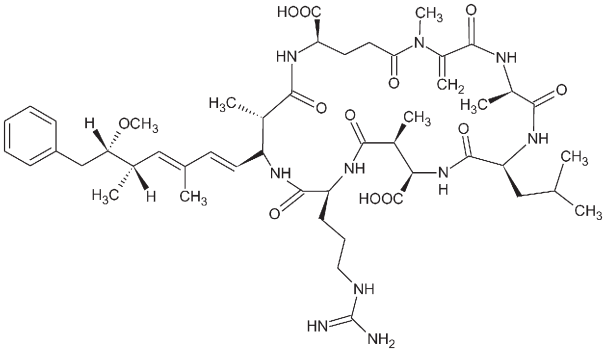

Fig. 1. Chemical structure of microcystin-LR.

et al., 2014). For this purpose, clam samples were collected for 6 months (from April 2015 to September 2015) in order to include different environmental and climatic variations. First, a preliminary screening with enzyme linked immunosorbent assay (ELISA) was used to evaluate MCs' presence in the samples. It is known that ELISA methods offer a fast screening tool but they cannot differentiate between congeners and they can suffer of false positives. Therefore, a positive ELISA result needs to be confirmed. For this reason, a chromatographic analysis was developed using a liquid chromatography-tandem mass spectrometer (LC–MS/MS) to confirm the ELISA results. In this second step we decided to focus our attention on the research of MC-LR considering that it is the most toxic and widely known cyanotoxin of its class. MC-LR standard was obtained from 3V Chimica S.r.l. (Rome, Italy). The EnviroGard® ELISA Kit was purchased from ECOTOX (Cornaredo, Milan, Italy). Deionized and distilled water was purified through a Milli Q water system (Millipore, Bedford, MA, USA). MS grade solvents were purchased from Sigma Aldrich (Milan, Italy). CHROMABOND C_{18} SPE columns (500 mg/6 mL) were purchased from Exacta Optech Labcenter (S. Prospero, Modena, Italy) and mounted on a Vacuum Manifold Set (Phenomenex, Castelmaggiore, Bologna, Italy). MC-LR stock solution (0.1 mg/mL) was prepared in methanol and was used to prepare working solutions by appropriate dilution.

The study area was chosen given that Sardinian coastal ponds are considered a reservoir of high natural value for their richness in flora and fauna. Situated in the boundary line between earth and sea, they represent ecosystems with high biodiversity and high variable water turnover.

The Cabras pond is the largest freshwater pond on the island of Sardinia (Fig. 2). It is powered by the "Mare'e Foghe" river and connected with the sea through four canals. Its deepness is variable from 40 cm on the bank to 3 m on its center.

The Tortolì pond (Fig. 3) is located on an alluvial plain. It is powered from the river "Girasole" on the north and from the sea, through the "Baccasara" channel, on the south. It has a total area of 250 ha and a deepness comprises between 1 and 2 m. Its salinity varies from 11% to 38% and its temperature is always comprised between 12 °C and 29 °C.

Samples of Tapes decussatus of the two ponds were partly obtained from the Zooprophylactic Institute of Sardinia (Sassari, Italy) and partly bought from local fishermen, twice a month in each site, from April 2015 to September 2015. Clams were immediately shelled, and homogenized using an electric homogenizer at room temperature and frozen at −20 °C till the following preparation for the analysis. Sample extraction was performed as already described by Mekebri et al., with some modifications. In details, 5 g of homogenated tissue was transferred into a centrifuge tube and 10 mL of methanol/0.2% aqueous formic acid (9/1) was added. After vortexing for 10 min, the mixture was sonicated for 1 h at room temperature and then centrifuged at 1400 g for 30 min. The obtained supernatant was collected and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 500 μL of 0.2% aqueous formic acid. Then, the extracts were cleaned by SPE using C_{18} columns previously activated and conditioned with 3 mL of methanol and 6 mL of MilliQ water. After the application of the sample, the column was washed with 3 mL of Milli Q water and 3 mL of water/methanol (80/20). The column was dried by passing a stream of air for 15 min and eluted with 5 mL of 0.1% formic acid in methanol and 5 mL of 0.1% formic acid in methanol/water (75/25). The eluate was collected in amber glass vials and evaporated to dryness under a stream of nitrogen. The residues were reconstituted with two different solvents depending on the technique used for the analysis. For ELISA test, samples were reconstituted with 500 μL of acidified water, while, samples to be analyzed through LC–MS/MS were reconstituted using 500 μL of mobile phase. Samples were concentrated by a factor of 20.

The ELISA assay was performed using the EnviroGard® Microcystin Plate Kit which is a direct competitive ELISA for quantitative detection of MCs, according to the manufacturer's instructions. The toxin concentration is visualized with a color development process and it is inversely proportional to the intensity of the color developed. In detail, 100 μL of negative control, calibrators and samples were added to their respective

Fig. 2. Map of Cabras pond with the geographic coordinates where clams were collected.

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