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# Human Fatalities from Cyanobacteria: Chemical and Biological Evidence for Cyanotoxins

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An outbreak of acute liver failure occurred at a dialysis center in Caruaru, Brazil (8°17' S, 35°58' W), 134 km from Recife, the state capital of Pernambuco. At the clinic, 116 (89%) of 131 patients experienced visual disturbances, nausea, and vomiting after routine hemodialysis treatment on 13–20 February 1996. Subsequently, 100 patients developed acute liver failure, and of these 76 died. As of December 1996, 52 of the deaths could be attributed to a common syndrome now called Caruaru syndrome. Examination of phytoplankton from the dialysis clinic's water source, analyses of the clinic's water treatment system, plus serum and liver tissue of clinic patients led to the identification of two groups of cyanobacterial toxins, the hepatotoxic cyclic peptide microcystins and the hepatotoxic alkaloid cylindrospermopsin. Comparison of victims' symptoms and pathology using animal studies of these two cyanotoxins leads us to conclude that the major contributing factor to death of the dialysis patients was intravenous exposure to microcystins, specifically microcystin-YR, -LR, and -AR. From liver concentrations and exposure volumes, it was estimated that 19.5 µg/L microcystin was in the water used for dialysis treatments. This is 19.5 times the level set as a guideline for safe drinking water supplies by the World Health Organization. **Key words:** cyanobacteria, cyanotoxins, cylindrospermopsins, microcystins, toxins. *Environ Health Perspect* 109:663–668 (2001). [Online 20 June 2001] <http://ehpnet1.niehs.nih.gov/docs/2001/109p663-668carmichael/abstract.html>

Poisonings by waterblooms of toxic cyanobacteria (cyanobacteria toxin poisonings; CTP) are a significant part of the concern over harmful algal blooms (1–4). The toxins, called cyanotoxins, are responsible for intermittent but repeated widespread poisonings of wild and domestic animals and aquacultured fish. Cyanotoxins include neurotoxic anatoxin-a and anatoxin-a(s), paralytic shellfish poisons (PSP; saxitoxin and analogues), and hepatotoxic microcystins, nodularins, and cylindrospermopsins (5). Human poisonings have, in the past, been suspected but not confirmed due to a lack of information regarding vectors or circumstances that would confirm the presence of cyanotoxins in human food or water supplies, plus a shortage of appropriate methods of detection. Because most CTP occur only when waterblooms accumulate as thick surface scums, humans do not experience acute intoxication because they generally avoid contact with such high cell concentrations. In addition, drinking water supplies usually receive a degree of treatment that prevents high concentrations of cyanotoxins from being present. This spares humans from severe poisoning episodes by the oral route. However, the oral route of exposure is not the only one possible for humans. This article provides biological and chemical evidence for the first documented lethality to humans from cyanobacterial hepatotoxins, occurring

via the intravenous route, in a dialysis clinic in the city of Caruaru, Brazil, during 1996.

Caruaru is a city of about 350,000 people 2 hr west of Recife, the capital of the Brazilian state of Pernambuco. Two renal dialysis centers (A and B) serve the population, with center A receiving water from the city's water treatment plant, which is delivered by truck. Center B obtains reticulated water from the city's water treatment plant. Beginning on 13 February 1996, center A patients receiving routine renal dialysis treatment began to complain of headache, eye pain, blurred vision, nausea, and vomiting. The first patient died suddenly on 20 February at center A. Fifty-one patients died by the middle of April, and a total of 76 deaths were reported in the course of our studies, which ended in October 1997. Through the cooperation of health and medical authorities in Pernambuco and the Centers for Disease Control (CDC) in Atlanta, Georgia, we obtained liver samples from 52 of these 76 victims. From these case reports, a pattern of disease was described that is now referred to as Caruaru syndrome. Caruaru syndrome is characterized by painful, extreme hepatomegaly, jaundice, and a bleeding diathesis manifested by ecchymosis, epistaxis, and methrorrhagia; elevated transaminases, variable hyperbilirubinemia, prolonged prothrombin time, and severe

hypertriglyceridemia; and disruption of liver plates, liver cell deformity, necrosis, apoptosis, cholestasis, cytoplasmic vacuolization, mixed leukocyte infiltration and multinucleated hepatocytes observed upon light microscopy and intracellular edema, mitochondrial changes, rough and smooth endoplasmic reticulum injuries, lipid vacuoles, and residual bodies observed upon electron microscopy (5–7).

This outbreak received much attention from the media and public health authorities in Brazil and has been reported in many countries. The background of this case and a summary of the epidemiology have been published (6,7). Over the intervening period, we have examined the limnological and case report data and analyzed most of the liver tissues provided and some of the serum samples. These results have allowed us to address some important questions regarding these human fatalities: What were the possible toxigenic cyanobacteria present in the reservoir that supplied water to Caruaru and the dialysis clinic? Which cyanotoxins were present, and at what concentrations? Were the concentrations of cyanotoxins present in the dialysis center high enough to produce liver failure and death?

## Materials and Methods

**Phytoplankton collections and identifications.** Water treatment personnel were not doing phytoplankton counts or identification during

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the month of February 1996, and so samples of phytoplankton were not available from the time of the initial outbreak. Subsurface phytoplankton samples from Tabocas Reservoir (Caruaru water supply) were, however, collected retrospectively at the end of March 1996, identified, and quantified. Subsamples used for phytoplankton identification and quantification were preserved with Lugol's iodine solution. Direct counting of preserved cells was carried out with Utermohl's counting techniques using a counting chamber and inverted microscope.

**Analyses for cyanotoxins.** Samples of carbon, sand, and cation/anion exchange resin from the in-house filters at both clinics A and B were analyzed using a polyclonal antibody against the cyclic peptide hepatotoxins, the microcystins, in an enzyme-linked immunosorbant assay (ELISA) (8,9). This antibody was raised against microcystin-LR but cross-reacts with many of the known microcystin analogues. Blood sera and liver from affected and control patients provided by state health personnel in Pernambuco and sent through the CDC were also examined by ELISA (see tissue extraction method, below). Samples of carbon, cation and anion exchange resin, and sand from clinic A and carbon and sand from clinic B were extracted 3 times with 10 volumes of 100% methanol for 12 hr. Extracts were dried and resuspended in distilled water and applied to C18 cartridges (6 mg). Deionized water and 20 and 100% methanol eluates were collected and dried. The 100% methanol extracts (both filter and tissue extracts) were resuspended in 1:1 methanol/H<sub>2</sub>O and diluted 10<sup>-1</sup>, 10<sup>-2</sup>, and 10<sup>-3</sup> with phosphate-buffered saline and assayed by ELISA.

We used 52 liver samples representing 39 victims (covering February–December 1996) in our analyses (13 replicate samples were also analyzed). Liver samples were homogenized in 100% methanol (2 times over 24 hr) using a volume of 10 mL for liver < 1 g and 10 mL/g for tissue > 1 g. Extracts were centrifuged at 7000 × *g*. Methanol supernatants were combined and mixed with a 3-times volume of hexane. The hexane layer was discarded and the methanol extract was placed in 40 mL distilled water and applied to C18 silica cartridges. Water and 20 and 100% methanol eluates were collected. The 100% methanol extracts were dried, resuspended in 1:1 methanol/H<sub>2</sub>O, and diluted 10<sup>-1</sup>, 10<sup>-2</sup>, and 10<sup>-3</sup> with phosphate-buffered saline and assayed by ELISA.

We used 17 serum samples representing 12 victims in our analyses reported here. Serum samples (200 µL–1.0 mL) were diluted 4 times with double-distilled H<sub>2</sub>O, and the diluted serum was loaded onto a C18 silica cartridge. The C18 cartridge was

processed as described above for liver samples and assayed by ELISA.

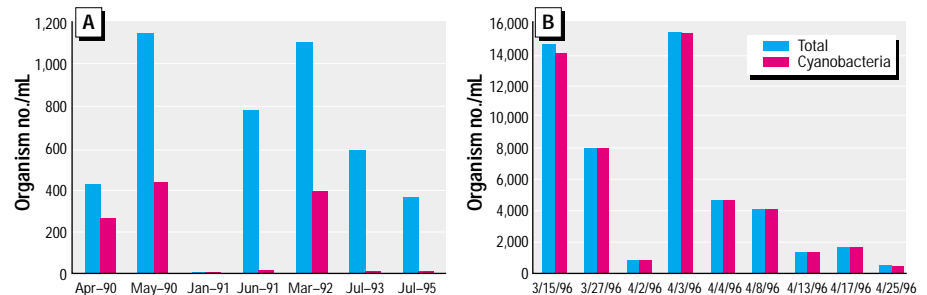
We obtained control liver samples from five persons from the pathology unit at the Federal University of Pernambuco. These samples represented persons not associated with dialysis treatment in Caruaru. Control sera (*n* = 12) were from volunteer health personnel in Caruaru, Recife, and Atlanta. These samples were extracted for analyses in the same way as victim samples were.

Chemical analyses for microcystins in liver and serum samples were done by ELISA-guided HPLC-photodiode array (PDA) separation of microcystins from liver tissues.

HPLC chromatograms were obtained on a Waters 2690 Millennium system (Waters, Milford, MA, USA). HPLC column conditions were: column, Waters µ Bondapak C18, 3.9 × 300 mm; mobile phase, isocratic-60% methanol/40% 0.05% trifluoroacetic acid; flow rate, 1 mL/min, Waters 996 PDA detector. The separated microcystins were further analyzed by matrix-assisted laser desorption/ionization/time of flight (MALDI/TOF) and electrospray ionization (ESI) mass spectrometry (MS). MALDI spectra were acquired on a VG TofSpec mass spectrometer (VG Analytical Fisons Instruments, Manchester, UK) in the linear mode, using a 337 nm N<sub>2</sub>

**Table 1.** Phytoplankton counts from Tabocas reservoir.

Samples from reservoir, March 1996	Total phytoplankton/mL	Cyanobacteria/mL (% of total phytoplankton)
Center	40,000	39,731 (99.3)
Margin	13,190	13,100 (99.3)
Raw water impounded to water treatment plant	21,420	20,882 (97.5)



**Figure 1.** Quantitative analysis of phytoplankton in raw water received at the Caruaru city water treatment plant. (A) Database from 1990 to 1995. (B) Database from March 1996 to April 1996. Data were provided by the State of Pernambuco Water Treatment Company (COMPESA).

**Table 2.** Microcystin (by ELISA) and cylindrospermopsin (by LC/MS/MS) content of carbon, ion-exchange resin, and sand filters from dialysis centers A and B in Caruaru, Brazil.

Center/sample source	Microcystin (µg/g)	Cylindrospermopsin (µg/g)
Center A, carbon	1.0	19.70
Center A, anion resin	0.5	0.02
Center A, cation resin	2.1	0.05
Center A, sand	ND	ND
Center B, carbon	3.4	ND
Center B, sand	ND	ND

ND, not detected.

**Table 3.** Microcystin values, by ELISA, of liver and serum from Caruaru dialysis patients.

Month of death (1996)	No. of victims/no. of samples	Mean microcystin (ng/g)
February	4/7	257.7
March	11/14	255.4
April	8/12	179.6
May	3/4	272.5
July	1/1	471.8
August	4/6	216.8
September	3/3	219.9
October	2/2	50.2
December	1/1	160.4
Patients without date of death	2/2	145.9
Total no. patients/no. samples	39/52	223
Mean of serum values <sup>a</sup>	12/17	2.2 (ng/mL)

<sup>a</sup>Values from victims who died and who also had positive liver values.

laser. The MALDI/TOF ionization method has emerged as a powerful analytical method (10), with a high sensitivity and tolerance for small amounts of salt and buffer that allow low-level detection of analyses (sub-femtomole) in complex mixtures. The thin-film coating method was used in these analyses (11). ESI spectra were acquired on a VG Quattro mass spectrometer (VG Biotech, Fisons, Manchester, UK). Cesium chloride was used for the calibration.

During the time in which microcystins were being analyzed, it became apparent that we should also examine the samples for other cyanotoxins. After the first analyses for microcystins, other studies demonstrated a wide occurrence of *Cylindrospermopsis* (*Anabaenopsis*) in Brazil. Because this cyanobacterium is capable of producing the alkaloid hepatotoxin cylindrospermopsin (CYN) and deoxy-cylindrospermopsin (deoxy-CYN), analyses for this cyanotoxin were begun in 1998. For CYN/deoxy-CYN analysis of sand, carbon, and ion exchange resins, methanolic extracts (5 g of sample in

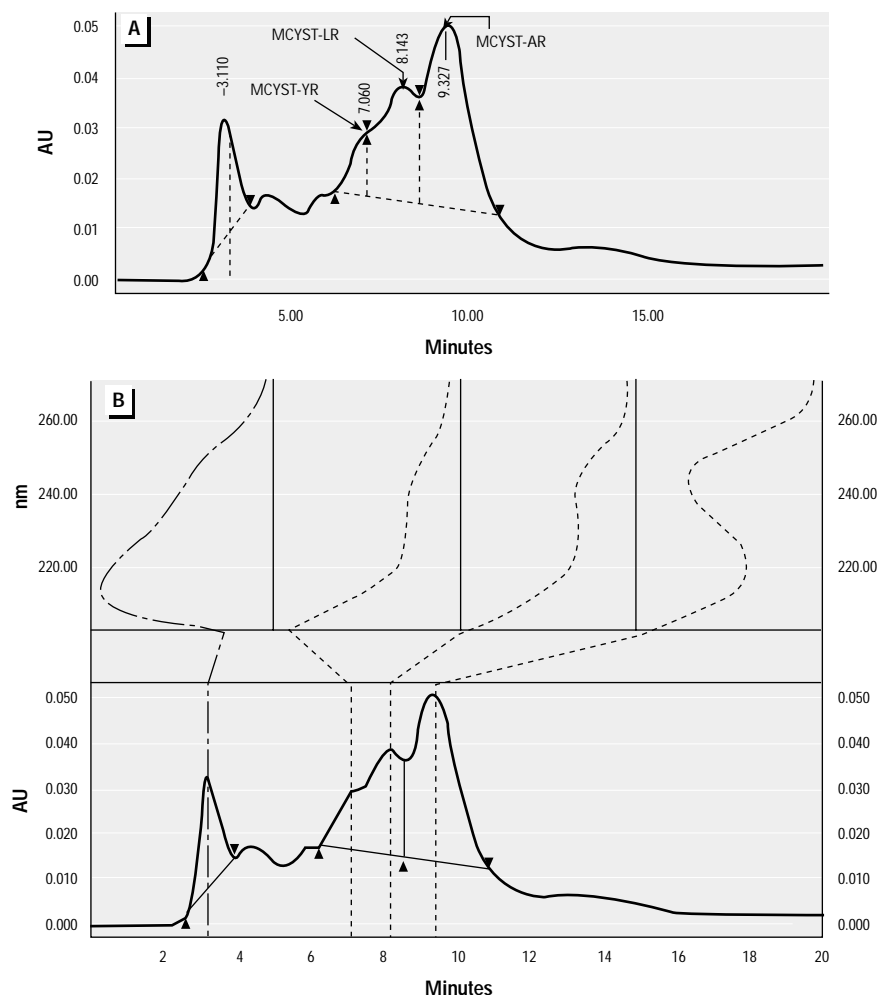
20 mL of 100% methanol) were dried under nitrogen gas and the residue taken up in 1:5 methanol/H<sub>2</sub>O. Due to limited amounts of liver and sera for a given victim, extracts for CYN analyses were the same as those used for microcystin (MCYST) analyses. This extract was filtered and dilutions performed as needed to maintain a response in the linear range of the calibration curve. Analyses were by HPLC/MS/MS using a PE/Sciex API 300 mass spectrometer with turbo-ion spray interface coupled to a Perkin Elmer series 200 HPLC system (Applied Biosystems, Foster City, CA, USA). HPLC separation was achieved on a 150 × 4.6 mm Alltima C18 column (Alltech Associates, Sydney, Australia) run at 35°C. The effluent was split to achieve a flow rate of 0.25 mL/min to the MS. The MS was operated in the multiple-reaction monitoring mode using nitrogen gas as the collision gas. The transition 416.2 to 194 Da was used to determine CYN concentration, and the transition 400.2 to 194 Da was used for deoxy-CYN. The limit of detection with the method was 0.01 ng/g (12).

## Results

**Phytoplankton analyses.** Analysis of phytoplankton from the Tabocas Reservoir at the end of March 1996 demonstrated that toxigenic cyanobacteria were the main phytoplanktonic group, representing 99% of the total density of phytoplankton. The average number of cyanobacterial organisms was 24,500/mL (Table 1). Some cyanobacterial picoplanktonic strains isolated from water samples of Tabocas Reservoir after the outbreak were identified as MCYST producers by HPLC and ELISA (13). However, because the water utility was not making phytoplankton counts at the time of the outbreak, toxic cyanobacteria producing microcystin or other cyanotoxins during the period of intoxication could not be examined. An evaluation of previous years' phytoplankton data showed that cyanobacteria were dominant in the reservoir during the summer months since at least 1990 (Figure 1). The most common genera during this time period were *Microcystis*, *Anabaena*, and *Anabaenopsis* (*Cylindrospermopsis*). Other studies confirm that toxic cyanobacteria, especially *Cylindrospermopsis*, are present in other Brazilian water supplies (14–16). *Microcystis* and *Anabaena* are known producers of microcystins, while *Cylindrospermopsis* can produce cylindrospermopsin. For this reason we analyzed samples for these two cyanotoxins. Although the literature reports the neurotoxins anatoxin-a and a(s) to be produced by *Anabaena* and certain PSPs to be produced by *Cylindrospermopsis* (3), the lack of reported neurotoxic symptoms in most of the Caruaru victims meant that these toxins were not likely present and therefore we did not analyze samples for those toxins.

**Analyses for cyanotoxins.** Examination of carbon, sand, and cation/anion resin exchange filters from the clinic using a polyclonal antibody in an ELISA against microcystin-LR showed microcystin in the microgram per gram range (Table 2). Further examination of methanolic extracts from these carbon, sand, and resins performed by HPLC-electrospray MS/MS provided chemical evidence for CYN contamination in this material. The highest concentration of this hepatotoxic alkaloid was found in the carbon extract (Table 2).

Blood sera and liver from affected and control patients provided by state health officials in Pernambuco and sent through the CDC were examined by ELISA. All patient sera and tissue were positive for microcystins (Table 3). ELISA-guided HPLC-PDA separation of microcystins from liver tissues provided a profile of microcystins present (Figure 2). MALDI/TOF (Figure 3) and ESI (Table 4) MS analysis were used to identify isolated microcystins from liver. Microcystins



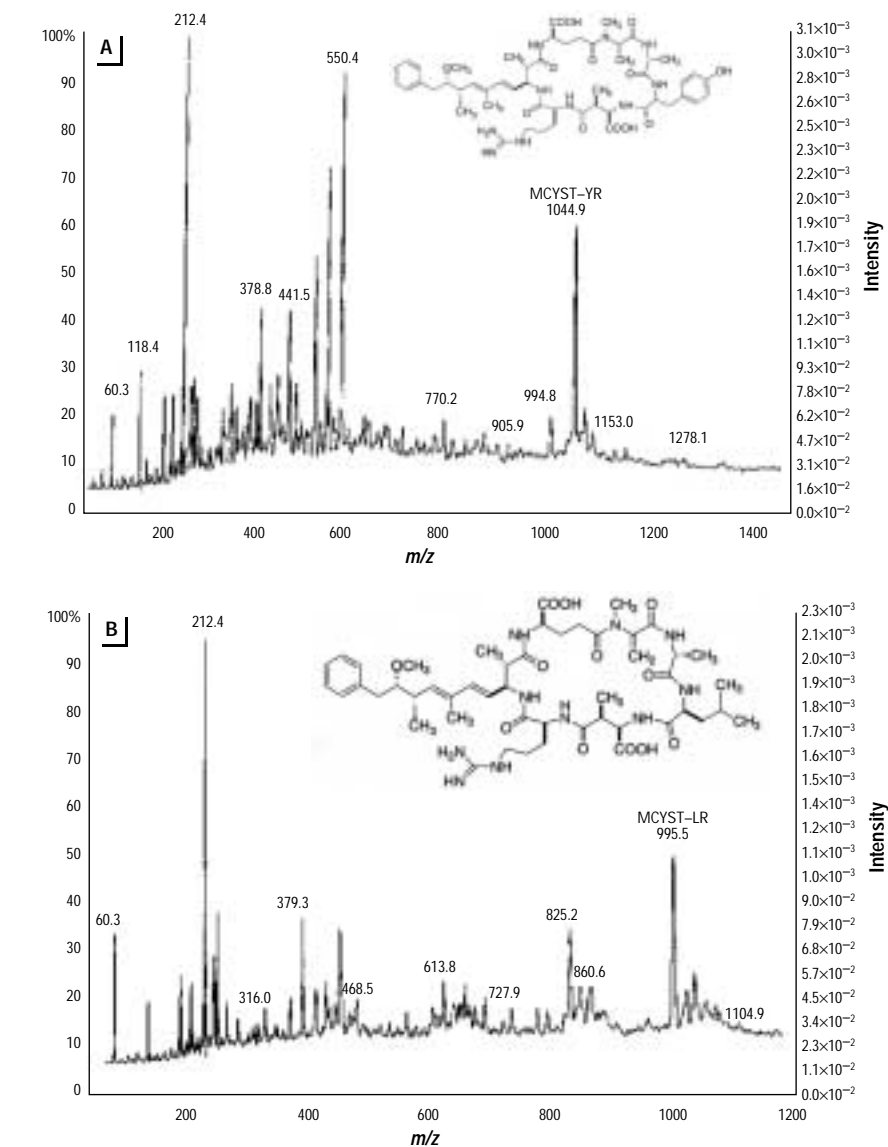
**Figure 2.** HPLC/PDA spectrum from liver extract of a Caruaru dialysis patient. (A) Retention times of labeled peaks correspond to retention times of authentic microcystin-YR, -LR, and -AR standards; detection was done at 238 nm. (B) Microcystin peaks showing their characteristic 238-nm ultraviolet chromophore.



identified were microcystins-YR, -LR, and -AR. MALDI MS analysis of finished water from dialysis center B also showed the presence of these microcystins (data not shown).

Analysis of liver samples for CYN by HPLC-MS/MS did not reveal the parent compound at molecular mass 416 (M+H). These liver extracts had been prepared for MCYST analyses, and the method may not have selected for the more polar alkaloid CYN. Due to limited sample availability, further analyses of remaining liver tissue and sera for CYN is pending while a method is developed for detecting this cyanotoxin in these matrices.

**Calculation of microcystin exposure.** The average microcystin value in 52 liver samples from 39 victims who died from February to December 1996 was 223 ng/g (Table 3). This compares well with values reported in laboratory test animals receiving acute exposure of microcystin-LR (17). ELISA detected 125 ng/g of microcystin-LR in the livers of mice exposed to a lethal intraperitoneal injection of 100 µg/kg (18). We used the average liver concentration of 223 ng/g to estimate the amount of microcystin present in the source water from clinic A. Using an average liver weight for an adult human of 1,500 g gives 335 µg of possible MCYST burden in an average liver from the Caruaru victims. We then used two correction factors that could affect the amount of MCYST in the source water. The first was the amount of MCYST in the liver as a percentage of the total exposure. Laboratory experiments indicate that from 10% to 90% of the total exposure can be found in the liver of mice and rats receiving an acute or lethal dose of MCYST (19,20). Therefore, an application factor of 2 (estimate of 50% uptake in the liver) was used. The second factor was based on the finding that since microcystin is covalently bound to protein phosphatase 1 and protein phosphatase 2A, as much as 80% of the MCYST exposure might not be recognized by the ELISA assay (21,22). Although this estimate is conservative, it means that the MCYST exposure might have been about 7 times higher (2 times and 5 times, respectively). Using this value and dividing by the amount of water used during a typical dialysis treatment (120 L), and assuming 100% dialysis of MCYST across the hollow fiber dialysis filter gives an estimated concentration in the source water of 19.5 µg/L. Although there are several assumptions in estimating this value, it does mean that an environmentally feasible level of MCYST in the source water would have been adequate to cause acute liver failure and death in humans by the intravenous route. If this concentration of MCYST is an accurate estimate, then there was no significant contribution to the



**Figure 3.** MALDI/TOF-MS of liver extract from a Caruaru dialysis patient. (A) HPLC/PDA fractions obtained at retention times at about 7 min showed a peak at  $m/z$  1045,  $[M+H]^+$ , the molecular ion of microcystin-YR. This spectrum also shows the 995 peak of microcystin-LR, which can be explained by the unresolved peaks from the HPLC profile in Figure 2. (B) Fractions collected at 8 min showed a peak at  $m/z$  995 (microcystin-LR) and its fragment ion peak at  $m/z$  860. The  $m/z$  860 peak was due to a loss of Adda  $[PhCH_2CH(OMe)]^+$  residue from the molecular ion,  $[M+H]^+ = 135$ . (C) Microcystin-AR, eluted last (9–10 min) and showed a peak at  $m/z$  954 plus its fragment ion at  $m/z$  818. Peaks observed at the low molecular weight region ( $m/z < 450$ ) are due to the matrix used for the analysis. The six other patient livers analyzed showed a similar pattern. The accuracy of the method was estimated to be within 0.1% (1 mass unit for a molecular weight of 1,000).

toxicity from CYN. This MCYST value is about 20 times higher than the 1 µg/L proposed by the World Health Organization as a safe level for oral consumption of MCYST in drinking water (23).

Histology and pathology of liver tissues revealed a pattern of liver plate disruption identical to that found in previous laboratory animal experiments of microcystin exposure (5,24–26). MCYSTs are potent inhibitors of eukaryotic protein serine/threonine phosphatases 1 and 2A (27,28). Substances that inhibit these protein phosphatases are

considered to be nonphorbol ester-type tumor promoters (29,30). This means that survivors of Caruaru syndrome could be at higher risk for liver cancer and should be monitored (3); however, the degree to which CYN contributed to the deaths of the dialysis patients is less clear. Until we can evaluate whether CYN is present in the victims' livers, we cannot estimate the possible toxicity due to this cyanotoxin. CYN is a hepatotoxic alkaloid first studied from Australian water supplies. In a retrospective study it has been held as the cause of hepatoenteropathy and

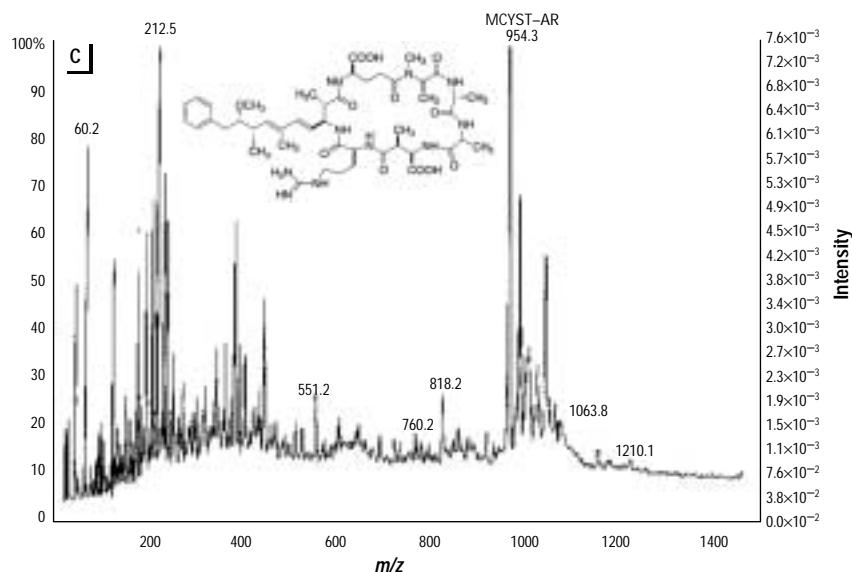


Figure 3. Continued.

**Table 4.** ESI MS fragment ions from a liver analysis for microcystins.

<i>m/z</i>	Fragment ions <sup>a</sup>
135	PhCH <sub>2</sub> CH(OMe) <sup>+</sup>
155	[MdhA-Ala + H] <sup>+</sup>
238	[Co-Glu-MdhA-2H] <sup>+</sup>

<sup>a</sup>These fragment ions are characteristic for microcystins with *m/z* 135 representing a key fragment ion (Adda) used in microcystin screening (34,35). ESI MS was unable to determine the structure of the microcystins present, probably due to interference by salts in the extracts, but it did confirm their presence.

renal damage to 148 indigenous peoples using an Australian water supply (37). Comparison of the Caruaru victims' livers with published studies in animals dosed with CYN did not reveal a pattern indicating that this toxin contributed to the toxicity. Because the victims in Caruaru were undergoing renal dialysis, we cannot evaluate whether CYN contributed to renal toxicity. A better understanding of CYN toxicology and biochemistry, both alone and in combination with MCYST, is needed before we can confirm (or rule out) a contributing toxicity factor from this toxin.

## Discussion

The available biological and chemical evidence supports cyanotoxins from the Tabocas Reservoir water supply as being the major factor in the deaths of dialysis patients at clinic A. One important question was how could lethal levels of MCYST (and possibly CYN) be present in waters receiving standard water treatment procedures including sedimentation, coagulation, filtration, and chlorination? The answer is that center A did not have water directly piped to its facility. Instead, they relied on water that was trucked to the center for treatment in its in-house water system—a process that involved sand filtration, carbon filtration, and cation/anion exchange

filtration, followed by a microfilter. Because removal of chlorine residues was poor, due most likely to inadequate maintenance of the dialysis center in-house water treatment system, center A maintenance staff used water from the city's water treatment system that had received only alum flocculation in order to avoid chlorine residues in the dialysis treatment water. This water, without the full benefit of water treatment, contained high levels of cyanotoxins that were not removed by the small, inadequately maintained, in-house treatment facility of dialysis center A. This center did not use reverse osmosis in its water treatment process (32,33). Reverse osmosis is used in several dialysis centers in Brazil and in the United States and Europe. We conclude that if reverse osmosis had been used, cyanotoxins would have been less likely to pass through the filtration process.

A newer dialysis center (center B) is also present in Caruaru. Center B received water piped from the municipal water plant that is fully treated, except for carbon filtration. No fatalities that could be contributed to Caruaru syndrome occurred at this center. However, several patients at center B were reported to have some symptoms of Caruaru syndrome after the center used trucked water delivered at the end of March. MCYST was identified in the filter system of center B (Table 2) and patients symptomatic for Caruaru syndrome had MCYST in their serum.

One of the questions we have not been able to address is the possibility of repeated exposure to these cyanotoxins. It was not until 7 March 1996, 15 days after the first death, that center A was closed and dialysis patients were sent to other center. Some of these patients were sent to center B, where there was also evidence of exposure to cyanotoxins. Therefore it is possible that multiple

exposures occurred. This makes it difficult to establish a dose–response effect. However, we do think that most of the exposure to cyanotoxins came from one or two shipments to center A, and we estimate that the primary exposure to cyanotoxins was due to one to three dialysis treatments. Another important question is whether there may have been some type of treatment that could have removed or detoxified the exposure from cyanotoxins that these dialysis patients experienced. Although there is no treatment for MCYST or CYN exposure, another study treated one clinic A patient with charcoal hemoperfusion (7). Serum analyses of the victim before and after hemoperfusion showed a reduction of MCYST levels, as measured by HPLC-photodiode array detector and ELISA, but levels rose after treatment, and the patient died about 1 week after the charcoal hemoperfusion. Liver samples from this victim were analyzed in our study, and the level was 250 and 430 ng/g (two separate liver samples).

We have learned significant information concerning human exposure from MCYST and CYN in this first confirmed outbreak of human poisoning involving cyanotoxins. Because many of the world's reservoir- and lake-based water supplies are subject to increasing levels of nutrients, it is highly probable that repeat episodes of cyanotoxin poisoning will occur unless measures are taken to better understand their role in water-based disease. These measures should consist of programs for watershed management to reduce nutrient inputs, cyanotoxin monitoring programs to alert authorities to the presence of cyanotoxins, and improvements in water treatment techniques to reduce or to remove cyanotoxins from potable water supplies.

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