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## RESEARCH ARTICLE

### The Relevance of Microcystin Monitoring in Dialysis Centers of Sicilians Cities: An Environmental Study

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#### Abstract:

#### Background:

Sicilian surface basins are among the most important water supply resources available on the island. They are often affected by harmful cyanobacteria blooms as *Planktothrix rubescens* and *Microcystis aeruginosa*. Since dialysates are produced using network water, they could contain cyanotoxins. No study has been conducted yet to evaluate the removal efficiency of osmotic systems for cyanotoxins in abnormal conditions at room temperatures of about 40°C. The aim of this study was to monitor the presence of microcystins in Sicilian dialysis center, network waters and, finally, dialysates produced from these waters in an Agrigento dialysis unit where environmental conditions are favorable for algal bloom.

#### Methods:

This clinic normally receives surface water from artificial basins, where several times, traces of cyanobacteria have been detected. Moreover, dialysates and underground supply waters of a clinic in Catania were also sampled as control. Samplings were performed in summer 2018, when room temperatures were above 38 °C. A total of 40 samples were analyzed by ELISA assay.

#### Results:

Results of our monitoring highlighted concentrations of MCs in waters of several basins among LOD - 155 ng/L, lower than WHO reference value for drinking waters (1,0 µg/L), that decrease up to undetectable levels whereas no MC contamination was detected both in supply waters and dialysates.

#### Conclusion:

Although our first set of data outcomes seem to be quite comforting, an improvement of law and a complete census of the water supplies of dialysate centers would be ideal.

**Keywords:** Dialysis, *Microcystis aeruginosa*, *Planktothrix rubescens*, Health Risk Management, Sicily (Italy), Microcystins.

#### Article History

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## 1. INTRODUCTION

Cyanobacteria (Cyanophyte) are aquatic organisms that can live and grow both in fresh and brackish waters, sea and thermal waters [1 - 4]. Microcystins are the most widespread and hazardous among cyanotoxins [5, 6]. In fact, the LD50 dose for microcystins-LR is 50 µg/kg [7, 8]. The acute or chronic microcystins exposure through diet may cause severe

and sometimes fatal hepatotoxicity and could be associated with the increased onset of cancer both in humans and animals [8, 9]. The main action mechanism is the proteic phosphatases inhibition, which causes changes in cytoskeleton, oxidative stress, apoptosis [10], an increase in certain transcription genes, induction of cell proliferation and liver hypertrophy [4, 11, 12]; they can represent their defense and/or attack mechanism against competitors or to improve resistance in the presence of oxidative stress [13, 14].

In favorable conditions, algal cells may reach high

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concentrations producing known “Harmful Algal Blooms” with strong microcystin release [3, 5, 6, 15]. Therefore, the use of surface freshwaters for medical related purposes may not be safe due to the presence of microcystins [4, 5].

An emergent issue is the use of these waters for the productions of dialysates in hemodialysis processes. In fact, according to the Pharmacopoeia standards [16], the water coming from the network supply is employed directly for dialysate production.

The reverse osmosis still represents the main and most powerful technique to remove the inorganic and organic compounds, bacteria, and pyrogens [17]. However, it is yet not clear if this process is always and fully sufficient in guaranteeing the total removal of such elements [18 - 21]. Waters with unusual conditions of pH or salinity can decrease the removal system efficiency [22, 23]. Also, in critical working conditions such as high room temperatures near to 40 °C or contexts showing poor records of regular maintenance of osmotic systems, organic compounds, such as biotoxins, could find easier access into dialysates due to the enlargement of membrane pores diameter and further increasing of permeate flux [24]. Indeed, although bacterial endotoxins are much bigger than cyanotoxins, the 9<sup>th</sup> Edition of European Pharmacopoeia (2016) recommends a stricter control of waters for injection of the analysis in order to help prevent pyrogens contamination. Microcystin contamination in dialysates has been occasionally reported even after reverse osmotic treatment [25], and their presence at high concentration during hemodialysis can still lead to poisoning and death, as occurred in Brazil [26, 27]. However, no study was performed with the aim to assess the risk of exposure at low concentrations in the middle-long term. This issue could represent a serious risk in hemodialysis because it can get worse health of patients with kidney failure and it has yet to be dealt with by both national and international law makers [16].

Several cases of algal blooms phenomena have periodically been reported in many lakes across Sicily (Arancio and Castello lakes in Agrigento, Trinità Lake in Trapani, Pozzillo in the province of Enna), an island in the south of Italy. In past occurrences, the main cyanobacteria species emerged particularly in lakes of Sicily where *Microcystis aeruginosa* and *Planktothrix rubescens*, microcystin producing species can also be found [5, 28, 29].

Furthermore, since Sicilian basins are not regularly monitored by local authorities or regional surveillance agencies, particularly regarding algal toxins measurement, it has now become key to monitor the presence of cyanotoxins in dialysate waters before their use for medical purposes.

The purpose of this study was to monitor the presence of microcystins in the mains water and, finally, in the dialysates produced by them in a dialysis unit in Agrigento where the reverse osmosis systems of the clinic were exposed to high temperatures in the summer (2018) above 40 °C together with ambient temperatures of the Agrigento clinic between 36-38 °C due to a technical failure of the air cooling system. As a control, the sampling was also performed in a dialysis department in Catania where the drinking water comes from

the subsoil and where the presence of cyanotoxins has never been found. This problem could pose a serious risk in hemodialysis because it can worsen the health of patients with kidney failure and is yet to be addressed by both national and international legislators.

## 2. MATERIALS AND METHODS

### 2.1. Reagents and Materials

Microcystins-ADDA ELISA of the Abraxis LLC (Warminster, PA 18974) and standard solutions of MC-LR for ELISA test were provided by Tecna s.r.l. (Trieste, Italy) whilst methanol for residual analysis and Trifluoroacetic acid (TFA) were purchased from Sigma Aldrich. Bond Elut SPE C18 cartridges were delivered from Agilent Technology (Santa Clara, United States). Deionized water (> 18 MΩcm<sup>-1</sup> conductivity) was produced by a MilliQ water purification system. Glass fiber filter of 1.2 μm and 0.2 μm, respectively were obtained from Millipore (Darmstadt, Germany). The Variable Volume Multichannel pipettes were Eppendorf Research plus. An EMD Millipore 47mm Glass Vacuum Filter Holder was used for the filtration of samples. Finally, a Thermo Scientific Multiskan FC microplate photometer was used to carry out the ELISA tests.

### 2.2. Sampling

In August 2018, preliminarily, we carried out sampling in the Agrigento network, and after in an Agrigento dialysis unit supplied by its surface freshwaters. This clinic was affected by high room temperatures about 36-38 °C due to the air conditioning system not functioning; in addition, membranes of the osmotic system had not been recently replaced. On the other hand, since Catania municipal network supply uses ground waters where the presence of biotoxins had not been detected at that time, samples collected from the private medical unit in this city were used as control samples. In both clinics, the water was sampled in each of the four steps used for the production of dialysates:

- “the tank” for network water before the osmotic treatment;
- “osmosis 1”, the output of the first osmotic treatment line;
- “osmosis 2”, the output of another osmotic treatment line;
- “dialysate”, the water after treatment and before the entry in dialysis machine.

The sampling frequency was weekly, during a period of five weeks up to no MCs were detected in Agrigento network waters. Five samples were collected from the municipal network on inlet of the dialysis system, forty samples were obtained from the two clinics. Five negative controls were further added during the ELISA tests. For each sampling point, two duplicate aliquots were collected using two glass bottles of 1 liter each. All samples were preserved at a temperature of 4°C until analysis. The sample analysis was carried out within 24 hours of sampling in the Environmental and Food Hygiene Laboratories of Catania University. We declare that our study

did not require any ethical approval because there are not ethical issues related to the consent of patients or experimentation on animals.

### 2.3. Extraction and Analysis

Extraction and analysis were carried out according to the main available guidelines [30 - 32]. Since in dialysates, we expect low microcystin concentrations, we tend to adjust the method of guidelines by Lawton et al. [33]. For each sample, the first step of process involved water filtration through glass filters of 1.2  $\mu\text{m}$  porosity. The filtered samples were then treated and analyzed to measure free microcystins while a new filter (blank filter) was treated and analyzed to verify the absence of the intracellular toxins. The filtered waters were purified through an Agilent Bond Elut SPE cartridge C18 activated according to the manufacturer's instructions and, finally, the samples were eluted with 5 ml of methanol (0.1% of TFA); the extracts were dried under nitrogen flow and reconstituted with a 20 ml of ultrapure water (concentration factor was 50). Filters were kept at  $-20\text{ }^{\circ}\text{C}$  for a night and, subsequently, defrosted at room temperature to help the cell lysis. Afterward, they were inserted in a Falcon test tube and extracted twice using a 5 ml of methanol solution in an ultrasonic bath for 15 min at  $20^{\circ}\text{C}$ . Two extracts were then pooled in a single new falcon test tube. Finally, extracts were dried under nitrogen flow and reconstituted with 20 ml of ultrapure water. Using this process, we were able to analyze each extract through the ELISA test.

ELISA test is unable to discriminate between nodularin and microcystins toxins, but it is often used as a preliminary screening since it is a rather quick and affordable test according to cited guidelines. If the sum of both toxins is detectable by the ELISA method, it will be necessary to analyze the dialysates through a specific method in LC-MS; this approach will allow discriminating only the most toxic microcystins by other toxins [27, 31, 35, 34].

ELISA test was performed according to the manufacturer's instructions. This is an indirect competitive immuno-enzymatic test for the detection of microcystins and nodularins. When toxins were present in a sample, they competed with the microcystins-protein analog that was immobilized on the plate through the binding sites of the microcystins/nodularins antibodies in solution. Then, the plate was washed and the second antibody-HRP label was added. The plate was again washed, and a color signal would eventually develop. The intensity of color was inversely proportional to the total concentration of toxins present in the sample. The intensity was detected using a Thermo plate reader with a 450-nm filter. The microcystin concentration was measured by interpolation using a standard curve performed at every analytical batch. A certified reference standard of MC-LR was also adopted for calibrating at concentrations of 0.15 - 0.40 - 1.00 - 2.00 - 5.00  $\mu\text{g} / \text{L}$ . According to instructions, all samples and standards were analyzed in duplicate. In every batch, a negative control sample and a certified spiked check sample of Abraxis were read. Spiked concentrations were  $0.75 \pm 0.185\text{ }\mu\text{g}/\text{L}$ . The LOD of the instrumental method was  $0.10\text{ }\mu\text{g}/\text{L}$ . Since extracts were concentrated fifty times, the real measurement field was among

3-500 ng/L and LOD was 2 ng/L. Microcystin concentrations highest to the WHO limit will be confirmed in the UPLC-MS ESI TQD Acquity system (Waters inc.).

### 3. RESULTS

The semi-logarithmic curve linearity ( $R^2$ ) was between 0.9982 and 0.9919. The values of certified spiked control samples were among 0.73 and 0.92  $\mu\text{g}/\text{L}$ . In all negative control samples, MC concentrations were less than 2.0 ng/L, the LOD of the method. In network waters and in all samples of the two clinics MC levels are below of method LOD. For each week, MCs concentrations were below the LOD.

### 4. DISCUSSION

Harmful cyanobacterial blooms represent a worldwide issue. Among cyanobacterial toxins, microcystins are the most toxic. Although in Italy, these cyanobacteria are frequently found, there are still no standards quality controls, or regulation limiting microcystins in fresh or seawaters [29]. The quality of drinking water from surface basins influences the dialysate quality because various parameters such as pH or salinity influence the treatment process [22 - 24]. In a critical environment or undesirable water conditions, such as that featuring the presence of high concentrations of toxins, the contamination of dialysates could be potentially possible. Thus, it seems that the osmotic treatment is not always an effective and safe barrier [25, 18, 19].

Microcystin contamination of dialysates would lead to serious adverse effects on patients' health, especially in people already debilitated by kidney failure (also called end-stage renal disease), with an increase in disease severity [26, 27].

The aim of this study was to precisely conduct health surveillance by monitoring the presence of microcystins in the dialysates produced from municipal network by surface freshwater at risk to an algal bloom.

Although in dam waters low levels of MCs were shown for four weeks in Summer 2015, results of our surveillance show that there was not any sign of contamination in the network and all samples were collected by the investigated clinics.

ELISA was used as a screening method [36, 37]. It allows the congener-independent detection of microcystins and nodularins. Therefore, if the results were above LOD, it was not confirmed whether that the response was related only to the presence of microcystin congeners or to sum among these and nodularins. Positive results should be confirmed by an alternative method such as LC-MS. For an accurate risk assessment, when the results are above the LOD, it is necessary to determine the presence and concentration of each microcystins congener because of each congener different toxicity. Since the extracts of this study were concentrated 50 times, the calculated LOD was 2 ng/l. All results lower than 2 ng/l, guarantee the safety of dialysate.

For raw waters coming from Agrigento dams, authors decided to investigate in LC-MS method only samples where there were concentrations above the WHO reference value for drinking waters (1.0  $\mu\text{g}/\text{L}$ ). Instead, LC-MS analysis would have been necessary for each positive result in dialysis waters. However, since in dam waters, the maximum concentration

recorded was 155 ng/L and in waters of dialysis center no MCs were detected (<2.0 ng/L), LC-MS analysis is not necessary.

As shown by our study, potabilization of surface water before an input in the water network seems to be adequate to eliminate a low concentration of MCs. For example, clariflocculation is carried out to facilitate the removal of lighter particles, biomass, and organic compound as cyanotoxins. Other extensive experimental works are necessary to prove this.

However, although our first set of data seems to be heartening, it has to be reminded that we analyzed only one dialysis unit that used raw waters where microcystin contamination occurred.

This study represents only the beginning of a larger project. The issue must not be underestimated because we expect an exponential increase in the frequency of cyanobacteria blooms on surface freshwaters in the region, which could directly lead to diffusive contamination of dialysates. This potentially harmful scenario must be prevented through continuous and thorough monitoring programs. A good starting point could be the complete census of the water supplies of clinics and hospitals producing the dialysates. For dialysis units supplied by surface waters, any future law should aim at enforcing the analysis of cyanotoxins in water before and after treatment.

We must remember that in 2001, in Brazil, microcystins (0.4 µg/L) were found in treated and distributed potable water in Rio de Janeiro. The monitoring conducted in a Brazilian dialysis clinic showed microcystin contamination of dialysates before their use. Therefore, forty-four patients have been potentially exposed to contaminated dialysates by microcystins. During hemodialysis, in 90% of patients, the toxin plasma concentrations ranging from 0.16 ng/ml to 0.96 ng/ml were detected. These showed a plasmatic peak after one month of therapy approximately [25]. A plausible cause of microcystin contaminations may be related to high environmental temperature (near 40°C) in rooms where the dialysates were prepared; such room temperatures were above the acceptable range instructed by the manufacturer of the osmotic system (recommending room temperatures of approximately 20 °C) [25, 38].

## CONCLUSION

It seems evident that the supply water is the main source of dialysates contamination by cyanotoxins, due in particular to the lack of any mandatory control. According to Hilborn et al. (2013), it has now become vital to carry out an in-depth risk assessment on this issue and then move on to filling the legislative gap and improving Pharmacopeia and current guidelines concerning cyanotoxins quality controls in dialysates and water used for their production [39].

In fact, recent studies show that concentrations of MCs above 2 µg/L are not safe to guarantee the complete elimination of toxins from dialysates [40].

Finally, technical and medical personnel must strengthen their knowledge and skills on the topic to operate with more awareness. They must be able to operate interventions both in the production process of dialysates and during hemodialysis

when patients show the first symptoms of acute poisoning. Indeed, this topic is so complex and vast that we must not simply entrust the risk management solely to nephrologists, especially without the necessary skills. We suggest close cooperation with health authorities, perfusionist technicians, hygienists, microbiologists, and nephrologists must be enforced.

The results of this study can be used as data to initiate a risk assessment protocol aimed at improving the management of microbiological quality of dialysates and as a tool helping prevent the worsening of health conditions in dialysis patients.

## LIST OF ABBREVIATIONS

<b>MCs</b>	= Microcystins
<b>ELISA</b>	= Enzyme-Linked Immunosorbent Assay
<b>LOD</b>	= Limit of Detection
<b>LD<sub>50</sub></b>	= Lethal Dose 50
<b>LC-MS</b>	= Liquid Chromatography Coupled with Mass Spectrometry

## AUTHORS' CONTRIBUTIONS

“Conceptualization”, M. Ferrante and G. Oliveri Conti; methodology, G. Oliveri Conti; M.G. Elfio; data collection and validation, M. Fiore; formal analysis, S. Saitta; A. Cristaldi; investigation, C. Copat; writing-original draft preparation, P. Zuccarello G. Oliveri Conti; writing-review and editing, G. Oliveri Conti and M. Ferrante; supervision M. Ferrante”.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

Not applicable.

## CONSENT FOR PUBLICATION

Not applicable.

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None.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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