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RESEARCH ARTICLE

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# Use of synchrotron radiation X-ray fluorescence and X-ray absorption spectroscopy to investigate bioaccumulation, molecular target, and biotransformation of volcanic elements

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Because environment pollutants have a strong impact on ecosystems, including human health, methods of their determination and mitigation have received special attention in recent years. Taking advantage of the wide range of data that can be obtained by synchrotron radiation X-ray fluorescence spectroscopy (SRXRF) in the field of environmental sciences, different instrumental setups were used to study the biological fates of toxic elements in volcanic environments. The elemental composition of plants, algae, and bacteria in Copahue and Domuyo volcanoes from Argentinean Patagonia was determined by SRXRF and the volcanic elements Ti, Fe, and Zn were abundant in these organisms. Interestingly, a high As concentration was found in cyanobacteria (26.2 μg/g) living in As contaminated stream (250 μg/ml). Because arsenic is toxic and human carcinogen, element-retention capacity, element-protein associations, and arsenic metabolism in this As resistant organism were analyzed by SRXRF. A high capacity (100–95%) of Ti > Fe > Cr > Sr > Ni > Cu > Mn > Zn > As retention was found after aqueous/alcoholic extraction assisted by ultrasonication. The cyanobacterial proteins were separated by SDS-PAGE, electro-transferred to nitrocellulose, and mapped by SRXRF. Defined protein bands containing Ca, Ti, Mn, Fe, and/or Zn were observed. Their ability to metabolize arsenic was revealed by combining SRXRF and X-ray absorption near edge spectroscopy and Dimethylarsenic was found. Based on results, we speculate that these cyanobacteria could be interesting candidates for water treatment. Finally, we conclude that SRXRF is a valuable tool to study the biological cycle of environmental pollutants, including their accumulation, molecular targets, and metabolism. The SRXRF may also assist in remediation researches.

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## 1 | INTRODUCTION

Most of the elements present in the environment are toxic/carcinogenic, either naturally or through human activity.[1] In view of this, it becomes extremely important to know sources and the fate of toxic elements, and hence biological carriers involved in their mobility inside trophic chain as an integrative view of environmental pollution. Occurrence and bioavailability information is essential to investigate the biogeochemical cycling of elements in ecosystems and reduce possible exposure that can affect natural ecosystems or human health. Furthermore, chemical and biological characterization of accumulating organisms will help in the selection and implementation of one or more appropriate technologies to bioremediation or biomitigation. Although many analytical techniques are available today to characterize elements in air, water, soils, and biota, the accuracy, precision, and meaningful data normally depend on quantitative extraction efficiency. Sample preparation procedures are mostly required for getting reliable results, although they often based on destructive methods. Otherwise, in instrumental neutron activation analysis, which is essentially a nondestructive technique, the samples will remain radioactive for many years after the initial analysis, requiring special handling and disposal protocols. An interesting alternative is synchrotron radiation X-ray fluorescence (SRXRF), because brilliant, penetrating, high-energy X-rays produced by a synchrotron allow direct trace level analysis of a wide range of elements with high spatial resolution, without destroying the sample. Indeed, we previously demonstrated that the bioaccumulation of toxic elements in the tissues of experimentally exposed animals can be spatially resolved with a resolution up to 20 μm and in few seconds (2D mapping, or 3D imaging) by SRXRF and X-ray fluorescence computed tomography at the X-Ray Fluorescence Facility (D09B-XRF) of the Brazilian Synchrotron Light Laboratory (LNLS).[2–7] As well, chemical speciation of elements in biological samples was carried out at the LNLS by synchrotron radiation total reflection X-ray fluorescence analysis combined with X-ray absorption near edge spectroscopy (XANES), allowing arsenic speciation without previous extraction procedures.[5] Another advantage of SRXRF lies in that samples can usually be analyzed without pretreatment.[2–8] This is particularly important when a large number of samples need to be analyzed, as often happens in environmental monitoring.[9] In order to highlight the wide range of data that can be obtained by SRXRF in the field of environmental sciences, volcanic environments were selected. Certainly, volcanic environments provide an interesting scenario to studies of occurrence and biotransference of toxic elements, because during and after eruptions, volcanoes release hazardous elements in solid, liquid, and gas phases.[10–14] In this regard, volcanic eruptions of the Southern Volcanic Zone on South Andean Range (between Argentinean and Chilean Northern Patagonia) have provided the highest volume of volcanic elements to a continental environment in the last 2 million years. This event caused a continuous exposure of pyroclastic products to weathering processes to slowly release volcanic elements to soils and waters.[15] Many of these elements, such as Al, Cr, Mn, Co, Ni, As, and Hg, are toxic to living organisms and carcinogens to human in several cases, and can be naturally transferred from soils and waters to the biota, bioaccumulated and magnified in the food chain.[4,16–22]

Considering the above comments, environments with high volcanic influence were identified in Argentinean Northern Patagonia, and analysis by SRXRF and x-ray absorption spectroscopy were combined with biological methods to investigate elemental composition, element-retention capacity, element-protein associations, and element metabolism in exposed biota.

## 2 | METHODS

### 2.1 | Areas under study

In order to establish areas of study that could help to remark advantages of the SRXRF techniques in the analyses of particular ecosystems, 125 lentic and lotic waters from Northern Patagonia were analyzed. In these samples, the common volcanic elements, such as fluoride (range 2–100 mg/L F), aluminum (5–500 mg/L Al), arsenic (range 0.005–0.500 mg/L As), manganese (range 0.01–0.50 mg/L Mn), zinc (0.02–0.20 mg/L Zn), and cadmium (range 0.01–1.22 mg/L Cd), were determined, as well as pH, conductivity, and water temperature. Based on results (not shown), hostile environments on Domuyo and Copahue volcanos, all placed in Neuquén province, Argentine, were selected (map in Figure S1). The Domuyo volcanic complex (S36.57989 W70.42036; peak elevation 4,709 m; current status: dormant), nicknamed as the "Roof of Patagonia," has the second highest advective heat flux from any geothermal system on Earth, after Yellowstone.[23] On the western slope of this volcano, several boiling thermal springs discharge into creeks, which in turn flow into Varvarco River (photo in Figure S2), tributary of Neuquén River (one of the main rivers in Northern Patagonia).

Copahue volcano (S37.85222 W71.16750; peak elevation 2,997 m) is an active stratovolcano. The last eruption began on July 19, 2012, up to December 2012, and minor steam/gases and ash emissions continued intermittently

up today (www.semageomin.cl). On the northern slope of the volcano, the main manifestations of geothermal activity of the region occur, as the hot springs of Copahue, La Máquina, and Las Maquinitas (photo in Figure S3). The Agrio River is an acidic river whose waters originate from the crater-lake of the Copahue volcano and flows (upper Agrio) into Caviahue Lake.[24] The lower Agrio flows out from the northeastern point of the lake, and after 400 km, it merges with Neuquén River.

## 2.2 | Sample collection

Samples were collected in protected natural areas, so the following permissions were obtained: Disposition ANP No. 0178/11 (Provincial Direction of Natural Protected Areas of Neuquén), COPADE Resolution No. 389/11 (Ministry for Territorial Development of the Province of Neuquén), and Permission APN No. 1160-1111 (National Park Administration).

## 2.3 | Water sampling and analysis

All plastic bottles used were previously washed for 15 days in 50% $HNO_3$ (Fisher trace-metal grade) and thoroughly rinsed in Type 1 (Ultrapure) Milli-Q water. In order to determine presence of common volcanic elements in hydrological systems from Northern Patagonia, water samples from major river systems were analyzed. The positional coordinates and elevation data were obtained by GPS measurements. The samples were maintained at 4 °C and transported to the laboratory, where were immediately analyzed. Concentration of manganese (range 0.01–0.5 mg/L Mn) and cadmium (range 0.01–1.2 mg/L Cd) were determined in water by spectrophotometric methods using AQUANAL™-plus tests from Sigma-Aldrich Co. Calibration curves were made in every case using TraceCERT®, certified standards for ICP from Sigma-Aldrich Co. Aluminum was estimated by Aluminum Test Sticks Quantofix® (range 0, 5, 20, 50, 200, 500 mg/L Al + 3) from Sigma-Aldrich Co.; arsenic by Merckoquant® Arsenic Test (range 0.005, 0.010, 0.025, 0.05, 0.10, 0.25, 0.50 mg/L As) from Merck. The accuracy of sticks-based tests (Al and As) was checked with TraceCERT® standards. Physical properties of water were determined *in situ* using ADWA AD31 EC/TDS (Waterproof Conductivity, total dissolved solid, and temperature Tester) and ADWA AD12pH meter (Waterproof pH and temperature Tester).

## 2.4 | Biological samples

At least 10 individuals (around 50 g) of representative organisms were collected from selected areas. The sampling sites are indicated in Table 2. The aquatic cyanobacteria (sp.) samples were collected in the geothermal region of Domuyo volcano. These samples consisted of tangled filaments adhered to submerged rocks (photos in Figures S4 and S5). Samples from Agrio River watershed or hot springs placed in the Copahue geothermal region named "Las Maquinitas" consisted of terrestrial vegetation and green filamentous algae (photos in Figure S6–S9). Green filamentous algae were sampled in lower Agrio River, at 13 km from volcano crater-lake, before and after Copahue eruption event. The samples were rinsed *in situ* with distilled water, cooled in ice, and immediately transported to the laboratory, where were exhaustively washed. Although SRXRF permits the analysis of fresh biological materials,[8] the samples were freeze-dried. To satisfy homogeneity condition of SRXRF analysis, the dried samples were pulverized manually to very fine powder with an agate mortar and pestle. Cylindrical pellets of 13.5 mm diameter were made from 0.5 g powder without binder at 8 tons of pressure with a hydraulic press. In order to preserve their physical and chemical properties, the samples were stored in hermetic containers in nitrogen saturated atmosphere until analysis in the LNLS. Three specimens were prepared as pellets and analyzed by SRXRF. One arsenic accumulator organism, containing 26.2 μg/g of As (cyanobacteria; see Table 2), was selected to study element-retention capacity, element-protein association, and arsenic metabolism.

## 2.5 | Multielemental analysis of biological samples by SRXRF

The elements Si, P, S, Cl, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Br, Rb, and Sr were measured by X-ray fluorescence spectrometry at the XRF station of the LNLS, an international synchrotron research facility located in Campinas, Brazil (details of the XRF beamline at http://www.lnls.cnpem.br/linhas-de-luz/xrf-en/overview/). Measurements were performed in air, whereas X-ray fluorescence spectra were collected using a Silicon Drift Detector. Samples were set in the horizontal plane at 90° from the incident beam to minimize scattering (conventional incidence geometry: 45–45°), as previously reported.[2–4,7] They were positioned in the image plane within an accuracy of 0.5 μm with a three-axis (*x, y, z*) remote controlled stage. Pellets were excited by a white beam that was focused down to 30 μm in diameter size using a pair of mirrors in a KB arrangement. To reduce the statistical error to an acceptable level, 5 points in each pellets were measured with a counting time per point of 200 s (relative standard deviation <1%). Spectra were processed by PyMca software developed by the software group of the European Synchrotron Radiation Facility.[25]

Elemental concentrations were determined by Fundamental Parameter method calibrated with certified reference materials, NIST 1640A (Natural water), SRM 15723A (Tomato leaves) from NIST, IAEA-336 (Lichen) from International Atomic Energy Agency, and SRM 1577c (Bovine Liver) from NIST. Accuracy of the calibration method was tested on pellet of TORT2 (Lobster Hepatopancrea containing 21.6 μg/g As) from the National Research Council of Canada Institute for National Measurement Standards.

## 2.6 | Analysis of element-retention capacity by SRXRF

In order to assess element-retention capacity of cyanobacteria from Domuyo, leaching of toxic elements was measured. The leaching procedure was performed by a modified method, based in procedures used in arsenic extraction.[26,27] Briefly, 8.3 g of cyanobacteria sample (dried and powered) were hydrated in 20 ml of ultrapure water and then 20 ml of absolute ethanol were added. The extraction was assisted by heating at 60 °C in a water ultrasound bath during 45 min. The soluble fraction (extract) was obtained by centrifugation at 2,000 *g* and then was dried in SpeedVac concentrator at 65 °C during 200 min. The insoluble fraction (residue) was dried overnight in oven at 60 °C. The elemental concentration in extract and residue was determined by SRXRF.

## 2.7 | Characterization of element-protein associations by SRXRF

According to Laemmli, 100 μg of dried cyanobacteria was solubilized in sample buffer.[28] Kaleidoscope™ Prestained SDS-PAGE Standards (BioRad) were used to determine the molecular weight of proteins (kDa). Proteins were separated by 10%-SDS-PAGE, transferred to a nitrocellulose membrane, and then stained with Ponceau S dye at 0.2% (*w*/*v*), acetic acid 5% (*w*/*v*) in ultrapure water. This reversible staining allows transient visibility of proteins on nitrocellulose, to identify line of proteins, avoiding their modifications. The nitrocellulose was positioned with computer-controlled X, Y, Z, z stages that was placed at 45° from the direction of the incident beam. The setup also included an optical microscope with motorized zoom that helped in selecting the region of interest on the nitrocellulose before the measurement processes (Figure S10). Then, the line of proteins, as well as a region without any proteins (blank), were scanned with a white synchrotron beam of 2,000 × 300 μm along line in 300 μm steps, totaling 135 determinations for line (proteins between 4 and 180 kDa). The measuring time per point was set to 20 s (relative standard deviation <1%). Elemental concentrations in each point was determined by Fundamental Parameter method calibrated with certified reference materials described above, then the respective blank values were subtracted and resultant concentrations for each element were graphed as a function of its corresponding molecular weight.

## 2.8 | Arsenic speciation by XANES in pelleted samples

The speciation of accumulated arsenic in cyanobacteria was carried out by XANES. The excitation energy was selected using a Si(111) channel-cut crystal monochromator and calibrated by defining the first derivate peak of an Au foil spectrum to be 11,919.0 eV (Au L3-edge). All spectra were recorded in fluorescence mode. The excitation energy was tuned across the As K-edge ($E_o$ = 11,867 eV) from 11,785 to 12,000 eV. Spectra were recorded from 11,785 to 11,843 eV using step sizes of 2 eV for the pre-edge region (11,785–11,843 eV), 0.5 eV for the edge and 2 eV above 11,963 eV. The count times were 3 s per point on the pre-edge and 9 s for the edge and post-edge regions. The main features of the XANES spectra from the sample were qualitative compared with those obtained from the following analytical standards: As(III) (Arsenic trioxide, catalog No. 72718 Sigma-Aldrich; http://www.sigmaaldrich.com/argentina.html), As(V) (Arsenic pentoxide, catalog No. 76686 Sigma-Aldrich), MMA(III) (monomethyl arsenic diiodide, catalog No. AR60013, Argus Chemicals SRL, http://www.arguschem.com/catalogue/arsenic-standards/), MMA(V) (monomethylarsonic acid disodium salt, catalog No. AR60009, Argus Chemicals SRL), and DMA(V) (Dimethylarsinic acid, catalog No. PS51 Sigma-Aldrich). Standards and pellets were analyzed performing three scans for standards and six for the sample. The final XANES spectra were processed after background subtraction and normalization to the post-edge intensity. Data analysis was performed using the Athena software in the computer package IFEFFIT.[29] The background was corrected by fitting a first-order polynomial to the pre-edge region between −80 and −20 eV relative to $E_o$ and a quadratic normalization function between 45 to 150 eV above $E_o$. White line peak positions were obtained from the point where the first derivative of each averaged near-edge spectrum crossed zero.

## 2.9 | Statistical analysis

Data were expressed as an average of five measurements for both analyses (SRXRF and XANES) in three specimens of 0.5 g (three pellets) of each vegetable, algae, cyanobacteria sample or standards. For simple results

visualization, standard deviation was omitted in Tables 1 and 2. However, statistical error of the technique is <0.1% and standard deviation was always <30%. *T* test comparison was used to evaluate significant differences between means. Differences were considered to be significant when $p < .05$.

## 3 | RESULTS

### 3.1 | Physic-chemical properties of lentic and lotic waters on Domuyo and Copahue volcanoes

Based on an extensive monitoring of the Northern Patagonia of Argentina (results not shown), affected by volcanism from its formation up to date, hostile environments on Domuyo and Copahue volcanos, all placed in Neuquén province, Argentinian Northern Patagonia, were selected because element anomalies related to volcanism were found in water. Indeed, Table 1 shows that Al, As, and/or Mn concentrations were above the guideline value for aquatic organism protection (2 mg/L, 50, and 100 μg/L, respectively; according to Argentinian National laws No. 24585 and 24051, and their modifications). Additionally, high conductivity (up to 16,210 μSi/cm in water of Upper Agrio River, on Copahue volcano) was found, with high temperature in hydrothermal systems (40 °C vs. 9–11 °C of ambient temperature). A low pH was found in the Rio Agrio watershed on Copahue Volcano (2.21) as previously described.[24]

### 3.2 | Multielemental analysis of bioindicators by SRXRF

Representative specimens of terrestrial vegetables close to water, algae, and aquatic cyanobacteria were collected in these environments, and the elemental composition was analyzed by SRXRF. High concentrations of elements related to volcanic activity such as Ti, Fe, and Zn were abundant in exposed biota (the concentration was significantly higher than average of 25 vegetable species from Northern Patagonia), whereas As was found to be accumulated in cyanobacteria (26.2 μg/g; Table 2) living in As contaminated stream in Domuyo volcano (250 μg/ml; DOM5 in Table 1). Figure 1, illustrates a representative SRXRF spectra for cyanobacteria from Domuyo. Although a high As concentration was found in the water system of Copahue volcano (up to 250 μg/ml As in Upper Agrio River; COP2 in Table 1), a low As concentration was found in organisms from this environment.

In order to evaluate the impact of volcanic ashes in the environment, green filamentous algae from lower Agrio River were sampled before (March 2011) and after (March 2013) Copahue eruption (December 2012). A significant increase of Ca, Mn, Fe, Cu, and Br concentrations in the aftermath of eruption was observed (Table 2).

### 3.3 | Analysis of element-retention capacity by SRXRF

In order to assess the use of cyanobacteria from Domuyo as a possible biosorbent in future metal bioremediation researches, the leaching of bioaccumulated elements was studied in these organisms to address they element-retention capacity. Although cyanobacteria were subjected to aggressive extraction conditions, a high retention of toxic elements was observed. The extracted elements included Cl (31.48) > K (14.72) > Br (10.98) > S (8.61) > Ca (5.04) > As (4.64) > Zn (3.37) > Mn (3.07) > Cu (1.60) > Ni (1.10) > Sr (0.53) > Cr (0.08) > Fe (0.01); the numbers inside the parenthesis denote percentage (*w*/w) of elements in extract with respect their content in bacteria (Table 3).

### 3.4 | Characterization of element-protein associations by SRXRF

In order to analyze the association between accumulated elements and proteins in cyanobacteria from Domuyo, those between 4 and 180 kDa were studied by electrophoresis followed by electroblotting and scanning by SRXRF. The blank measurement, used as control, was collected from a region of nitrocellulose without any protein band. Analysis of the blank showed the presence of S, Cl, Ca, Cr, Fe, Cu, and Zn (721.7, 1,011.4, 270.3, 2.6, 5.2, 2.2, and 4.4 μg/g, respectively) in the nitrocellulose. The results obtained for S and Cl in proteins were not included because they were related to its presence in the electrophoresis buffers. To avoid problems related to contamination from nitrocellulose, the results showed in Figure 2 were already subtracted to those obtained from blank. The results showed in Figure 2 revel metalloprotein bands with Ca into 22, 25, 34, 38, and 70 kDa proteins, Ti into 34, 41, 88, and 154 kDa proteins, Mn into 77, 90, and 121 kDa proteins, Fe into 34, 48, 50, 60, 154, and 162 kDa proteins, and Zn into 34 and 154 kDa proteins. On the other hand, As-protein bands were not detected. Because SRXRF is a nondestructive methodology, the future characterization of these metal-binding proteins on nitrocellulose could be carried out by additional methodologies.

**TABLE 1** Physicochemical properties of lentic and lotic waters on Domuyo and Copahue volcanoes

| Sample site | Date of sampling | Hour | Description of site | Coordinates WGS 84 | | Altitude [m.a.s.l.] | pH | Temperature of water [°C] | Conductivity [μSi/cm] | Al LOD: 5 mg/L [mg/L] | As LOD: 5 μg/L [μg/L] | Mn LOD: 10 μg/L [μg/L] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Long | Lat | | | | | | | |
| Hydrological system on Copahue volcano | | | | | | | | | | | | |
| COP1 | 03/30/2011 | 10:00 | Dulce River, which drains into Caviahue Lake | −37.8629 | −71.0469 | 1,565 | 5.59 | 6.8 | 367 | - | < | - |
| COP2 | 03/31/2011 | 14:50 | Upper Agrio River (Cascada del Gigante), which drains into Caviahue Lake | −37.8868 | −71.0714 | 1,705 | 2.21 | 13.1 | 16,210 | < | 250 | 570 |
| COP3 | 03/31/2011 | 15:30 | Portezuelo Srteam, which drains into Caviahue Lake | −37.8944 | −71.0244 | 1,606 | 5.77 | 12.4 | 50.2 | < | < | < |
| COP4 | 03/30/2011 | 10:30 | Stream, which drains into Caviahue Lake | −37.8553 | −71.0284 | 1,622 | 6.28 | 7.1 | 46.6 | < | < | 50 |
| COP5 | 03/31/2011 | 12:30 | Caviahue Lake | −37.8933 | −71.0235 | 1,607 | 2.88 | 15.3 | 1,187 | 150 | 50 | 760 |
| COP6 | 03/30/2011 | 10:50 | Caviahue Lake | −37.8665 | −71.0492 | 1,577 | 2.8 | 13.7 | 1,347 | 150 | 35 | 950 |
| COP7 | 03/30/2011 | 11:43 | Lower Agrio River (after Caviahue Lake) | −37.8283 | −70.9684 | 1,530 | 2.95 | 9.7 | 1,065 | < | 25 | 690 |
| COP8 | 03/30/2011 | 13:20 | Las Mellizas Lakes | −37.8371 | −71.1005 | 1,991 | 6.54 | 9.8 | 429 | < | < | 10 |
| COP9 | 03/30/2011 | 14:00 | Las Maquinitas hot spring (mud) | −37.8192 | −71.0868 | 2,005 | 2.6 | 25.9 | 6,480 | < | 50 | 2,370 |
| COP10 | 03/30/2011 | 14:15 | Las Maquinitas hot spring | −37.8194 | −71.0869 | 2,006 | 3.76 | 39.1 | 1,274 | < | 35 | 130 |
| COP11 | 03/30/2011 | 14:50 | Las Maquinitas hot spring (other) | −37.8183 | −71.0867 | 2000 | 5.98 | 42.7 | 877 | < | 10 | 1,370 |
| COP12 | 03/30/2011 | 15:15 | Stream (closed to Las Maquinitas hot spring) | −37.8185 | −71.0869 | 2,006 | 3.75 | 16.3 | 185.1 | < | < | 20 |
| Hydrological system on Domuyo volcano | | | | | | | | | | | | |
| DOM1 | 05/05/2012 | 14:47 | Stream | −38.0488 | −70.6367 | 1,020 | 6.5 | 14.5 | 321 | 5 | < | 26.4 |
| DOM2 | 05/05/2012 | 15:30 | Salado River | −38.2132 | −70.0938 | 726 | 7.75 | 18.1 | 5,570 | 5 | < | 10 |

(Continues)

**TABLE 1** (Continued)

| Sample site | Date of sampling | Hour | Description of site | Coordinates WGS 84 | | Altitude [m.a.s.l.] | pH | Temperature of water [°C] | Conductivity [μSi/cm] | Al LOD: 5 mg/L [mg/L] | As LOD: 5 μg/L [μg/L] | Mn LOD: 10 μg/L [μg/L] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Long | Lat | | | | | | | |
| DOM3 | 05/05/2012 | 18:00 | Pichi Neuquén stream | −37.7382 | −70.1331 | 843 | 8 | 18 | 2,400 | 5 | < | 43 |
| DOM4 | 05/06/2012 | 13:40 | Covunco River, which merges with Varvarco River | −36.7047 | −70.6331 | 1,529 | 6.5 | 18.5 | 2,510 | 5 | 250 | < |
| DOM5 | 05/06/2012 | 14:35 | Stream, which merges with Varvarco River | −36.6799 | −70.6080 | 1,736 | 7.75 | 40.7 | 3,610 | 5 | 250 | < |
| DOM6 | 05/06/2012 | 12:45 | Atreucó River, which merges with Varvarco River | −36.7180 | −70.6198 | 1,649 | 4.75 | 7.8 | 28.7 | < | < | < |
| DOM7 | 05/06/2012 | 11:20 | Neuqén-Varvarco River, confluence | −36.8598 | −70.6766 | 1,174 | 5.5 | 9.1 | 459 | 5 | 100 | < |
| DOM8 | 05/07/2012 | 09:45 | Neuquén River | −37.4068 | −70.2301 | 830 | 5.5 | 9.2 | 290 | 5 | 35 | < |
| DOM9 | 05/05/2012 | 18:30 | Triquimilán stream, which merges with Neuquén River | −37.4699 | −70.2132 | 817 | 6.5 | 14.4 | 3,540 | 5 | < | < |

*Note*. The values are the means of three determinations. LOD = limit of detection; < = less than the lower limit of detection; - = not measured.

**TABLE 2** Concentration of elements in samples from Domuyo geothermal region, Agrio River watershed, and Copahue geothermal region obtained by SRXRF

| Sample | Sample site according to Table 1 | Element concentration μg/g DW | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | S | Cl | K | Ca | Ti | V | Cr | Mn | Fe |
| Green filamentous algae March 2011 | COP7 (photo Figure S6) | 7,800 ± 800[b] | 450 ± 50 | 27,000 ± 3,000[b] | 1,400 ± 100 | 190 ± 20 | 100 ± 10[b] | NQ | 89 ± 9 | 2,700 ± 300 |
| Green filamentous algae March 2013 | COP7 | 2,800 ± 300[a] | NQ[a] | 6,500 ± 700[a] | 2,700 ± 300[a] | 270 ± 30[b] | 62 ± 6[a] | NQ | 260 ± 30[a] | 4,500 ± 400[ab] |
| Leaves of *Araucaria araucana* (Pehuen tree) | Closed to the COP2 (photo in Figure S7) | 1,200 ± 100 | 3,100 ± 300 | 8,200 ± 800 | 1,700 ± 200 | NQ | NQ | NQ | 96 ± 9 | 19 ± 2 |
| Fruit of *Maihuenia patagonica* | Closed to the COP2 (photo in Figure S8) | 490 ± 50 | 17,000 ± 2,000 | 27,000 ± 3,000[b] | 2,000 ± 200 | 14 ± 2 | NQ | NQ | 190 ± 20 | 3,100 ± 300 |
| Seeds of *Maihuenia patagonica* | | 1,200 ± 100 | 6,700 ± 700 | 5,200 ± 500 | 270 ± 30 | NQ | NQ | NQ | 79 ± 8 | 330 ± 30 |
| Grass | Closed to the COP10 (photo in Figure S9) | 1,600 ± 200 | 650 ± 70 | 13,000 ± 1,000 | 640 ± 60 | 55 ± 6 | NQ | NQ | 40 ± 4 | 1,400 ± 100 |
| Grass | Closed to the COP11 | 1,500 ± 200 | 2,400 ± 200 | 2,300 ± 200 | 6,500 ± 600 | 150 ± 20 | 1.4 ± 0.2 | NQ | 470 ± 50 | 1,200 ± 100 |
| Cyanobacteria | DOM5 (photo in Figures S4 and S5) | 2,500 ± 200 | 2,200 ± 200 | 2,500 ± 200 | 1,2000 ± 1,000 | 250 ± 30[b] | 11 ± 2 | 41 ± 4[b] | 340 ± 30 | 6,800 ± 700[b] |
| MDL: Minimum detection limit (μg/g dried weight) | | 49 | 32 | 15 | 7.4 | 0.8 | 0.2 | 0.17 | 0.2 | 0.15 |
| Mean concentration in north Patagonian species | | 1,600 ± 1,700 | 7,600 ± 16,200 | 10,000 ± 7,200 | 7,409.5 ± 7,760.8 | 75.6 ± 88.3 | 13.0 ± 28.8 | 5.4 ± 12.5 | 182.8 ± 183.5 | 1,179.0 ± 1,683.8 |

**TABLE 2** (Continued)

| Sample | Sample site according to Table 1 | Element concentration μg/g DW | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Co | Ni | Cu | Zn | Ga | Ge | As | Se | Br | Rb | Sr |
| Green filamentous algae March 2011 | COP7 (photo Figure S6) | NQ | NQ | 18 ± 2 | 55 ± 6 | 1.1 ± 0.1 | NQ | 1.0 ± 0.1 | NQ | 7.0 ± 0.7 | 20 ± 2 | NQ |
| Green filamentous algae March 2013 | COP7 | NQ | NQ | 44 ± 4[ab] | 61 ± 6 | 0.7 ± 0.1 | NQ | 1.7 ± 0.2 | NQ | 11 ± 1[a] | 23 ± 2 | 1.4 ± 0.2 |
| Leaves of *Araucaria araucana* (Pehuen tree) | Closed to the COP2 (photo in Figure S7) | NQ | NQ | 4.6 ± 0.5 | 6.3 ± 0.6 | NQ | NQ | NQ | NQ | 3.0 ± 0.3 | 23 ± 2 | 1.5 ± 0.2 |
| Fruit of *Maihuenia patagonica* | Closed to the COP2 (photo in Figure S8) | NQ | NQ | 35 ± 4[b] | 48 ± 5 | NQ | NQ | NQ | NQ | 350 ± 40[b] | 190 ± 20[b] | NQ |
| Seeds of *Maihuenia patagonica* | | NQ | 11 ± 1 [b] | 12 ± 1 | 110 ± 10[b] | NQ | NQ | NQ | NQ | 16 ± 2 | 31 ± 3 | NQ |
| Grass | Closed to the COP10 (photo in Figure S9) | 1.9 ± 0.2 | NQ | 10 ± 1 | 29 ± 3 | NQ | NQ | NQ | NQ | 2.7 ± 0.3 | 6.9 ± 0.7 | 5.0 ± 0.5 |
| Grass | Closed to the COP11 | 1.3 ± 0.2 | NQ | 14 ± 2 | 35 ± 4 | NQ | NQ | 0.9 ± 0.1 | 1.3 ± 0.1 | 6.7 ± 0.7 | 24 ± 2 | 6.2 ± 0.6 |
| Cyanobacteria | DOM5 (photo in Figures S4 and S5) | NQ | 11 ± 1[b] | 6.6 ± 0.7 | 14 ± 1 | 2.6 ± 0.3 | 5.0 ± 2[b] | 26 ± 3[b] | NQ | 15 ± 2 | 9.0 ± 0.9 | 17 ± 2 |
| MDL: Minimum detection limit (μg/g dried weight) | | 0.14 | 0.15 | 0.16 | 0.17 | 0.21 | 0.22 | 0.25 | 0.38 | 0.51 | 0.72 | 0.83 |
| Mean concentration in north Patagonian species | | 4.5 ± 5.8 | 4.0 ± 3.2 | 12.6 ± 11.8 | 29.8 ± 27.7 | 1.5 ± 1.0 | NQ | 1.9 ± 5.1 | 7.0 ± 0.6 | 28.3 ± 68.2 | 18.1 ± 36.1 | 9.9 ± 9.4 |

*Note*. The values represent micrograms of the relevant element per gram of dry weight (μg/g DW) in the samples and are the means of five determinations in three pelleted samples. Values in last row represent the mean elemental concentrations of 25 vegetable species from North Patagonia, analyzed by SRXRF (not shown species were included). ± *SD*. NQ = not quantifiable (signal < MDL); MDL = minimum detection limit.

[a]significant difference between 2011 and 2013.

[b]significant difference with respect to mean concentration in Patagonian species.

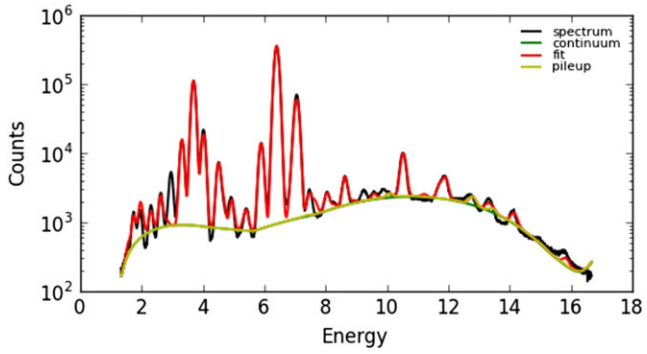


**FIGURE 1** Representative X-ray fluorescence spectroscopy spectra for cyanobacteria from Domuyo obtained by PyMCA software. Energy in keV. Pick at 10.5 keV corresponds to As

### 3.5 | Arsenic speciation in pelleted sample by XANES

A qualitative comparison of XANES spectra for cyanobacteria and previously collected in a range of arsenic standards that include As(III) (arsenite), As(V) (arsenate), MMA(III) (monomethylarsonous), MMA(V) (monomethylarsonic), and DMA(V) (dimethylarsinic), Figure 3 denotes the presence of two white line picks in cyanobacteria corresponding to As(V) at 11,873.5 eV and DMA(V) at 11,870.5 eV. This result suggests that DMA(V) would be the main As metabolite in the accumulator cyanobacteria from Domuyo.

## 4 | DISCUSSION

### 4.1 | Physic-chemical properties of hostile volcanic environments in northern Patagonia

The concentrations of Al, As, and Mn were above the reference value for the protection of aquatic organisms in the water of the Domuyo and Copahue volcanoes. These results are in agreement with previous reports in Copahue and other geothermal systems of Latin America, as processes that release As with Al, Mn, and other associated trace elements.[30–32] Considering that these geological abnormalities were accompanied by high temperatures and low pH in some cases, the biota of these

**TABLE 3** Total trace elements in 8.3 g of cyanobacteria sample, their water/ethanol extract and residue by SRXRF

| | Element concentration in cyanobacteria (μg/g) | Element concentration in extract (μg/g) | Element concentration in residue (μg/g) | Elements in 8.30 g cyanobacteria (μg) | Elements in 0.53 g extract (μg) | Elements in 7.02 g residue (μg) | Extracted elements (%) |
|---|---|---|---|---|---|---|---|
| Si | 13,000 ± 3,000 | NQ | 12,000 ± 1,000 | 100,000 ± 30,000 | NQ | 88,000 ± 8,000 | - |
| S | 2,500 ± 200 | 3,300 ± 800 | 4,000 ± 900 | 21,000 ± 3,000 | 1,800 ± 400 | 28,000 ± 6,000 | 8.61 |
| Cl | 2,200 ± 200 | 11,000 ± 3,000 | 2,300 ± 200 | 18,000 ± 2,000 | 6,000 ± 1,000 | 16,000 ± 1,000 | 31.48 |
| K | 2,500 ± 200 | 6,000 ± 1,000 | 2,600 ± 900 | 21,000 ± 5,000 | 3,100 ± 800 | 18,000 ± 7,000 | 14.72 |
| Ca | 12,000 ± 1,000 | 10,000 ± 3,000 | 9,000 ± 400 | 100,000 ± 20,000 | 5,000 ± 2,000 | 64,000 ± 2,000 | 5.04 |
| Ti | 250 ± 30 | NQ | 330 ± 30 | 2,080 ± 70 | - | 2,300 ± 200 | - |
| V | 11 ± 2 | NQ | 10 ± 3 | 90 ± 20 | - | 70 ± 20 | - |
| Cr | 41 ± 4 | 0.5 ± 0.3 | 60 ± 20 | 340 ± 80 | 0.3 ± 0.2 | 400 ± 100 | 0.08 |
| Mn | 340 ± 30 | 160 ± 40 | 380 ± 70 | 2,800 ± 700 | 90 ± 20 | 2,600 ± 500 | 3.07 |
| Fe | 6,800 ± 700 | 8 ± 4 | 7,000 ± 1,000 | 60,000 ± 30,000 | 4 ± 2 | 48,000 ± 9,000 | 0.01 |
| Ni | 11 ± 1 | 1.9 ± 0.4 | 10 ± 4 | 90 ± 20 | 1.0 ± 0.2 | 70 ± 20 | 1.10 |
| Cu | 6.6 ± 0.7 | 1.7 ± 0.1 | 12.5 ± 0.4 | 50 ± 10 | 0.88 ± 0.05 | 87 ± 3 | 1.60 |
| Zn | 14 ± 1 | 7 ± 1 | 16 ± 3 | 110 ± 30 | 3.8 ± 0.5 | 110 ± 20 | 3.37 |
| Ga | 2.6 ± 0.3 | NQ | 0.55 ± 0.05 | 21 ± 6 | - | 3.8 ± 0.4 | - |
| Ge | 5 ± 2 | NQ | 6 ± 1 | 40 ± 10 | - | 39 ± 8 | - |
| As | 26 ± 3 | 19 ± 3 | 25 ± 4 | 220 ± 40 | 10 ± 1 | 170 ± 30 | 4.64 |
| Br | 15 ± 2 | 27 ± 4 | 15 ± 3 | 130 ± 50 | 14 ± 2 | 100 ± 20 | 10.98 |
| Rb | 9.0 ± 0.9 | NQ | 11 ± 1 | 70 ± 20 | - | 75 ± 7 | - |
| Sr | 17 ± 2 | 1.4 ± 0.1 | 13 ± 3 | 140 ± 40 | 0.73 ± 0.05 | 90 ± 20 | 0.53 |

*Note*. The values are the means of five determinations ± *SD*. The values of extracted elements represent percentage (*w*/*w*) of elements in 0.53 g extract with respect to elements in 8.3 g cyanobacteria. NQ = not quantifiable; - = not measured.

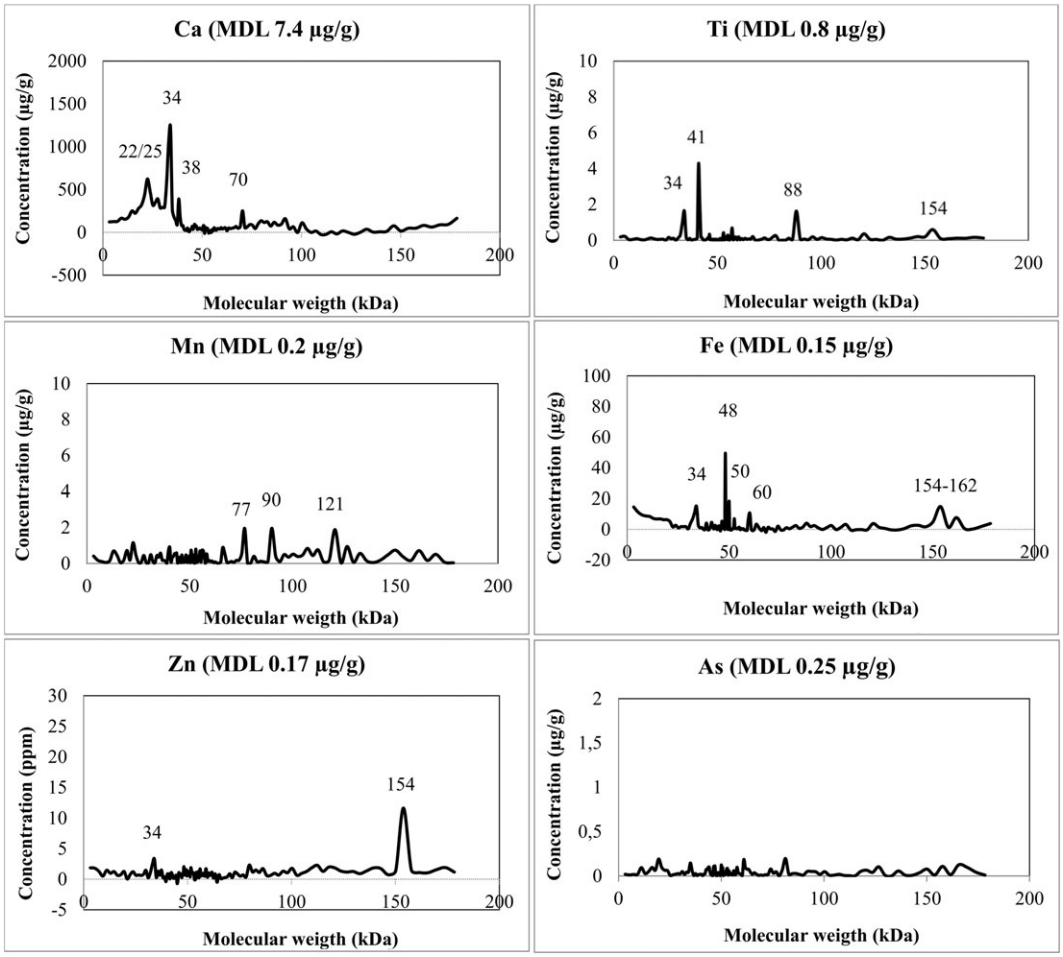


**FIGURE 2** Metal-binding proteins by μ-X-ray fluorescence spectroscopy. The proteins in cyanobacteria from Domuyo were separated by SDS-PAGE, electro blotted to a nitrocellulose membrane, and then scanned by synchrotron radiation-induced micro X-ray fluorescence analysis with a spatial resolution of 300 μm. The *x* axes represent the molecular weights (kDa) of proteins on nitrocellulose; the prominent metalloprotein peaks are indicated. The *y* axes represent the concentration of elements in proteins expressed as microgram/gram of dried sample, where the respective values of blank were subtracted. The minimum detection limit (MDL) for each element into proteins is indicated. The values are the mean of two determinations. Graphics without detected elements into proteins are shown in Figure S11

environments was analyzed by SRXRF as bioindicators of hostile volcanic ecosystems.

## 4.2 | Multielemental analysis of bioindicators by SRXRF

In a previous study, composition and physicochemical parameters were analyzed in Agrio River and geothermal waters of Copahue.[24,32] However, limited research is available on biotransference of these volcanic elements. On the other hand, only medicinal properties to cyanophyceae species from the Domuyo have been studied.[33] In this work, elemental composition of exposed biota was determined by SRXRF. The extreme hydrogeological anomalies found in Copahue and Domuyo waters do not allow abundance, or presence in several cases, of aquatic species as macrophytes or fishes. Thus, representative specimens of terrestrial vegetables close to water, algae, and aquatic cyanobacteria were included in this analysis. As expected, volcanic elements were abundant in exposed biota. However, As accumulation was only observed in cyanobacteria from Domuyo (26.2 μg/g; Table 2). Cyanobacteria are the only group of prokaryotes able to conduct oxygenic photosynthesis and spread out in almost any ecological niche, from fresh and salt water to terrestrial and extreme environments, including metal-contaminated areas.[34] Thus, it is not surprising to find a thermophile cyanobacteria that grows in high concentrations of toxic elements (at least Al and As; Table 1), and also able to accumulate large amounts of As and heavy metals. On the other hand, the low As bioaccumulation in organisms from Copahue may be related to high P concentrations introduced by this volcano into the ecosystem.[35] Although arsenate and phosphate are chemically analogue to cells, and both can be taken up by the same transporters, phosphate is

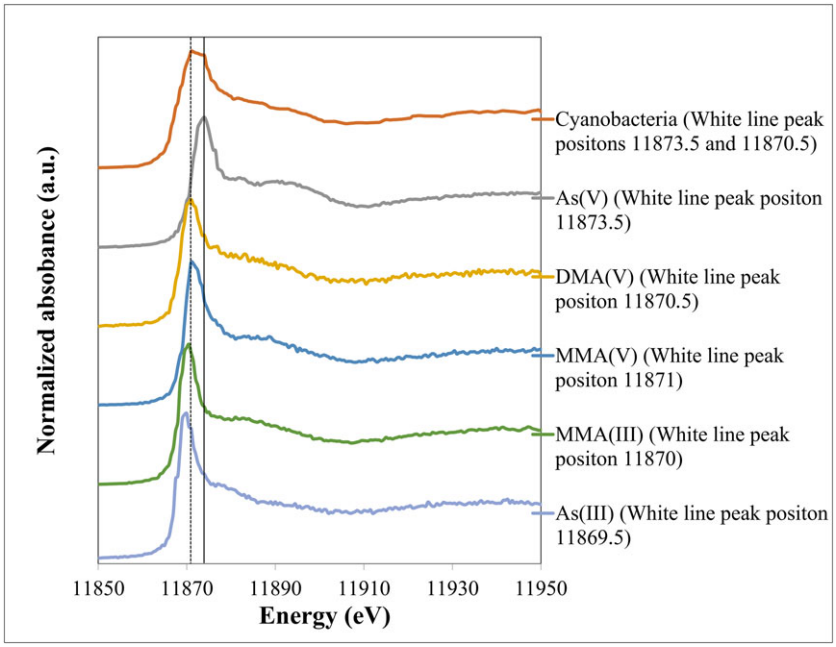


**FIGURE 3** X-ray absorption near edge spectroscopy spectra at arsenic K-edge obtained in pellet of cyanobacteria from Domuyo and arsenic standards: As(III), MMA(III), MMA(V), DMA(V), and As(V). The final X-ray absorption near edge spectroscopy spectra were processed after background subtraction and normalization to the post-edge intensity. Dotted vertical line: DMA(V) (11,870.5 eV). Solid vertical line: As(V) white line position (11,873.5 eV). The values are the mean of five determinations in two sample pellets or standards. The spectra are displaced vertically for clarity

normally preferred by nature.[36,37] Then, we show that SRXRF allows to study the biological carriers of elements in particular environments and to identify accumulating organisms, with minimum sample preparation.

On the other hand, an increase of Ca, Mn, Fe, Cu, and Br concentrations was found in green filamentous algae from lower Agrio after Copahue eruption. This result is congruent with the geochemical release of these elements from Copahue ashes, as described by Ruggieri et al.[38] This means these algae could be appropriate local bioindicators showing a substantial capability to react to environmental disturbances in short periods of time. So, we also concluded that SRXRF is a valuable tool to assess environmental changes.

## 4.3 | Analysis of element-retention capacity by SRXRF

Unlike organic contaminants, which can be degraded to harmless chemical species, heavy metals (or metalloids) cannot be destroyed. Therefore, the mitigation of contaminated water by heavy metals can only be envisioned as their immobilization in a nonbioavailable form or respeciation into less toxic forms. Although these approaches do not solve the problem all together, they can help to protect population from noxious effects, and isolate the contaminants as a contained and sometimes recyclable residue. In this regard, the final fate of metals is a question of concern, because long-term applications could lead to secondary contamination of the environment. Because a high retention of toxic elements was observed in cyanobacteria from Domuyo, we speculate that these biomaterials could be interesting candidates for water treatment. Similar findings have led to biotechnological developments using other tolerant and accumulating bacteria in metal abatement strategies.[39–41] Thus, the analysis of retained and extracted elements by SRXRF allows a rapid screening of potential bioadsorbents to bioremediation applications.

## 4.4 | Characterization of element-protein associations by SRXRF

Since early history, mutual coexistence between microorganisms and metals happens. It appears in the wide range of divalent or transition metals present in active centers of many enzymes.[39] Recently, it has been shown that up to a third of the total microbial proteome contains a metal cofactor, but there are limitations in metalloprotein prediction that make it difficult to predict if a protein will contain metals or not. Metal homeostasis is especially important in cyanobacteria because the photosynthetic machinery imposes a high demand of metals, which act as cofactors of several proteins.[42] In this regard, SRXRF result an advantageous tools in protein analysis. Mainly because no other multielement analytical technique allows detection of element associated with proteins directly on nitrocellulose without destroying it, permitting posterior immune-detection and identification. Thus, in order to analyze the association between accumulated elements and proteins in cyanobacteria from Domuyo, electrophoresis was combined with SRXRF. The results shown metalloproteins containing at least one of the followed elements: Ca, Ti, Mn, Fe, Zn. As described by Waldron and Robinson,[43] in *in vitro* conditions, most of metalloproteins in bacteria bind

metals according to the order of affinity, following the Irving–Williams series for divalent cations ($Mg^{2+} < Ca^{2+} < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$). In agreement, Figure 2 shows that protein band at 34 kDa is associated with cations in similar order: Ca (1,230 μg/g), Fe (10.8 μg/g), Zn (2.4 μg/g), and also with Ti (1.6 μg/g). Similarly, protein band at 154 kDa is associated with Fe (14.9 μg/g), Zn (11.6 μg/g), and Ti (0.6 μg/g). Additionally, data obtained by SRXRF could support the future characterization of metal-binding proteins. For instance, the pick of 34 kDa containing Ca, Fe, and Zn could be related to proteins of similar molecular weight and metal affinity described in Photosystems or periplasmic iron binding protein of cyanobacteria.[44,45]

On the other hand, although these cyanobacteria are arsenic bioaccumulators, As-protein bands were not detected. Taking into account that arsenic toxicity is exerted in part through As binding to protein dithiols,[46,47] the results suggest that if there are As-binding proteins in the cyanobacteria, the As-protein bands would be below the minimum detection limit (As MDL: 0.25 μg/g). Based on showed results, proteins would be biotargets for a fraction of biotransferred metals from the environment. However, the results also suggest that there would be other immobilization mechanisms involved in heavy metals and arsenic resistance in this organism. This hypothesis is not surprising because the complexation of heavy metals and arsenic with fatty acids and carbohydrates has been described as an important mechanism to chelate metals and metalloids in cyanobacteria.[40,48]

### 4.5 | Arsenic speciation in pelleted sample by XANES

In the arsenic biotransformation, a detoxification system that is present from bacteria to humans, first, the arsenate is converted into arsenite and then transformed into monomethylated, dimethylated, and trimethylated products.[49–52] Thus, studies about the chemical form of arsenic, especially its transformation, allow to understand the biogeochemical cycling of this element in peculiar geo-systems, and offer a molecular explanation of how exposed organisms tolerate arsenic in their environment. Our qualitative results suggest that arsenic metabolites are present in the arsenic accumulator cyanobacteria from Domuyo, indicating that, as expected, arsenic metabolic pathway is active in this organism, which tolerate high concentrations of arsenic in their geothermal surrounding. Interesting, arsenic metabolites were identified *in situ* by XANES, avoiding the limitations and/or effects of sample preparation required with conventional methods.[53] We conclude that results obtained by XANES using Synchrotron radiation represent an accurate arsenic profile of the original organism. Thus, future quantitative analysis could be interesting in ecotoxicological researches.

## 5 | CONCLUSION

In this work, we demonstrate that SRXRF allows chemical, biological, and molecular characterization of organisms exposed to hostile volcanic environments. Additionally, analyses by different synchrotron radiation techniques have also been useful to guide for possible biotechnological applications of accumulating organisms. For instance, based on its high tolerance to hostile habitats, enhanced metal/metalloid-retention capability, and bioaccumulation properties, we presume that the cyanobacteria from Domuyo could be useful to bioadsorb heavy metals/arsenic in water. However, no direct evidence of this hypothesis exists yet.

Besides, the same sample was analyzed by both SRXRF and XANES, and then treated with electrophoresis or fractionated with solvents. Because these techniques are nondestructive, after SRXRF analysis, their molecular constituents may be purified and identified. In this regard, the metalloproteins into nitrocellulose may be identified by Western blot or the protein band can be excised for sequencing after SRXRF analysis.

As a summary, SRXRF and XANES studies have provided an accurate and comprehensive picture of the environment under study, making broad data available about the elemental composition of exposed biota, the molecular targets of elements, and the metabolic pathway involved in pollutant tolerance.

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## CONFLICT OF INTEREST

The authors declare they have no conflicts of interest.

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## SUPPORTING INFORMATION

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