Isolation of Toxic Marine Cyanobacteria and Detection of Microcystins in Thermaikos Gulf in Central Macedonia in Greece

Maria Kalaitzidou[1], George Filliousis[2], Evanthia Petridou[3], Vangelis Economou[4], Alexandros Theodoridis[5], Panagiotis Angelidis[6]

[1]Region of Central Macedonia Greece, General Directorate of Rural Economy and Veterinary, Directorate of Rural Economy and Veterinary of Regional Unit of Thessaloniki, Veterinary Department, Greece, e-mail: M.Kalaitzidou@pkm.gov.gr
[2]Laboratory of Microbiology and Infectious Diseases, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Greece, e-mail: georgefilious@vet.auth.gr
[3]Laboratory of Microbiology and Infectious Diseases, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Greece, e-mail: epetri@vet.auth.gr
[4]Laboratory of Hygiene of Food of Animal Origin, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Greece, e-mail: boikonom@vet.auth.gr
[5]Laboratory of Animal Production Economics, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Greece, e-mail: alextheod@vet.auth.gr
[6]Laboratory of Ichthyology, School of Veterinary Medicine, Faculty of Health Sciences Aristotle University of Thessaloniki, Greece, e-mail: panangel@vet.auth.gr

Abstract. The presence of toxic marine cyanobacteria and secondary metabolites, microcystins, were studied in Thermaikos Gulf in Central Macedonia in Greece, during the period from March 2013 to March 2014. Toxic marine cyanobacteria were isolated in marine agar and identified with PCR using primers based on 16S rDNA. The presence of microcystins in water extraction was detected by immunoassay (competitive ELISA). The concentration was ranged from 0.15 to 5ppm. It was observed that populations of toxic marine cyanobacteria were increasing during spring and early winter and there was a correlation to the physical and chemical parameters of the water. The percentage of microcystins was 20.8 % and there were significant differences ($p<0.05$) between the areas and the seasons.

Keywords: cyanobacteria, microcystins, water bloom, Thermaikos Gulf.

1 Introduction

Cyanobacteria (blue-green algae) occur in freshwater, brackish and marine environments (Lawton and Cood, 1991). Toxic species can be potentially hazardous for animal and public health, since the toxins known as cyanotoxins are produced during eutrophication periods (Hitzfeld *et al*.; 2000). Poisoning by cyanotoxins has been described in humans and animals (Carmichael, 1997; Chorus *et al*., 2010). Cyanotoxins act as inhibitors of protein phosphatases 1and 2A (PP1 and PP2A),

Proceedings of the 7th International Conference on Information and Communication Technologies in Agriculture, Food and Environment (HAICTA 2015), Kavala, Greece, 17-20 September, 2015.

inducing apoptosis and necrosis of the hepatocytes (Dawson, 1998). Moreover they cause diarrhea and skin irritations (Chernoff *et al*., 2002; Martins *et al*., 2005).

Microcystins, which are cyclic heptapeptides, are cyanotoxins that exhibit toxic activity due to a unique amino acid, Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-dienoic acid) (Codd *et al*., 1999; Hitzfeld *et al*., 2000). More than 70 analogues of microcystins have been described (MacElhiney & Lawton, 2005). Although microcystins have been widely studied in fresh and brackish waters, the bibliography is limited concerning microcystins in marine waters (Lawton and Cood, 1991). The presence of microcystins in the marine environment has been reported in seawater from the Atlantic Ocean, the Caribbean, the Pacific, the Indian Ocean, the Arabian Sea the Marmara Sea, and the Mediterranean Sea (Martins *et. al*., 2005; Taş *et al*., 2006). In Greece there is only a report from Amvrakikos Gulf, which confirmed the presence of microcystins in marine waters at levels ranging from 0.003 to 19.8 ng 1^{-1} (Vareli *et al*., 2012).

Although intense human activity such as fishing, shellfish farming and tourism take place in the Thermaikos Gulf, there are no data concerning toxic marine cyanobacteria. Thermaikos is a semi-closed gulf located in the northwest of Aegean Sea, with a surface of 5.100 km^2. It is one of the major productive areas of Central Macedonia and generally Greece. The north limit is the bay of Thessaloniki with maximum depths up to 27m (Nikolaidis *et al*., 2006), the west is the region of Pieria and the east is the peninsula of Kassandra. In the south limit the gulf opens to Aegean Sea with a maximum depth up to 90m. Thermaikos Gulf is enriched by three big rivers, Axios, Loudias, and Aliakmonas and a small one, Gallikos. The annual runoff mainly from November to May amounts to 150 m^3s^{-1} (Ganoulis, 1988). The sediment fluxes from the surrounding areas are 500 tn km^{-2} per year (Poulos *et al*., 2000). These include nitrate and phosphate salts due to fertilizer application in the adjacent crops.

The possible impact of cyanotoxins on people practicing recreational or occupational activities in Thermaikos gulf is of concern. Many cases of skin and eyes irritations, diarrhea, asthma and allergic reactions have been reported after swimming in marine waters, where cyanobacteria bloom had occurred (Chorus *et al*., 2000; Stewart *et al*., 2009). In addition toxic marine cyanobacteria constitute an occupational hazard for fishermen, water sports teachers, cleaners and maintainers of coasts, fish and shellfish farmers and divers (Stewart *et al*., 2009). Also it is reported that toxic marine cyanobacteria inhibit grazing of zooplankton (Figueiredo *et al*., 2004) and may be toxic to it and to crustaceans (Carmichael, 1992). Studies in mussel's embryos report that cyanobacterial extracts from *Synechocystis* spp. and *Synechococcus* spp. caused total inhibition of embryogenesis (Martin *et al*., 2007). This report is of importance for shellfish production of Thermaikos gulf since it provides 90% of mussel production annually of Greece.

The morphological characteristics of Thermaikos Gulf, the enrichments and the climate conditions of North Greece, especially of Thessaloniki can induce eutrophication problems mostly near the shallow coastal zones (Koukaras, 2004; Nikolaidis *et al*., 2006). The scope of this research was to identify the toxic marine cyanobacteria and to detect the presence of microcystins in Thermaikos Gulf.

2 Materials and methods

2.1 Field Sampling and Handling

During 2013, 120 water samples were collected from Thermaikos gulf every three months, with the exception of the winter months. The sampling points were selected according to their ecological and economic importance, with selected points having intense fishing, shellfish farming and recreational activities. The sites were: **1**. Chalastra area in Thessaloniki region (40º 32′ 20.12′′N and 22º 44′56.63′′E) **2**. Aggelochori area in Thessaloniki region (40º 29′30.05′′N and 22º 49′11.79′′E) **3.** Makrigialos area in Pieria region (40º24′57.98′′ N and 22º37′14.93′′E) **4.** Klidi area in Imathia region (40º28′37.03′′N and 22º39′58.94′′E). Sampling was performed by a portable hose sampler, which was submerged at a depth of 2 m. After receiving the water column, 500ml of marine water were transferred into a sterile flask. Salinity, oxygen saturation, pH and temperature of the water were measured in the field with an YSI 556 handheld multiparameter instrument (YSI Incorporated, Ohio, USA). The samples were transferred in the laboratory in insulated cold boxes. 150 ml were filtered through filters with 0.45μm pore diameter (PALL CORPORATION, 600 South Wagner Road Michigan). One filter was used for culture and one was stored in freezer at -80ºC until microcystin detection.

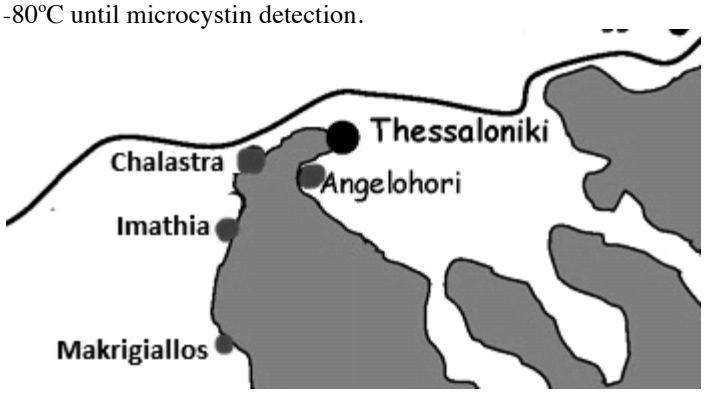

Fig. 1. Sampling areas in Thermaikos Gulf

2.2. Cyanobacteria isolation and identification

After filtration the filters were placed on Marine Agar growth medium (CONDA S.A. Torrejon de Ardoz, Madrid Spain) supplemented with imipenem (50mg l^{-1}, BIO-RAD, München, Germany) (Ferris and Hirsch, 1991) and kanamycin (50mg l^{-1}, BIO-RAD, München, Germany). The petri dishes were incubated up to 21 days at 25ºC under cool white fluorescent light (700LUX), with a 14:10 light:dark cycle and monitored for growth at the seventh, tenth, fourteenth and twenty first day of incubation. Colonies showing typical morphology were observed in an optical microscope (Olympus CH30) after Gram staining. The morphology characteristics were used to characterize cells as to *Synechocystis* spp. according to Anagnostidis

and Komárek (1985). In brief cells that were coccoid, nanoplaktonic morphology with a diameter of 1 to 4 μm, organized as single cells, doublets of dividing cells, and cloverleaf-type cell aggregates were characterized as presumptive.

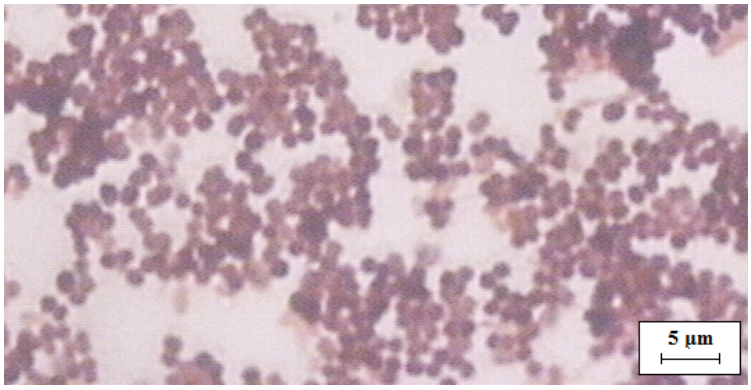

Fig. 2. Marine cyanobacteria cells after Gram staining.

Colonies showing typical morphology were harvested and passaged in Marine Agar. The cultures were considered axenic after two passages, and further identified with PCR, using specific primers for the amplification of the cyanobacterial 16S rDNA fragments (Forward primer 27f 5'-AGA GTT TGA TCM TGG CTC AG, reverse primer 1525r 5'-AAG GAG GTG WTC CAR CC) (Svenning *et al*., 2005).

2.3 Sample Analysis for Microcystins

For microcystins detection the frozen filters were dissected and extracted with methanol (Merck, Germany). The extraction was performed with a 75% aqueous solution of methanol, since it is reported to be the most suitable for microcystins extraction (Rodriguez *et al*., 2005). The filters were cut in squares pieces of 2-3 mm acne and put in 10 ml methanol overnight. The supernatant was transferred into 50 ml tubes, 10 ml of methanol 75% were added in the pellets. Then they were heated at 55ºC for 15 min, centrifuged at 3000rpm for 20min at 4ºC and the supernatant was collected and transferred into the 50ml tube. This procedure was repeated once more, the extracts were pooled and concentrated using rotary evaporation until dry, dissolved in 1ml methanol 75% and filtered through syringe filters.

The extracts were examined for microcystins with the commercial immunoassay method Microcystins (Adda specific) ELISA kit according to `manufacturer's instructions (Enzo Life Sciences Inc, USA). The detection limit was 0.10ppb microcystin-LR analogues. The absorbance was read at 450nm using a microplate ELISA photometer (DAS model A3, Italy). Calculation of microcystin concentration was performed according to the standard curve plotted against standard concentrations of 0.15, 0.40, 1.00, 2.00, 5.00 ppb of microcystin-LR analogues.

2.4 Statistical Analysis

The presence of cyanotoxins was measured as the percentage of microcystins at concentrations higher than 1 ppb, within the selected samples. The relation of the toxins frequencies to region and season was evaluated using the chi-square test of independence (χ^2-test). The chi-square test of independence was applied to determine whether there is a significant association between two variables or not. Analysis of Variance (ANOVA) was performed to evaluate possible effects of season and region, as well as their interaction, on toxins concentration, temperature, ph and salinity. Differences between mean values of specific factors were evaluated using the Duncan's new multiple range test. The statistical analysis was conducted using the SPSS software program and significance was declared at $p \leq 0.05$, unless otherwise noted.

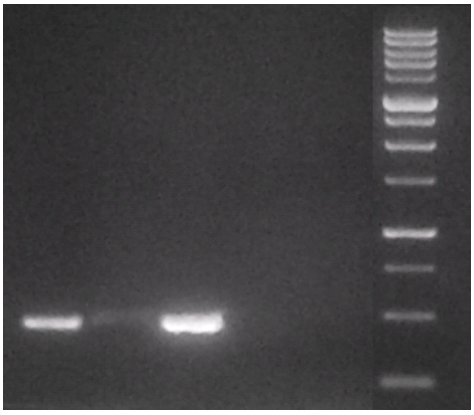

Fig. 3. PCR detection of 16S rDNA of cyanobacteria.

3 Results and Discussion

Our research confirmed the presence of toxic marine cyanobacteria in Thermaikos gulf by culturing of cyanobacteria in a novel medium, marine agar supplemented with imipenem and kanamycin. We found that the combination of imipenem and kanamycin at the final concentrations of 50mg l^{-1} helped to reduce saprophytic microbial flora and greatly enhanced the easiness and possibility of obtaining axenic cultures. ELISA showed that concentrations of microcystins varied from 0.15 ppb in late summer and late autumn to 5.00 ppb in spring and early autumn. April, May and June were the months with higher toxin concentrations. It was found that 20.8% of the samples had concentrations higher than 1 ppb (Table 1). The percentage of positive samples were higher in Aggelochori and Makrigialos areas (36.7% and 26.7% respectively), while Chalastra and Imathia had lower ones (13.3% and 6.7% respectively).

The number of positive samples varied had shown a seasonal variation (Table 2). In Chalastra and Aggelochori the number of positive samples was higher during

spring (3 and 6 positive samples respectively). On the other hand in Makrigialos the number of positive samples was higher during autumn (5 samples). This can be explained by the position of the sampling points in regard of the discharges of Axios River. The outfall of Axios is near the Chalastra and Aggelochori sampling points which are situated in the greater vicinity of it. Whereas the Imathia (approximately 6 km) and especially Makrigialoas (approximately 14 km) . The sampling points in these areas are located near to Delta of Axios, which is the second most polluted river in Greece (Skoulidis, 1993). The discharges of Axios are higher during spring reaching the 279 m^3 sec^{-1} (Poulos *et al*., 2000; Koukaras, 2004) since they are connected to the snowmelt from mountains in FYROM (Poulos *et al*., 2000). In addition the gulf streams which are south-south east during spring and summer (Koukaras, 2004), transfer big amounts of water masses from Chalastra to Aggelochori.

Table 1. Presence of toxins in marine waters for each region

Regions	Presence of toxins N (%)
Chalastra	4/30 (13.3%)
Makrygialos	8/30 (26.7%)
Imathia	2/30 (6.7%)
Aggelochori	11/30 (36.7%)
Total	25/120 (20.8%)

Table 2. Presence of toxins in marine waters in each region according to seasons

Regions	Seasons			
	Spring	Summer	Autumn	Total
Chalastra	3/10	1/10	0/10	4/30
Makrigialos	2/10	1/10	5/10	8/30
Imathia	0/10	1/10	1/10	2/30
Aggelochori	6/10	3/10	2/10	11/30
Total	11/40	6/40	8/40	25/120

The test of independence show that there is a significant association between the concentration of the toxins and the region (p-value= 0.02). Significant differences in microcystins concentrations were observed within areas in spring and autumn as it is shown in Table 3.

Larger microcystin concentrations were observed in samples from Aggelochori sampling point. This can be explained by the proximity of the sampling point to urban waste treatment facility, where all the sewage of Thessaloniki, the second bigger city in Greece, is processed.

Regarding the physical and chemical characteristics of the water, temperature ranged from 13ºC to 23ºC depending on the season, ph from 7.80 to 8.79 and salinity from 34.8 to 38‰. Oxygen saturation was 75-80%. Tables 4 and 5 show the mean temperature values between areas in different seasons and significant differences of ph and salinity according to seasons respectively. Statistical analysis has revealed

significant effects of the physicochemical characteristics to the growth of toxic cyanobacteria, especially during spring and autumn.

Table 3. Microcystin concentration differences within areas according to the season (ppb)

Seasons	Regions			
	Chalastra	Makrygialos	Imathia	Aggelochori
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Spring	1.55±2.05^{ac}	1.06±1.42^{ab}	0.35±0.22^{b}	2.25±1.53^{c}
Summer	0.58±0.71^{a}	0.75±0.21^{a}	0.41±0.29^{a}	1.15±0.88^{a}
Autumn	0.60±0.29^{a}	1.75±1.72^{b}	0.67±0.39^{a}	0.97±0.38^{a}
Total	0.91±1.31^{ab}	1.18±1.31^{a}	0.47±0.33^{b}	1.45±1.16^{a}

* Different uppercase letters indicate statistically significant differences.

Our results show a seasonal growth of toxic marine cyanobacteria in Thermaikos gulf during spring and early autumn. Seasonal distribution of cyanobacteria especially in these seasons have been reported previously (Magalhães *et al.,* 2003; Taş *et al.,* 2006; De Pace *et al.,* 2014) that also report that cyanobacterial blooms in fresh, brackish and marine waters are observed mainly during late spring and early winter, especially in May or September.

Table 4. Temperature differences among seasons (°C) in each area

Seasons	Regions			
	Chalastra	Makrygialos	Imathia	Aggelochori
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Spring	13.30±0.67^{abc}	12.70±0.48^{b}	14.50±0.53^{c}	14.00±1.33^{abc}
Summer	19.70±2.58^{a}	20.00±1.76^{ab}	18.70±2.58^{a}	21.50±1.78^{b}
Autumn	16.00±3.16^{a}	19.50±1.58^{c}	20.60±2.76^{b}	18.40±0.52^{c}
Total	16.33±3.53^{a}	17.40±3.64^{b}	17.93±3.35^{b}	17.97±3.38^{b}

* Different uppercase letters indicate statistically significant differences.

The high water temperatures generally reported in Greece can contribute in marine cyanobacteria bloom, confirming that temperature is the most important physical parameter which affects growth rates of bloom forming cyanobacteria (Davis *et al.,* 2009). Moreover the four rivers enrich the gulf through the runoff of large quantities of nutrients, including nitrate and phosphate salts from adjacent farmlands, increasing the risk. Discharges of freshwater (rivers or lakes) into coastal marine environments inducing toxic cyanobacteria bloom, have been described in Marmara Sea (Taş *et al*., 2006) and in Adriatic Sea from lake Occhito (De Pace *et al*., 2014).

Microcystin concentration in Thermaikos gulf is of great concern, since the upper mean limit was 2.25±1.53 ppb in Aggelochori area during May. The larger concentration observed were above the Tolerable Daily Intake (TDI) recommended by the World Health Organization (0.04μg $kg^{-1}d^{-1}$). This concentration is higher than the one in Amvrakikos Gulf (19.8 ppt), despite that Amvrakikos is a shallow gulf, low salinity, with two major rivers (Louros and Arachthos) draining into this gulf and considered the most polluted gulf in the Ionian Sea (Economou *et al*., 2007). In Amvrakikos gulf high concentrations of microcystins were observed in mussels

Mytilus galloprovincialis, at levels ranging from 45±2 to 141.5±13.5 ppt w/w (Vareli *et al*., 2012). Other studies from Mediterranean Sea confirmed the presence of microcystins in marine environment. In Adriatic Sea the concentration of microcystin in water was 0.61 ppb in May 2009, after a cyanobacterial bloom in Lake Occhito (De Pace *et al*., 2014).There are several reports of microcystin accumulation in seafood from the Baltic Sea (Luckas *et al*., 2005). In Sebetiba Bay in Brazil the concentration of microcystins in marine waters due to *Synechocystis* spp. bloom in June 1999 was 0.12 μg l^{-1}.Microcystins were detected also in fisheries and crustaceans with the largest concentration (0.198μg $kg^{-1}d^{-1}$) observed in a crab sample in September and was 13 times above the upper limit posed by the World Health Organization (Magalhães *et al*., 2003).

Table 5. ph and salinity (‰) differences according to seasons

Seasons	Parameters	
	ph	Salinity
	Mean±SD	Mean±SD
Spring	8.28±0.28[a]	35.10±0.76[a]
Summer	8.09±0.12[b]	34.50±2.08[b]
Autumn	8.00±0.25[c]	35.50±1.40[a]
Total	8.12±0.25	35.03±1.54[b]

* Different uppercase letters indicate statistically significant differences

In conclusion from our results it is found that marine cyanobacteria can be isolated in marine agar medium with the addition of imipenem and kanamycin at a final concentration of 50mg l^{-1}. The presence of toxin producing marine cyanobacteria in Thermaikos gulf is seasonable, especially during spring and winter. The physical and chemical parameters of the water (temperature, ph and salinity) conducive to the development of marine cyanobacteria. The high levels of microcystins especially during late spring and early autumn are of awareness, since they are considered hazardous for public health, aquatic animals and the marine ecosystem.

References

1. Anagnostidis, K. and Komárek, J. (1985) Modern approach to the classification system of Cyanophytes 1-Introduction. Algological Studies, 38–39, p.291–302.
2. Carmichael, W.W. (1992) Cyanobacteria secondary metabolites- the cyanotoxins. Journal of Applied Bacteriology, 72, p.445-459.
3. Carmichael, W.W. (1997) The cyanotoxins. Advances in Botanical Research, 27, p.211–256.
4. Chernoff, N., Hunter, E.S., Hall, L.L., Rosen, M.B., Brownie, C.F., Malarkey, D., Marr, M., Herkovits, J. (2002) Lack of teratogenicity of microcystin-LR in the mouse and toad. Journal of Applied Toxicology, 22(1), p.13–17.

5. Chorus, I., Falconer, I.R., Salas, H.J., Bartram, J. (2000) Health risks caused by freshwater cyanobacteria in recreational waters. Journal of Toxicology and Environmental Health, 3, p.323-347.
6. Chorus, I., Falconer, I.R., Salas, H.J., Bartram, J. (2010) Health risks caused by freshwater cyanobacteria in recreational waters. Journal of Toxicology and Environmental Health, 3, p.323-347.
7. Codd, G., Bell, S., Kaya, K., Ward, C., Beattie, K.,, Metcalf, J. (1999) Cyanobacterial toxins, exposure routes and human health. European Journal of Phycology, 34, p.405-415.
8. Davis, T.W., Berry, D.L., Boyer, G.L., Gobler, C.J. (2009) The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of Microcystis during cyanobacteria blooms. Harmful Algae, 8(5), p. 715–725.
9. Dawson, R.M. (1998) The toxicology of microcystins. Toxicon, 36(7), p.953-962.
10. De Pace, R., Vita, V., Bucci, M.S., Gallo, P., Bruno, M. (2014) Microcystin Contamination in Sea Mussel Farms from the Italian Southern Adriatic Coast following Cyanobacterial Blooms in an Artificial Reservoir. Journal of Ecosystems, 2014, p. 1-11.
11. Economou, V., Papadopoulou, C., Brett, M., Kansouzidou, A., Charalabopoulos, K., Filioussis, G., Seferiadis, K. (2007). Diarrheic shellfish poisoning due to toxic mussel consumption: The first recorded outbreak in Greece. Food Additives and Contaminants, 24 (3), p. 297–305.
12. Ferris, M.J and. Hirsch, C.F. (1991) Method for Isolation and Purification of Cyanobacteria. Applied and Environmental Microbiology, 57(5), p.1448-52.
13. Figueiredo, D.R., Azeiteiro, U.M., Esteves, S.M., Gonçalves, F.J.M., Pereira, M.J. (2004) Microcystin-producing blooms-a serious global public health issue. Ecotoxicology and Environmental Safety, 59(2), p. 151–163.
14. Ganoulis, J. 91988) Oceanographic conditions and environmental impact from the sewage works in Thessaloniki. Report to the Greek Ministry on the Environment, Town Planning and Public Works, Thessaloniki, p.1-244.
15. Halinen, K., Jokela, J., Fewer, D.P., Wahlsten, M., Sivonen, K. (2007) Direct Evidence for Production of Microcystins by Anabaena Strains from the Baltic Sea. Applied and Environmental Microbiol ogy, 73(20), p.6543–6550.
16. Hitzfeld, B.C., Hoger, S.J., Dietrich, D.R. (2000) Cyanobacterial Toxins: Removal during Drinking Water Treatment, and Human Risk Assessment. Environmental Health Perspectives, 108, p. 113–122.
17. Koukaras, K. (2004) Temporal and spatial distribution of Dinophysis ehrenbery harmful blooms in Thermaikos gulf. PhD thesis, Aristotle University of Thessaloniki.
18. Lawton, L.A. and Codd, G.A. (1991) Cyanobacterial (Blue-Green Algal) Toxins and their Significance in UK and European Waters. Water and Environment Journal, 5, p.460-465.

19. Luckas, B., Dahlmann, J., Erler, K., Gerdts, G., Wasmund, N., Hummert, C., Hansen, P.D. (2005) Overview of key phytoplankton toxins and their recent occurrence in the North and Baltic Seas. Environmental Toxicology, 20(1), p.1–17.

20. MacElhiney, J. and Lawton, L.A. (2005) Detection of the cyanobacterial hepatotoxins microcystins. Toxicology and Applied Pharmacology, 203(3), p. 219–230.

21. Magalhães, V.F., Marinho, M.M., Domingos, P., Oliveira, A.C., Costa, S.M., Azevedo, L.O., Azevedo, S.M.F.O. (2003) Microcystins (cyanobacteria hepatotoxins) bioaccumulation in fish and crustaceans from Sepetiba Bay (Brasil, RJ). Toxicon, 42, p.289–295.

22. Martins, R., Pereira, P., Welker, M., Fastner, J., Vasconcelos, V.M. (2005) Toxicity of culturable cyanobacteria strains isolated from the Portuguese coast. Toxicon, 46, p. 454–464.

23. Nikolaidis, N.P., Karageorgis, A.P., Kapsimalis, V., Marconis, G.,Drakopoulou, P., Kontoyiannis, H., Krasakopoulou, E., Pavlidou, A., Pagou, K. (2006) Circulation and nutrient modeling of Thermaikos Gulf, Greece. Journal of Marine Systems, 60 (1–2), p.51–62..

24. Poulos, S.E., Chronis, G.T., Collins, M.B., Lykousis, V (2000) Thermaikos Gulf Coastal System, NW Aegean Sea: an overview of water/sediment fluxes in relation to air–land–ocean interactions and human activities. Journal of Marine Systems, 25 (1), p.47-76. Sangolkar,LN., Maske, SS., Chakrabarti, T. (2006) Methods for determining microcystins (peptide hepatotoxins) and microcystin-producing cyanobacteria. Water Research, 40 (19), p.3485-3496.

25. Rodriguez, R.A. Tillmanns, A., Benoit, F.M., Pick, F.R., Harvie, J.H., Solenaia, L. (2005) Pressurized liquid extraction of toxins from cyanobacterial cells. Environmental Toxicology, 20(3), p. 390–396.

26. Stewart, I., Carmichael, W.W., Sadler, R., McGregor, G.B., Reardon, K., Eaglesham, G.K., Wickramasinghe, W.A., Seawright, A.A., Shaw, G.R. (2009) Occupational and environmental hazard assessments for the isolation, purification and toxicity testing of cyanobacterial toxins. Environmental Health, 8(52), p. 1-12.

27. Svenning, M.M., Eriksson, T., Rasmussen, U. (2005) Phylogeny of symbiotic cyanobacteria within the genus Nostoc based on 16S rDNA sequence analyses. Archives of Microbiology, 183, p.19-26

28. Taş, S., Okuş, E., Yılmaz, A.A. (2006). The blooms of a cyanobacterium, Microcystis cf. aeruginosa in a severely polluted estuary, the Golden Horn, Turkey. Estuarine, Coastal and Shelf Science, 68(3–4), p. 593–599.

29. Vareli, K., Zarali, E., Zacharioudakis, G.S.A., Vagenas, G., Varelis, V., Pilidis, G., Briasoulis, E., Sainis, I. (2012) Microcystin producing cyanobacterial communities in Amvrakikos Gulf (Mediterranean Sea, NW Greece) and toxin accumulation in mussels (Mytilus galloprovincialis). Harmful Algae, 15, p.109–118.