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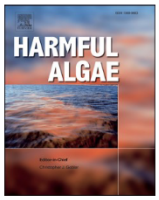

Can cyanotoxins explain the clinical features of the thermal crisis in balneotherapy?

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ABSTRACT

Microbial biofilms communities in mineral waters and hot springs have a particular composition with species belonging to different groups such as epsilonproteobacteria and gammaproteobacteria or different siderobacteria and other chymoautrophic organisms, in addition to certain bacillaryophytes, chlorophytes and especially cyanobacteria. Balneotherapy can cause adverse reactions to the usual doses of application of treatments, that consists of a non-specific clinical picture, the so-called "thermal crisis" or "balneointoxication". Despite its clinical similarity (gastric discomfort, hepatic congestive outbreaks, cutaneous reactions, etc.) with that observed in acute cyanotoxin poisonings, thermal crisis has never been associated with the abundant growth of potentially toxic cyanobacteria in the mineral water sources. The aim of this work was to verify the hypothetical involvement of cyanotoxins in this clinical picture. Samples from mostly sulphurous water sources, with thermal characteristics ranging from cold to hyperthermal waters were analysed. ELISA (both in solution and in cellular matrix samples), LC-ESI-HRMS (in cellular matrix samples), and analysis of potential toxicity by means of a standardized bioassay were carried out. The toxic effect observed in the toxicity bioassays in one third of the sources may be related to the existence of microcystins and nodularins and even with other cyanobacterial peptides detected. In addition, several responses observed in the toxicity analyses reflect a pattern, probably linked to a type of hormetic response (hormesis is an adaptive response to low levels of stress, characterized by a biphasic dose-response curve).

1. Introduction

Microbial mats and biofilms communities in mineral waters and hot springs have a diversity and specific composition that is linked to the geochemical nature of the water source. Under these conditions, the most abundant taxonomic groups are epsilonproteobacteria and gammaproteobacteria or different siderobacteria and other chymoautrophic organisms, in addition to certain bacillaryophytes, chlorophytes and especially cyanobacteria (Engel et al., 2004; Camacho et al., 2005; Ward et al., 2012). Within cyanobacteria, morphospecies of genera such as *Chroococcus, Fischerella, Merismopedia, Phormidium, Oscilatoria* or *Microcystis* can be identified, although morphological identification masks the recognition of species with notable adaptive differences. Abundance of species groups and community structure of benthic cyanobacteria in mineral waters and hot springs depends on temperature changes, dissolved sulphide, and metabolic requirements.

Thermal Medicine or Medical Hydrology can be defined as the study of the mineral-medicinal, marine and ordinary drinking waters, and their actions on the human organism in the state of health and illness. The Crenotherapy or Balneotherapy, deals, therefore, with the study of the mineral-medicinal waters and its possible therapeutic or preventive use (Gutenbrunner et al., 2010). The Declaration of St. Petersburg on the Thermal Medicine (Storozhenko et al., 2013) defines the thermal medicine as "an organized system that provides benefits for the health in the spas by means of the use of mainly natural therapeutic resources, the climatic properties and the education and the treatment of the patients,

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Table 1
Physicochemical characterization of the thermal sampling sites analysed.

Sampling sites	Prov	T (°C)	Classif	pH	Conduct (μS/cm)	Sulph	Fluor	Silic	Bicarb	Sod	Litin	Rad
Site 01	Co	17.5	Cold	9.29	483.7	x				x		
Site 02	Co	17.9	Cold	8.75	259.1	x			x	x		x
Site 03	Lu	15.0	Cold	9.16	365.8	x	x		x	x		x
Site 04	Ou	16.6	Cold	8.00	157.2	x	x					
Site 05	Ou	18.2	Cold	9.09	259.6	x						
Site 06	Ou	18.0	Cold	8.73	190.9							
Site 07	Ou	25.6	Hypoth	8.72	250.7				x	x		
Site 08	Ou	27.8	Hypoth	8.59	278.0	x			x			
Site 09	Ou	41.1	Hyperth	8.77	634.0	x	x	x	x	x		
Site 10	Ou	31.8	Hypoth	8.59	577.0	x	x		x	x	x	
Site 11	Ou	37.0	Mesoth	8.75	452.0		x	x	x	x		
Site 12	Ou	58.4	Hyperth	8.45	1080.0		x	x	x			
Site 13	Ou	57.4	Hyperth	8.29	1754.0		x	x			x	
Site 14	Ou	40.9	Hyperth	7.91	1577.0			x	x	x		x
Site 15	Ou	41.0	Hyperth	8.26	1343.0	x			x	x		
Site 16	Ou	63.8	Hyperth	9.22	754.0				x	x		
Site 17	Po	49.7	Hyperth	8.36	526.0	x	x	x		x	x	
Site 18	Po	43.2	Hyperth	8.92	1528.0				x	x		

Prov: province; Co: A Coruña; Lu: Lugo; Ou: Ourense; Po: Pontevedra; Classif: classification; Hypoth: hypothermal; Hyperth: hyperthermal; Mesoth: mesothermal; Conduct: conductivity; Sulph: sulphurated; Fluor: fluoridated; Silic: silicate; Bicarb: bicarbonate; Sod: sodium; Litin: litinic; Rad: radioactive.

Table 2
Microcystins and related cyanobacterial peptides detected by LC-ESI-HRMS in the samples analysed.

Reference samples	Number of MCs identified	Proposed identification
Site 01	1	CP-2
Site 02	4	CP-2, MC-Waba or analogue ($[M+H]^+$, tR 9.7 min), MC—OiaA, MC—OiaAba
Site 03	1	CP-2
Site 04	1	MC-Waba or analogue ($[M+Na]^+$, tR55.1 min)
Site 05	3	CP-2, MC—OiaA, MC—OiaAba
Site 06	1	CP-2
Site 07	1	CP-2
Site 08	3	CP-1, CP-2, MC-1
Site 09	1	CP-2
Site 10	1	MC-KynA
Site 11	1	CP-2
Site 12	1	CP-2
Site 13	3	CP-2, MC—OiaA, MC—OiaAba
Site 14	1	[L-Ser7]MC-E(OMe)E(OMe)
Site 15	2	CP-2, MC-2
Site 16	1	CP-2
Site 17	1	CP-2
Site 18	–	No signal

MC: Microcystin; CP: Cyanobacterial Peptide (to our knowledge, these signals are not referenced in the literature. According to the observed HRMS mass spectra, they could be microcystins or related peptides).
MC-1: [Asp3]MC-LF or MC-VF (two microcystin with identical *m/z* but different hypothetical retention time). As there are no commercial standards of these microcystins, their retention time and identity cannot be unequivocally confirmed.
MC-2: [D-Asp3, Dhb7]MC—HtyR, [D-Asp3]MC—HtyR, [Dha^7]MC—HtyR or MC-RY (four microcystin with identical *m/z* but different hypothetical retention time). As there are no commercial standards of these microcystins, their retention time and identity cannot be unequivocally confirmed either.

Table 3
Nodularins detected by LC-ESI-HRMS in the samples analysed.

Reference samples	Number of NODs identified	Proposed identification
Site 01	–	No signal
Site 02	–	No signal
Site 03	–	No signal
Site 04		No signal
Site 05	–	No signal
Site 06	–	No signal
Site 07	–	No signal
Site 08	–	No signal
Site 09	–	No signal
Site 10	–	No signal
Site 11	–	No signal
Site 12	1	[seco-2/3]NOD-R
Site 13	1	[seco-2/3]NOD-R
Site 14	1	[seco-2/3]NOD-R
Site 15	1	[seco-2/3]NOD-R
Site 16	2	[seco-2/3]NOD-R, [(6Z)-Adda3]NOD-V or NOD-R
Site 17	–	No signal
Site 18	–	No signal

NOD: Nodularin.
[*seco-2/3*]NOD-R, [(6Z)-Adda3]NOD-V or NOD-R are NODs with identical *m/z* but different hypothetical retention time. Nor are there commercial standards of these nodularins, their retention time and identity cannot be unequivocally confirmed.

promoting the healthy life, prevention and rehabilitation".

The mechanism of action of medicinal mineral waters includes specific actions, a direct consequence of their mineralization and the route of administration, and non-specific actions, related to the capacity of response to stimuli, the psychotropic effect or placebo and possible abnormal reactions. The most frequent routes of administration are the oral route, the atmiatric route and the topical route (Maraver, 2003). Megías (2015) described how the usual doses of application of treatments can cause adverse reactions. Some of these reactions are related to the exacerbation of chronic processes and others depend on the individual response. These include the so-called "thermal crisis", which consists of a non-specific clinical picture that presents gastric discomfort, diarrhoeal performances, hepatic congestive outbreaks, cutaneous reactions, etc. Despite the clinical similarity with that observed in acute cyanotoxin poisonings, thermal crisis has never been associated with the abundant growth of potentially toxic cyanobacteria in the water sources. The aim of this work was to verify the hypothetical involvement of cyanotoxins in this clinical picture.

2. Materials and methods

2.1. Materials

For this work, 21 samples of 18 sources, representative of the different mineral-medicinal waters present in Galicia (Northwest Spain) and mostly used for balneotherapy, were analysed. Samples (water and cell matrix) were taken during September 2018. Their main

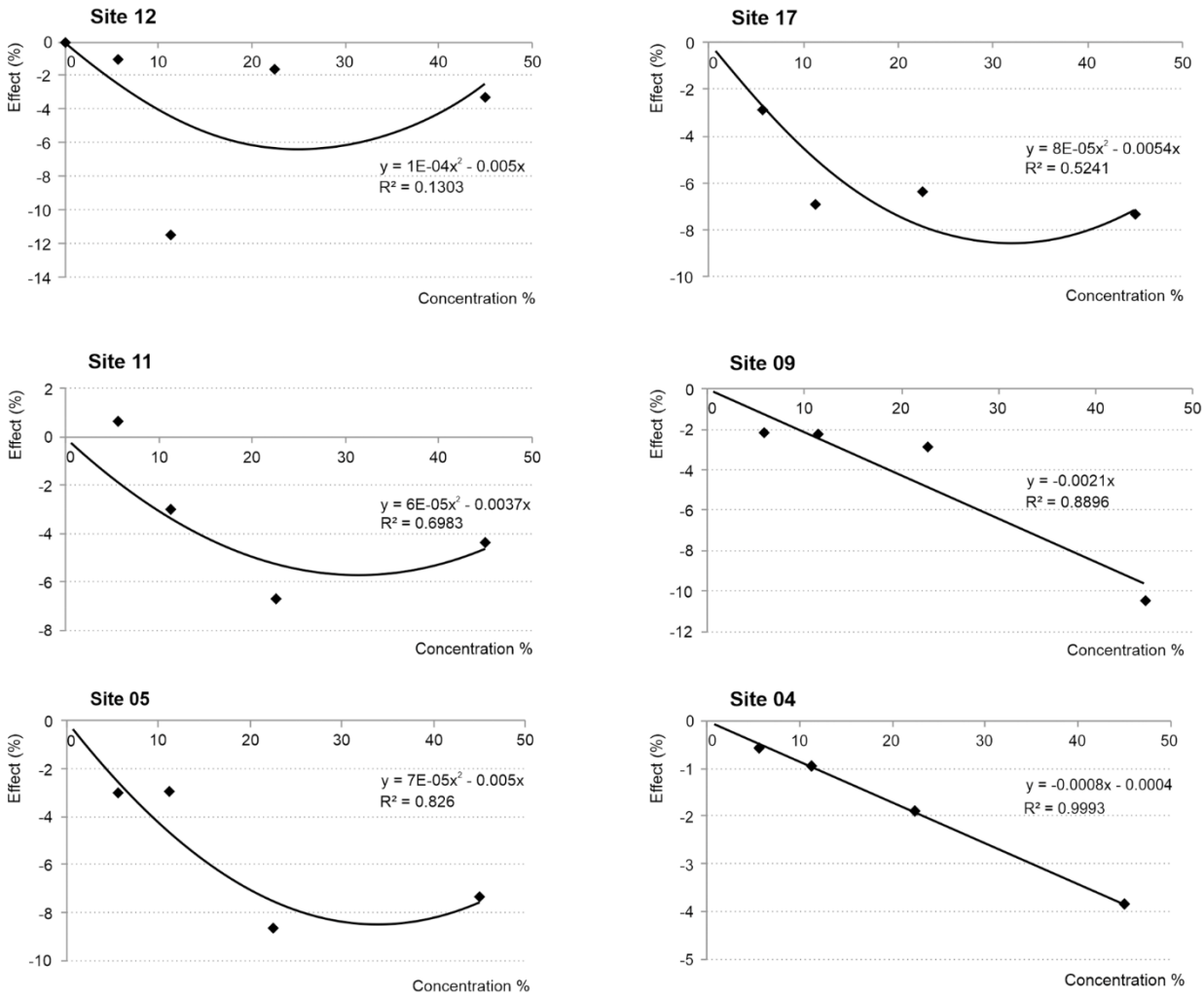

Fig. 1. Water sources that showed toxicity at all concentrations.

physicochemical characteristics are summarized in Table 1.

Water samples were collected in sterile polypropylene bottles with a capacity of 250 ml. Bottles were filled without leaving an air chamber, to keep possible volatile materials present in the solution. They were transported to the laboratory, refrigerated at 5 ± 1 °C, for subsequent analysis. Temperature, pH, and conductivity values were taken on site with a multiparametric probe. The cell matrix samples were collected with tweezers and transferred to 60 ml polypropylene jars. Once in the laboratory they were filtered with fibreglass filters (0.45 μm; Millipore, Bedford, MA, USA).

2.2. Standards and reagents

All reagents employed were of analytical or high-performance liquid chromatographic (HPLC) grade. Acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Formic acid was from Panreac (Montcada i Reixac, Barcelona, Spain). High purity water produced with a Milli-Q Synergy UV system (Millipore, Bedford, MA, USA) was used. MC-LA, -LR, -RR, -WR, -YR, and nodularin standards were purchased from Alexis Biochemicals (San Diego, CA, USA). Stock standard solutions of each analyte (100 or 500 μg mL^{-1}) were individually prepared by weight in methanol and stored at −20°C. Intermediate solutions were prepared weekly from the stock standard solution by appropriate dilution in methanol. MC-LF, -LW and -LY 5–10 μg mL^{-1} methanolic solutions were supplied from Sigma–Aldrich (St Louis, MO, USA). MC-dmRR 10 μg mL^{-1} in methanol was acquired from Cyano Biotech GmbH (Berlin, Germany). Finally, MC-dmLR 10 μg mL^{-1} methanolic solution was purchased from DHI (Hørsholm, Denmark). Mixed calibration standard solutions of all microcystins were prepared daily. Nitrogen (purity > 99.999%) supplied by Air Liquide (Madrid, Spain) was used for the ESI source and as a collision-induced dissociation (CID) gas in the Orbitrap mass spectrometer.

2.3. Sample extraction

For LC-ESI-HRMS analysis, algal samples collected in glass fibre filters (0.45 μm; Millipore, Bedford, MA, USA) were frozen-thawed threefold in order to cell lysis and extracted according to Barco et al. optimized methodology (Barco et al., 2005). Freeze-dried cells (25 mg) from each sample were extracted three times by sonication for 15 min with 3 mL of methanol acidified (0.16% HCOOH). The three extracts were consecutively centrifuged (10 min at 3000 rpm) and the supernatants were concentrated together to dryness with a stream of nitrogen and temperature (40 °C). Finally, the extracts were re-suspended in 500 μL of methanol and were filtered through Uptodisc PTFE syringe filters (4 mm, 0.20 μm; Interchim, Montluçon, Cedex, France).

Before LC-ESI-HRMS analysis, enzyme-linked immunosorbent assays (ELISA) were conducted, both in solution and in the cellular matrix of samples. The extraction of the sestonic phase prior to the ELISA assays was carried out as follows: first 200 ml of water was filtered, the filter (with the cells retained on it) was placed in a tube with 10 ml of cold 90% methanol, then the cells were broken up by sonication, finally the

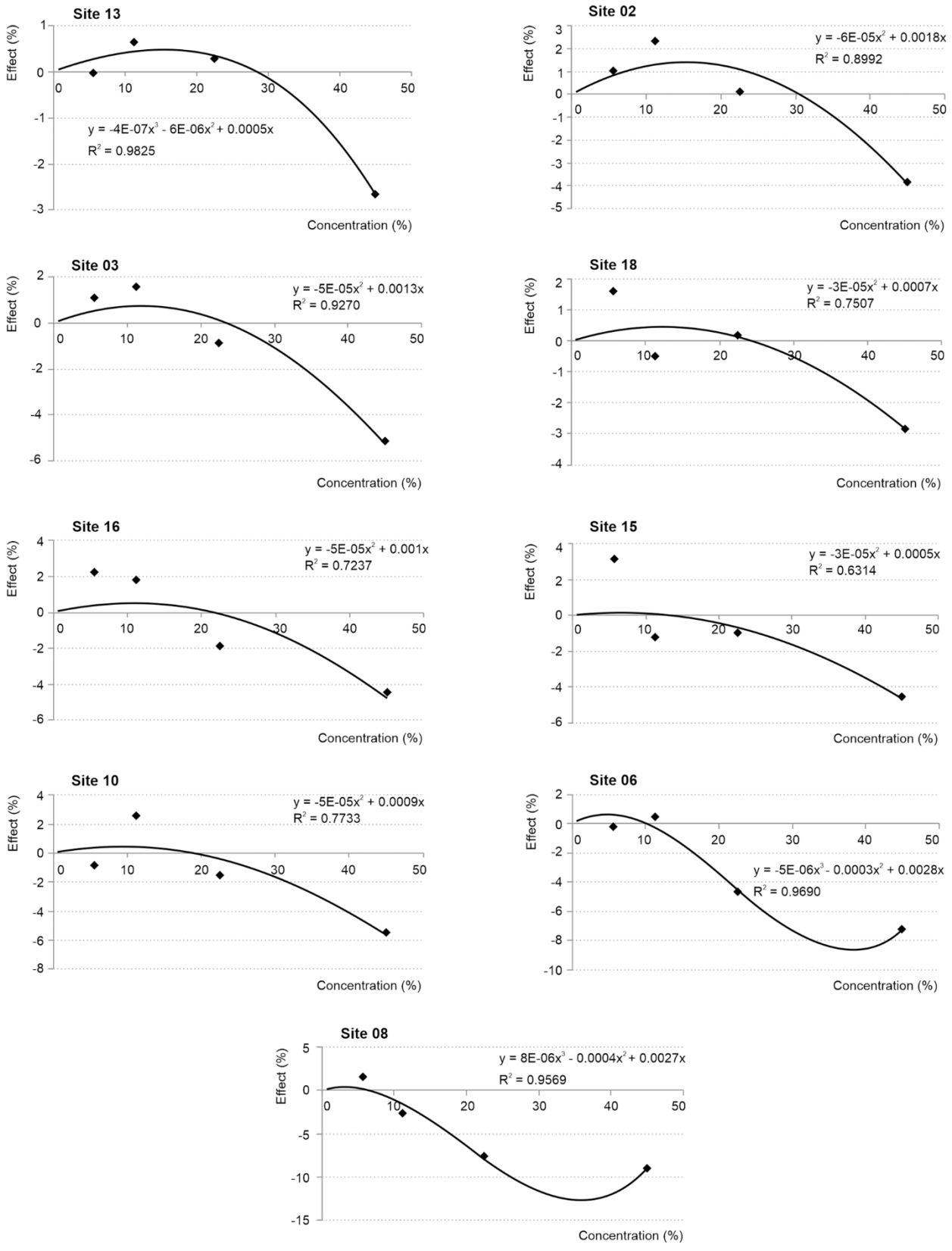

Fig. 2. Water sources with hormetics dose / response models associated with a biological dysfunction or toxic damage.

10 ml of methanol containing the extracted sestonic phase was filtered adding 190 ml of distilled water to keep the proportion of methanol to a minimum thus it does not interfere with the analyses.

2.4. Immunoassay

ELISA assays were carried out with the Abraxis® Microcystins Tube Kit Enzyme-Linked Immunosorbent Assay for the Determination of

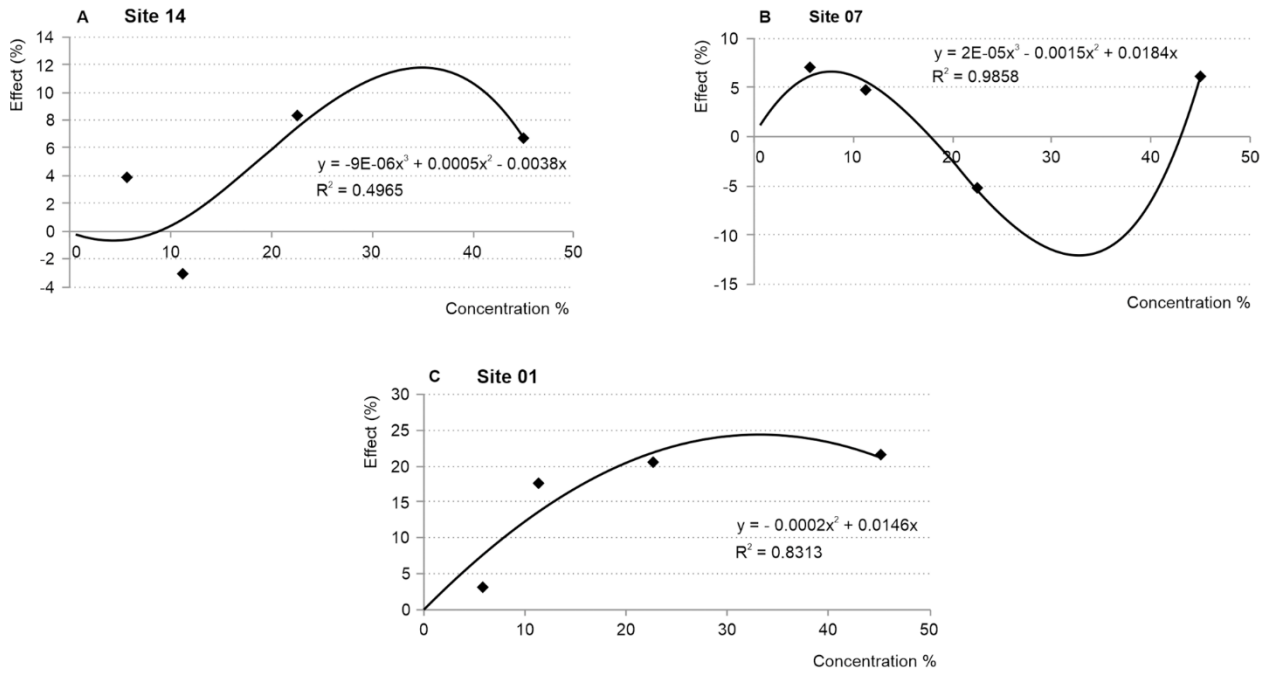

Fig. 3. A) Water sources with "J" shaped hormetic models. B) Bimodal curve with luminescence stimulation below and above the average dose. C) Growth in the entire dosage range.

microcystins and nodularins in Water Samples (Product No. 520012A). This test is a direct competitive ELISA based on the recognition of microcystins, nodularins and their congeners by specific antibodies. Microcystins, if present in the sample, compete with a microcystin-enzyme conjugate for the binding sites of a primary detection antibody in solution (rabbit anti-microcystins antibody). Then a second antibody is added (anti-rabbit) that binds to the primary antibody in an inverse relationship to the original concentration of microcystins in the sample. After a washing step and addition of the substrate solution, a colour signal is generated. The intensity of the blue colour is inversely proportional to the concentration of microcystins present in the sample. The colour reaction is stopped after a specified time and the colour is evaluated using an ELISA reader. The concentrations in the samples are determined by interpolation using the standard curve constructed with each run. The results are expressed as microcystin-LR equivalent concentrations (Limit of detection = 0.2 μg L^{-1}).

2.5. LC-ESI-HRMS analysis

An Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer equipped with heated electrospray source (H-ESI II), a Surveyor MS Plus pump and an Accela Open AS autosampler kept at 6 °C (Thermo Fisher Scientific, San Jose, California) were used for the LC-ESI-HRMS analysis (Flores and Caixach, 2015). The chromatographic separation was performed on a reversed-phase Phenomenex Luna C18(2) column (150 mm × 2.0 mm, 5 μm). The mobile phase was composed of Milli Q water as solvent A and acetonitrile as solvent B, both containing 0.1% (v/v) formic acid at a flow rate of 200 μL min^{-1}. The linear gradient elution program for the analysis was: 10–30% B 10 min, 30–35% B 20 min, 35–55% B 15 min, 55% B 5 min, 55–90% B 2 min, 90% B 3 min and return to initial conditions for re-equilibrate (10% B 10 min). The injection volume was 10 μL and the total duration of the method was 65 min.

Analyses were carried out in ESI positive ionization mode and the instrument was daily calibrated. Nitrogen was used as sheath gas, auxiliary gas and collision gas. The source parameters and voltages used were: capillary temperature of 250 °C, heater temperature of 30 °C, sheath gas flow rate of 42 psi, auxiliary gas flow rate of 10 (arbitrary units) and sweep gas flow rate of 0 (arbitrary units), spray voltage of 4.25 kV, capillary voltage of 35 V, tube lens voltage of 186 V and skimmer voltage of 35 V. Data was acquired simultaneously in full scan and all ion fragmentation (AIF) modes (at 30 and 70 eV). The mass range was m/z 400–1200 in full scan and m/z 60–1200 in AIF mode. The automatic gain control (AGC) was set as "balanced" (1e6) with a maximum injection time of 250 ms. High resolution defined as R: 50,000 (m/z 200, 2 Hz, FWHM) was set in all scan events. External calibration and mass accuracy expressed as parts per million (ppm) were used. Mass accuracy in all mass range (m/z 60–1200) was < 5 ppm. Therefore, a maximum of ±5 ppm extraction window was allowed for peak identification. Data was processed with Xcalibur 2.1 and Trace Finder EFS 3.3 software's (Thermo Fisher Scientific, Bremen, Germany). A home-made database of 157 microcystins, 10 nodularins, cylindrospermopsin and 29 cyanobacterial peptides (10 anabaenopeptins, 8 oscillapeptins, 4 microviridins, 2 agardhipeptins, 2 oscillapeptilides, oscillamide Y, oscillaginin and oscillacyclin) was used for identification purposes. Ion species $+H$, +NH4, +Na; $+K$ and +2H were considered. The identification of analytes was performed according to their experimental exact mass (m/z), accurate isotopic pattern, evidence from the fragmentation data and the retention time. The combination of high resolution and restrictive criteria was crucial for identification of target and unknown compounds. To ensure the reliability of the identifications, convenient HRMS and accuracy were employed in addition to the following criteria: elements considered were restricted in accordance with microcystin, nodularin, cylindrospermopsin and cyanobacterial peptides molecular formulae (C:10–60, H:15–90, O:0–20, N:0–20, S:0–1); the experimental isotopic pattern was matched regarding the theoretical in silico isotopic pattern (≥ 70%); and, the charge, the ring plus double bond equivalents (RDBE) and nitrogen rule were taken into account. Finally, the database include diagnostic fragment of each bioactive substance from literature. The AIF acquisition mode by Exactive mass spectrometer occurs in a special collision chamber (High energy Collision Dissociation, HCD cell) essentially comparable with those used in QqQ but without precursor mass selection. A comprehensive chromatographic separation allowed a reliable HCD fragmentation without isolating the precursor. The positive identification of suspect analytes was confirmed by detection of diagnostic fragments in AIF spectra and chromatogram such as m/z

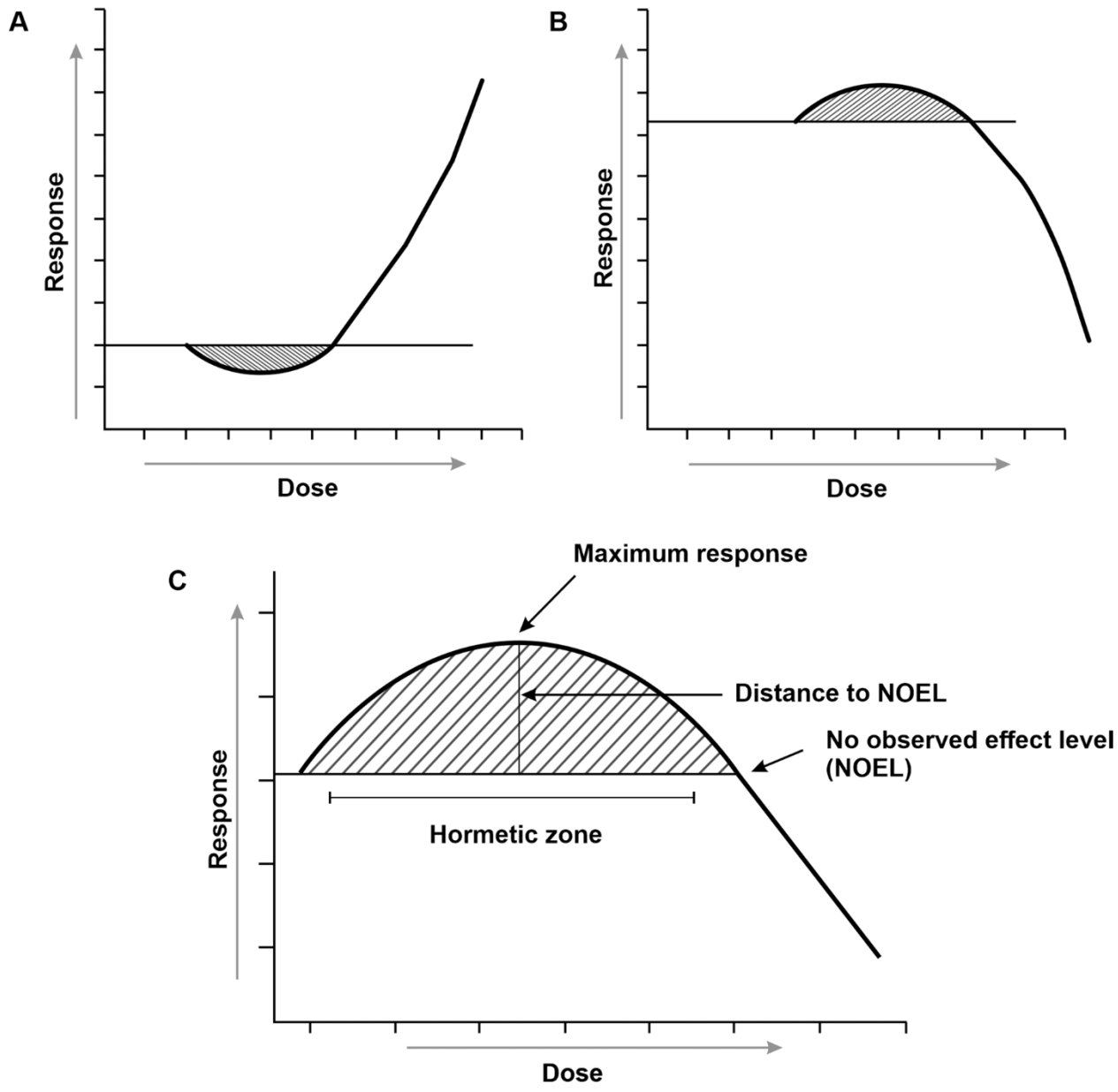

Fig. 4. Biphasic dose-response hormetic curve. A: "J" shaped curve representing a normal biological function such as growth B: inverted "U" curve associated with biological dysfunction or toxic damage. C: detail of the hormetic zone in the inverted "U" curve. Modified from López-Diazguerrero et al., 2013.

135.0804, characteristic Adda fragment, for microcystins. The fragmentation spectra of all signals were obtained as structural fingerprint for confirmation purposes.

2.6. Toxicity test

Analysis of potential toxicity were carried out by means of a standardized bioassay (standard method UNE-EN ISO 11,348–3). This test uses a specific clonal strain of bioluminescent bacteria (*Vibrio fischeri*) prepared in a unique lyophilized vial format as the toxicity indicator organism, and a Microtox® M500 as screening tool that includes an incubator, a luminometer and a data analysing software package. This is a self-calibrating temperature-controlled photometer that measures the relative toxicity of a sample as a percentage of bioluminescence inhibition.

For the elaboration of dose/response curves, in addition to the control, four dilutions of each sample were made at: 45.5%; 22.75%; 11.38% and 5.68% and incubated for 30 min, at 15 ± 0.5 °C. The analysed samples consist of water taken directly from the thermal sources. A negative control (diluent only) was included in all the performed analyses.

3. Results

3.1. Target analysis of nodularin and microcystins

Samples were analysed with both enzyme linked immunosorbent assays (ELISA) and liquid chromatography coupled to electrospray ionization high resolution mass spectrometry (LC-ESI-HRMS). ELISA tests are widely employed as screening tool for the analysis of microcystins. As more fully described in Section 2.4, ELISA assay is a colorimetric method that uses specific antibodies to detect the presence of characteristic amino acid Adda of microcystins. This bioassay is a sensitive, fast, easy-to-use and economically affordable method. Unfortunately, it is unselective toward specific microcystin variants, so results are usually expressed as MC-LR equivalents. ELISA tests do not respond

in the same way to the different variants of microcystins. Some ELISAs only respond or have been tested for the MC-LR. Also, they can respond to other toxins (false positives) and not all microcystin variants have the same analytical response (false negatives). LC—HRMS is a selective and specific methodology that can provide reliable detection, differentiation and quantification of all cyanobacterial metabolites. Most commonly, in the analysis of microcystins, first an ELISA screening is done and then some samples are selected and analysed by LC—HRMS. Therefore, LC—HRMS and ELISA results are complementary.

Results of ELISA test were negative (Limit of detection = 0.2 μg L^{-1}), both in the solution and the cellular matrix samples. LC-ESI-HRMS analysis of the cellular matrix samples (Limit of Detection = 0.01–0.05 μg L^{-1}) did not show nodularin or any of the microcystins (MCs) for which standards are available (MC-dmRR, RR, dmLR, YR, LR, WR, LA, LY, LW, LF).

Table S1 shows the limit of detection and quantification of the target analysis by LC-ESI-HRMS of individual microcystins and nodularin.

3.2. Suspects screening of microcystins and nodularins

A suspect screening of cyanotoxins by LC-ESI-HRMS, based on a home-made database of 157 microcystins, 10 nodularins, cylindrospermopsin and 29 cyanobacterial peptides previously described in the literature, was performed (Sivonen and Jones, 1999; Furey et al., 2008; Del Campo and Ouahid, 2010; Bortoli and Volmer, 2014; Bogialli et al., 2017; Spoof and Catherine, 2017; Natumi and Janssen, 2020). Many authors have proved a gradual increase in the number of microcystin variants described. In the latest comprehensive lists of microcystins, Furey et al. (2008) compile 77 microcystin homologs, Del Campo and Ouahid (2010) describe 87 and, subsequently, Bortoli and Volmer (2014) tabulate 94 variants. Additionally, cyanobacterial peptides are also receiving increasing attention due to high frequency of their detection associated with cyanobacterial blooms (Bogialli et al., 2017; Natumi and Janssen, 2020). Tables 2 and 3 summarize the microcystins, nodularins and related cyanobacterial peptides (CPs) identified by LC-ESI-HRMS in our samples. Signals are not referenced in the literature, have been identified as cyanobacterial peptide. According to the observed HRMS mass spectra, they could be microcystins or related peptides. MC-Waba signal was detected with different retention times in site 02 and site 04 samples. Since there is no standard to this microcystin, it is not possible to confirm which signal is MC-Waba's. The alternative signal would be from an analogue microcystin, but with different structural conformation. The signal referred as CP-2 in Table 2 was detected in 14 samples (all samples except sites 04, 10, 14, and 18) and it was confirmed by HRMS as a signal from a cyanobacterial peptide but with more unsaturations than analogous microcystins. The presence of MC—OiaA and MC—OiaAba in three samples (sites 02, 05 and 13) it is also noteworthy. In addition, [seco-2/3]NOD-R was detected in five samples (sites 12, 13, 14, 15 and 16).

In the present study, to ensure the reliability of the identification, convenient HRMS and accuracy were employed in addition to the criteria described in Section 2.5. The combination of high resolution and restrictive criteria was crucial for the identification of toxins. The list of all tentatively identified cyanopeptides in algal samples by HRMS analysis is reported in Table S2. This table includes retention times, ion signal type, proposed molecular formula, theoretical m/z, samples where each signal was detected, mass accuracy of measures and its isotopic pattern score. Notably identification in all samples was performed with excellent accuracy in the exact mass measurements of analyte m/z signals (in more than 92% of the identifications the mass accuracy was < 1.0 ppm, and it never exceeded 3.3 ppm) and the experimental isotopic profile (IP) matched with the theoretical one (% IP > 85, only three species showed an IP of 74%). Furthermore, elements in use, ring plus double bond equivalents (RDBE) and nitrogen rule were corresponded to analytes identified. Finally, characteristic fragments of cyanopeptides such as Adda fragment (m/z 135.0804) of microcystins were checked in their experimental all ion fragmentation (AIF) spectra. The Adda group is characteristic of microcystins; all microscystin variants have in common the presence of this specific amino acid in their structure. As is known, the conjugated diene of the Adda group contributes to the microcystin toxicity. Alternating full scan and AIF acquisition modes allows quick and easy detection of signals that can be identified as cyanobacterial metabolites, extracting from the AIF chromatogram the exact mass (m/z) of its specific fragments. The fragmentation by Exactive mass spectrometer occurs in a special collision cell by high collision dissociation (HCD) without precursor mass selection (AIF mode). The diagnostic fragment of m/z 135.0804 was observed in all fragmentation spectra of detected signals. As an example, Figures S1 and S2 show the extracted ion chromatogram (XIC) and mass spectra of cyanotoxins detected by LC-ESI-HRMS in sample of site 02 . The extracted ion chromatogram signal of analyte (m/z) in full scan acquisition mode could be usedfor quantification purposes. The cyanotoxins detected were not the usual ones, but these signals had been previously described in the literature. Unfortunately, there are no standards for the proposed cyanotoxins and therefore, their quantification has not been done.

3.3. Toxicity test

The analytical approach used for toxicity analysis in this work is a commonly used method for waters containing biological toxins or for bioreactivity tests. Due to its characteristics, this method eliminates the effect of the physical factors. Our results point to different types of response in toxicity bioassays. Thus, six of the water sources analysed (Fig. 1) showed toxicity at all concentrations; ten reflected hormetics dose/response models, nine of them with a stimulation of bioluminescence at low doses and subsequent inhibition at increasing doses, inverted "U" curves associated with biological dysfunction or toxic damage (Fig. 2) and one with a J-shaped response (Fig. 3A); in one case (Fig. 3B) a bimodal curve was obtained with stimulation of luminescence below and above the medium dose and finally, in a single case the growth of *Vibrio fischeri* was stimulated over the whole dosage range (Fig. 3C).

4. Discussion

Balneotherapy can produce local or generalised physiological responses in the organism, which originate both in the physical mechanisms, related to temperature and hydrodynamic pressure, and in the chemical or biological properties of water (Gomes et al., 2013; Gálvez et al., 2018). Most of the sources analysed here are sulphur waters, slightly or moderately mineralized. Although sulphur is toxic to most microorganisms with oxygenic photosynthesis (Castenholz, 1976; Castenholz and Utkilen, 1984), some cyanobacteria are common or even dominant in sulphur-rich environments (Camara, 1950; Van Gemerden, 1993; Stal, 1995; Camacho et al., 2000; Ward et al., 2012).

The concept of thermal crisis, thermal reaction, bath reaction or balneointoxication has been controversial for a long time, and there is a clear lack of consensus about what should be considered a thermal crisis and its meaning (Megías, 2015). It has been assumed that the frequency of the thermal crises relies on the mineralization and the thermality of the water sources. Even the so-called "yuatari" (balneophenomenon, bath reaction or balneointoxication) has been interpreted as a phenomenon that develops in the psychosomatic confusion resulting from the sudden release of tension after the start of balneotherapy (Nagata et al., 2007, 2014).

The toxicity bioassays carried out in this work show a toxic effect in one third of the water sources. In addition, the suspects screening of microcystins, nodularins and related cyanobacterial peptides by HRMS were also positive for these samples. Thus, for the first time, thermal crises in balneotherapy are possibly associated with the presence of cyanobacteria.

The detected microcystins are not the most commonly detected or studied. Unfortunately, there are no standards for the proposed cyanotoxins and their quantification has not been done. There are no toxicity studies for these unusual MCs either. As previously mentioned, it has to be considered that, the conjugated diene of the Adda group contributes to the microcystin toxicity. Therefore, knowledge about its toxicity and appearance in thermal waters would be highly valued.

Finally, it is remarkable that the response observed in the toxicity analyses reflects a pattern most probably linked to a type of hormetic response. This is reinforced by the fact that the thermal crisis usually occurs between the third and eighth day of treatment and rarely before or after (Megías, 2015). Hormesis is an adaptive response to low levels of stress, characterized by a biphasic dose-response curve (see Fig. 4) (Renner, 2004; López-Diazguerrero et al., 2013; Calabrese, 2013).

Author contributions

All authors have read and agree to the published version of the manuscript. Conceptualization, Fernando Cobo; Data curation, Fernando Cobo, Sandra Barca, Cintia Flores, Josep Caixach, M. Carmen Cobo and Rufino Vieira-Lanero; Formal analysis, Sandra Barca and Cintia Flores; Investigation, Fernando Cobo, Sandra Barca, Cintia Flores, Josep Caixach, María del Carmen Cobo and Rufino Vieira-Lanero; Methodology, Fernando Cobo and Josep Caixach; Software, Rufino Vieira-Lanero; Supervision, Fernando Cobo and Josep Caixach; Validation, Fernando Cobo and Josep Caixach; Visualization, Fernando Cobo; Writing – original draft, Fernando Cobo, Sandra Barca, Cintia Flores and María del Carmen Cobo; Writing – review & editing, Fernando Cobo, Sandra Barca, Cintia Flores, Josep Caixach, María del Carmen Cobo and Rufino Vieira-Lanero.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2022.102240.

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