Harmful Algae 15 (2012) 109–118

Contents lists available at SciVerse ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Microcystin producing cyanobacterial communities in Amvrakikos Gulf (Mediterranean Sea, NW Greece) and toxin accumulation in mussels (*Mytilus galloprovincialis*)

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ARTICLE INFO

Article history:
Received 24 June 2010
Received in revised form 19 December 2011
Accepted 20 December 2011
Available online 29 December 2011

Keywords:
Cyanobacteria
Synechococcus sp.
Synechocystis sp.
Cyanobacterial toxins
Microcystins
Microcystin-LR
Microcystin-YR
Amvrakikos Gulf
Mediterranean Sea
Marine ecosystem

ABSTRACT

Various cyanobacterial species have the capacity to produce different types of toxins. Microcystins, the most prominent cyanotoxins are considered health hazards because of their potential hepatotoxic effects. They are well known to contaminate freshwater ecosystems but their presence in marine ecosystems has been reported only occasionally. We investigated seasonal changes of microcystin concentrations both in water and in the edible species of mussels *Mytilus galloprovincialis* collected from Amvrakikos Gulf (salinity ranging from 30‰ to 34‰), the biggest semi-enclosed basin in Greece. The microcystin concentrations in the water ranging from 0.003 to 19.8 ng l^{-1}, were below the World Health Organization (WHO) upper limit for recreational activities. In contrast, we found that microcystin concentrations in *M. galloprovincialis* mussels (ranging from 45 ± 2 to 141.5 ± 13.5 ng g^{-1} ww) exceeded the upper limit of the tolerable daily intake (TDI) of microcystin as determined by WHO.

Genotype composition of the total cyanobacterial community of the Gulf was analyzed by using denaturing gradient gel electrophoresis (DGGE) profiling of the rRNA internal transcribed spacer region (rRNA-ITS). The cyanobacterial community was found to be dominated almost exclusively by the cosmopolitan species *Synechococcus* – *Synechocystis*. In order to determine genes involved in the production of microcystins, a range of both specific and degenerate molecular primers against microcystin synthetase gene cluster (*mcyS*) was used.

To our knowledge this is the first report of the presence of the hepatotoxic microcystins in the Mediterranean Sea, the first study on the accumulation of these toxins in mussels from a Mediterranean marine ecosystem and one of the few published works suggesting a potential association of microcystins with *Synechococcus* and/or *Synechocystis* cyanobacteria.

The importance of our study is strengthened by the fact that Amvrakikos Gulf is among the most productive Greek "seafood" areas and a Mediterranean wetland of international significance according to Ramsar Convention.

1. Introduction

Cyanobacterial blooms are increasingly a major problem in freshwater ecosystems. There have been increasing public health concerns, since 60% (on average) of these cyanobacterial blooms are toxic (Watanabe and Oishi, 1982; Hawkins et al., 1985; Repavich et al., 1990; Carmichael and Falconer, 1993; Gobler et al., 2007; Vareli et al., 2009a,b). The most studied cyanobacterial toxins belong to a family of cyclic heptapeptide hepatotoxins called microcystins (Carmichael, 1994; van Apeldoorn et al., 2007). More than 60 micocystins have been identified to date, all of which have the amino acid ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) combined with six other amino acids (Dawson, 1998; McElhiney and Lawton, 2005).

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doi:10.1016/j.hal.2011.12.005

Microcystins are synthesized non-ribosomally by the thiotemplate functions of large multifunctional enzyme complexes containing both non-ribosomal peptide synthase (PS) and polyketide synthase (PKS) (Pearson et al., 2008). The microcystin biosynthesis gene cluster (*mcyS*), has been sequenced and partially characterized in several cyanobacterial species (Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004). In *Microcystis aeruginosa*, the microcystin biosynthesis gene cluster (*mcyS*) spans 55 kb, comprises 10 genes arranged in two divergently transcribed operons (*mcyA–C* and *mcyD–J*) and includes genes encoding for peptide synthetases (*mcyA*, *mcyB* and *mcyC*), polyketide synthetases (*mcyD*), hybrid PS–PKS enzymes (*mcyE*, *mcyG*), and enzymes putatively involved in the tailoring (*mcyJ*, *mcyF*, and *mcyI*) and transporting (*mcyH*) of the toxin (Tillett et al., 2000).

Microcystin production in toxic cyanobacteria is thought to be influenced by a number of different physical and environmental parameters such as, nitrogen, phosphorus, trace metals, growth temperature, light, and pH (van derWesthuizen and Eloff, 1985; Sivonen, 1990; Lukac and Aegerter, 1993; Song et al., 1998). Interestingly, Long et al. (2001) suggested that the observed toxin fluctuations under different environmental conditions were probably due to the indirect effects on cell-growth rate. A closer examination of microcystin regulation at the molecular level revealed that high light intensities and red light were correlated with increased transcription, while blue light led to reduced transcript levels (Kaebernick et al., 2000).

The production of toxins by cyanobacteria, poses a serious problem to human health in relation to the consumption of contaminated drinking water or food. The cyanotoxins are collectively responsible for continued widespread poisoning of wild and domestic animals and human fatalities (Nishiwaki et al., 1994; Jochimsen et al., 1998; Carmichael et al., 2001; Briand et al., 2003; Jacquet et al., 2004; Falconer and Humpage, 2005; Wang et al., 2005; Xie et al., 2005; Soares et al., 2006; Yuan et al., 2006). Avian mortalities from cyanotoxins have been reported since the early 1900s (Schwimmer and Schwimmer, 1968). More recent reports of microcystin induced avian mortalities are from great blue herons (Driscoll et al., 2002) and flamingos (Ballot et al., 2002).

Although intoxication of aquatic organisms involving cyanobacterial toxins are documented worldwide in freshwater ecosystems (Codd et al., 2005), such intoxications of marine organisms have only occasionally been reported (Chen et al., 1993; Miller et al., 2010). As described for cyanobacteria blooms in freshwater and brackish waters, the increase in cyanobacteria bloom formation reported in coastal areas has been attributed to factors such as high irradiation, high temperatures and increased nutrient loading, as a consequence of human population growth near these locations (Camargo and Alonso, 2006; Ahern et al., 2007; Plinski et al., 2007). In the Baltic Sea, blooms of *Nodularia spumigena* are a common issue during the summer months and the production of the hepatotoxin Nodularin is common (Sivonen et al., 1989; Repka et al., 2004). Recently it was found that microcystin-LR levels are also high in the Baltic Sea (Karlsson et al., 2005a) and the most probable candidate organism for microcystin-LR production is thought to be *Anabaena flos-aquae* rather than *Anabaena lemmermannii* (Kankaanpaa et al., 2009).

Martins et al., 2007 showed that crude extracts of marine cyanobacterial species such as *Synechocystis* and *Synechococcus* had a negative effect on the survival and embryogenesis in a number of marine invertebrates. In a recent study (Carmichael and Li, 2006) it was found that in the inland hyper saline lake, the Salton Sea (California, USA), the genera producing measurable levels of microcystin included mainly *Synechococcus* and *Oscillatoria*. The production of microcystins by a *Synechococcus* strain closely related to marine *Synechococcus* indicates that microcystins may be more common in saline environments than previously thought.

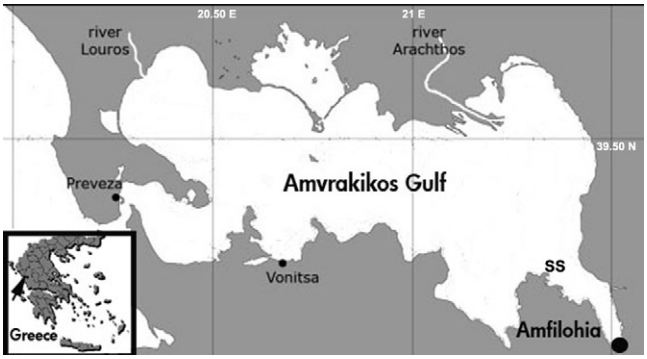

Fig. 1. Amvrakikos Gulf location in Northwestern Greece and a map of the Gulf. (SS: sample station.)

Cyanotoxins are also suggested to have a negative impact on selected aquaculture organisms (Carmichael and Li, 2006). The most well known is the loss of Atlantic net-pen reared salmon from microcystins produced by as yet unknown organisms (Andersen et al., 1993). These findings add concerns for potential negative impacts of cyanotoxins in the emerging business sector of fresh and marine aquaculture.

Mediterranean estuaries and enclosed basins, such as Amvrakikos Gulf, are areas of great ecological and economic importance. The Amvrakikos Gulf (Fig. 1) with an area of about 400 km^2 (salinity ranging from 24‰ to 36‰) is the biggest enclosed basin in Greece, among the most productive Greek "seafood" areas (Panayotidis et al., 1994; Economou et al., 2007), as well as a key wetland of international importance under the Ramsar Convention (www.ramsar.org). However, the area is at risk due to pollutants carried by the Louros and Arachthos Rivers and the wastewaters from the processing of agricultural products by small industries established in the area (Vassilopoulou et al., 2002). Anthropogenic inputs from agriculture, industry, and municipal wastes coupled with heavy nutrient loads from aquaculture industries that are stimulating factors for phytoplankton blooms and also putative toxigenic cyanobacterial blooms (Ignatiades and Gotsis-Skretas, 2010). Data on the trophic state of Amvrakikos Gulf are limited, usually concerning environmental parameters (e.g. nutrient loads) or sporadic biological parameters (e.g. chlorophyll measurements and fauna distribution) (Panayotidis et al., 1994). We studied microcystin concentrations for a one-year period in water (dissolved and particulate) and also in mussels collected from the SE less eutrophied region of the Gulf (Panayotidis et al., 1994). We chose to study mussels not only as bioindicators for the environmental quality assessment (Langston and Spence, 1995), but also due to their economic importance as edible products of the Gulf. We also mapped cyanobacteria species to identify potential toxic species in Amvrakikos Gulf. To our knowledge this is the first study to characterize cyanobacterial species in a Mediterranean semi-enclosed embayment by using molecular techniques and also the only study which has recorded microcystin concentrations in a Mediterranean marine ecosystem.

2. Materials and methods

2.1. Study area and sampling

Amvrakikos Gulf is a shallow (maximum depth: 60 m) semi-enclosed embayment in the Ionian Sea. It is connected with the Ionian Sea through a narrow channel (width 800 m, depth 12 m). At the northern part of the Gulf is situated the extensive deltas of Louros (mean annual discharge 19 $m^3 s^{-1}$) and Arachthos Rivers (mean annual discharge 70 $m^3 s^{-1}$) (Panayotidis et al., 1994).