



Determination and Analysis of Metals in Freshwater Microalgae (*Chlorella vulgaris* and *Spirulina platensis*) through Total Reflection X-ray Fluorescence Spectroscopy (TXRF)

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Authors' contributions

This work was carried out in collaboration between both authors. The lab work was carried by author JIB and wrote the first draft while author LLM performed the statistical analysis. Both authors read and approved the final manuscript.

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ABSTRACT

This work investigates the concentration of some trace elements in freshwater microalgae (*Chlorella vulgaris* and *Spirulina platensis*) through total reflection x-ray fluorescence spectroscopy (TXRF). Slurry samples were prepared from 3.4 ± 0.5 to 9.8 ± 0.5 mg of freeze-dried algae biomass for direct metals analysis. Gallium was used as internal standard at concentration of 500 $\mu\text{g/L}$ and the solution was mixed for 15 second using vortex. The concentrations of different metals including Magnesium (Mg), Zinc (Zn), Phosphorus (P), Sulphur (S), Copper (Cu), Potassium (K), Calcium (Ca), Manganese (Mn) and Iron (Fe) were determined in the ranged $6.8 \pm 1.7 - 15709$ mg/kg. A similar study investigated the total macro and micronutrient profile *Chlorella vulgaris* and the published data agree with current study. Statistical analysis shows that only Ba has significant difference between *Chlorella vulgaris* and *Spirulina platensis* ($P = 0.05$) The two investigated algal species shows a measurable concentration using TXRF of metals such as manganese, iron,

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copper, and zinc. TXRF shows good accuracy with ability to determine sample in small quantities (μl) In conclusion, the sensitivity of TXRF technique can also be seen in determining elements with lower atomic number such as Ca, P and K.

Keywords: Freshwater microalgae; metals; *Chlorella vulgaris*; *Spirulina platensis*; total reflection x-ray fluorescence spectroscopy.

1. INTRODUCTION

Algae has been reported to accumulate both essential and non-essential metals. The application of algae for environmental monitoring has been researched since in 1970s [1]. The excess presence of some metals in the water bodies may have a significant effect on the microalgae growth which constitute the major food source of other plants and animals during their growth stages [2]. Microscopic photosynthetic algae are found both in freshwater and marine environments and their mode of photosynthetic activities are similar to terrestrial plants. However, as a result of a simpler cellular structure and being submerged in an aqueous environment where CO_2 , water and other valuable nutrients are readily available they can more easily convert solar energy into biomass [3]. Microalgae are considered to be the first rung in aquatic systems food chain and they tend to accumulate extracellular polysaccharides including sheaths or capsules and envelope (mass enclosing their cells) [3]. Microalgae are comprised of fatty acids and lipids as membrane components- which act as sources of energy, metabolites and storage products [3]. In global ecology, both marine and freshwater microalgae are extremely efficient at scavenging the metals and they are taxonomically diverse, with over 500 million years of evolutionary existence. Many microalgal strains constitute valuable nutrients that can be used at low concentrations in microbial metabolic activities [3]. Macro algae such as green macroalgae, seaweed and their alginate derivatives have potential affinity towards many metals ions [4].

There are many research publications on the determination of the levels of heavy metal contamination or pollution using various analytical methods. Inductively coupled plasma atomic emission spectrometry (ICP – AES) have been applied to investigated the levels of metals toxicity in some biological samples by using microcentric nebulisation [5]. Inductively coupled plasma mass spectrometry (ICP – MS) and Inductively coupled plasma atomic emission spectrometry (ICP – AES) was applied in the

work of Marcos et al. [6] to determine the geographic origin of tea samples. It was also reported that total reflection X-ray fluorescence (TXRF) spectrometry was used to evaluate variable concentrations of metals profiles in fresh water microalgae [7]. Slurry – TXRF was also applied successfully in the assessment of metals air pollution on cemetery mosses, the work determined the concentration of many metals including Al, As, Br, Cl, Cu, K, Cr, Pb, V, Zn among others [8]. TXRF has been shown to be applicable in trace metals monitoring for cloud water and particulate matter due to it enhanced sensitivity [9]. TXRF samples analysis was conducted for sediments and soils to assess the environmental pollution in which the researchers performed studies to compare the results from TXRF to inductively coupled plasma, optimal emission spectroscopy and flame atomic absorption spectroscopy [10]. Highly dangerous heavy metals of lead, chromium, arsenic and mercury in dietary supplements has been determined using TXRF [11]. Toxic metals uptake and removal through biosorption process from collected wastewater by two different strains of freshwater microalgae (*Chlorella vulgaris* and *Spirulina platensis*) has been investigated [12]. Varying adsorbent dosage (0.20 – 2.50 g), contact time (5 – 100 min) and pH range (4- 8) were studied. The results show 95.0 \pm 0.3 % toxic metals removal of Al, 87.0 \pm 0.2 % for Ni and 63.0 \pm 0.3 % Cu by *Spirulina platensis* at pH range of 5.50, 6.00 and 7.00 with biomass dosage of 2.50 \pm 0.10 g/L respectively [12]. Lower metal removal was observed with *Chlorella vulgaris* with 87.0 \pm 0.2 %, 79.1 \pm 0.4 % and 80.0 \pm 0.2 % in Al, Ni and Cu respectively [12]. Bioremediation of textile water was reported using *Chlorella vulgaris* in batches of cultures [13]. The application of *Chlorella vulgaris* in this work suggest that the strain was potential for polishing of textile water before it being discharged [13]. *Spirulina platensis* has been used to monitor the environmental pollutant such as heavy metals [14]. Different metals including Pb, Cd, Cu and Cr were tested by *Spirulina platensis* for it ability in remediating these metals under varying concentration of 1.0, 3.0 and 5.0 mg/L. The work indicated that *Spirulina platensis*

is a good absorbent and potential strain in metals removal [14].

The impact of various environmental pressures has significantly changed the physiological and metabolic processes in algal communities and this by extension has reflected in the amount or content of nutrients and trace elements in algae [15]. Therefore, it is imperative to access the concentration of both micro-nutrients and toxic metals in the algae. TXRF has been reported to be a unique analytical technique in multi-elemental determination of plant, soil and water due to its lower detection limit [15]. Pettersson and colleagues have developed a direct method using TXRF to investigate periphyton communities. According to the experiments they conducted where samples of periphytons were placed on soda glass discs that were used as TXRF carrier plates as well as sample bottom holder, in that study the researchers confirmed that the analytical data of both direct and wet digestion methods for Fe, K, Mn, Zn, Sr, Ca, Cu, Rb and As were in good agreement [16]. TXRF was previously used directly to determine the concentrations of metals (Cu, Fe, Zn and Mn) using slurry samples in microalgae viz., *Chlorella keslerii*, *Synechococcus* sp. and *Cylindrospermopsis raciborskii* [15]. Slurry sampling method was efficient when compared to the result obtained by total digestion method and the procedure involves the use of TXRF on marine periphytons by colonization on soda glass disc to quantify the metals concentrations [15]. Impact of fuel oil on freshwater microalgae using *Selenastrum capricornutum* have also been investigated for chlorophyll and protein content, growth rate and carbohydrate [17].

2. MATERIAL AND METHODS

2.1 Instrumentation

The fundamental of X-ray fluorescence (XRF) involves hitting of an inner electron shell of a sample by the quantum of an X-ray thereby removing an electron that causes the atom to be excited. This excited atom will now be substituted by an electron from an outermost shell. Thus, the differences in energy between the outer and inner shells is balanced off through emission of a photon light or x-ray fluorescence radiation [18]. Every element shows a unique line pattern in spectrum depending on the orbitals involved [18]. TXRF can detect elements from Sodium (Na) up to Uranium (92) and elemental sensitivities depend on the atomic number [18]. The working

principle of S2 PICOFOX used in this study (produced by Bruker AXS Inc. Madison, WI, USA) is based on the total reflection X-ray fluorescence (TXRF) sample analysis.

2.2 Sample Preparation

The algae strains used in this work (*S. platensis* and *C. vulgaris*) were generously provided by Dr Piotr Zymski of Department of environmental medicine, Poznan University of Medical Sciences, Poland. The two species are freshwater microalgae (*S. platensis* and *C. vulgaris*) were cultivated separately and stored under a controlled condition as per package labels. Laboratory based photobioreactor method in an enclosed system was used to culture the algal strains under highly controlled conditions. The two species are valued for their evidence-based bioactive and nutritional properties. The strains were mostly cultivated for the purpose of production of food supplements. The method used in the preparation of the sample was adapted from the work of Katalin et al. [15] Slurry samples were prepared from 3.4 ± 0.5 to 9.8 ± 0.5 mg of freeze-dried algae for direct metal analysis. Gallium standard was used at concentration of 500 µg/L (adding 5 µl 100 mg/L Ga internal standard to 1 ml of algae sample). The solution was mixed using a vortex for 15 seconds. For each sample 10 µl was pipetted onto the centre of an individual siliconized quartz glass disc and then dried on a hotplate for 15 minutes at 50°C. A 1000 mg/L gallium standard disc was also measured in the TXRF to provide the gain correction.

3. RESULTS AND DISCUSSION

To obtain a representative result from both *C. vulgaris* and *S. platensis* samples, ten (10) and twelve (12) number of replicate samples were analysed (n = 10 and 12) respectively. The minimum quantity of both algal species analysed was 3.4 ± 0.5 mg. Fig. 1 shows the graphical mean concentrations of various metals concentrations in both *C. vulgaris* and *S. platensis* samples. The results obtained are summarised and presented in Table 1 and 2 and statistically shown in Figs. 1a and 1b, respectively. As mentioned above, Ga was used as internal standard, and this was the fact that TXRF sample specimen is small and is impractical to assess its areal density. Therefore, absolute sensitivities are seldom used in TXRF and instead relative sensitivities are prepared for calibration. In order to determine the

Table 1. TXRF mean calculated concentrations of metals (mg/kg) of *C. vulgaris* (n = 10) and *S. platensis* (n= 12) and standard error (mg/kg)

Species	Mg	Zn	P	S	Cu	K	Ca	Mn	Fe
<i>C. vulgaris</i>	3186.2 ± 476	27.9 ±5.4	15709.1±2335	11537.7±1331	6.8 ± 1.7	12326.3±1755	3412.4± 605	52.6 ±10.5	1067.9 ±309
<i>S. platensis</i>	3958.9±1272	24.7 ± 7	19237.7±4173	11982.2±1649	3.1 ± 0.9	17144.4±2504	7665.7±5554	39.1 ± 7.2	862.4 ±199
T-test	0.109	0.702	0.470	0.836	0.094	0.131	0.462	0.305	0.585
P values									

Table 2. TXRF mean calculated concentrations of other metals (mg/kg) detected only in *S. platensis* (n= 12) and standard error (mg/kg)

Species	Ti	Sr	Br	Cr	Ba	Pb	Y	Ni	As
<i>S. platensis</i>	22. 4 ±6.9	19.8 ± 4.9	2.5 ±0.8	1.9 ± 1.3	1.5 ±0.6	1.4 ± 0.6	0.9 ±0.2	0.9 ±0.3	0.6 ± 0.3
T-test	0.883	0.340	0.634	0.279	0.030	0.580	0.944	0.168	0.955
P values									

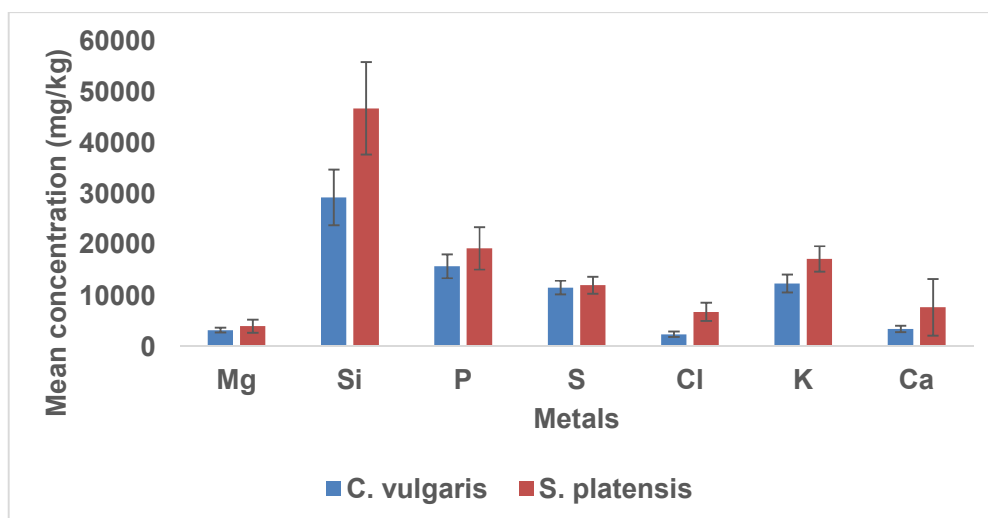


Fig. 1a. Metals mean concentrations of *C. vulgaris* and *S. platensis* slurry measured by TXRF, *cv* = *Chlorella. vulgaris*, *n*= 10 and 12 respectively standard error shown as error bars, elements from Mg – Ca

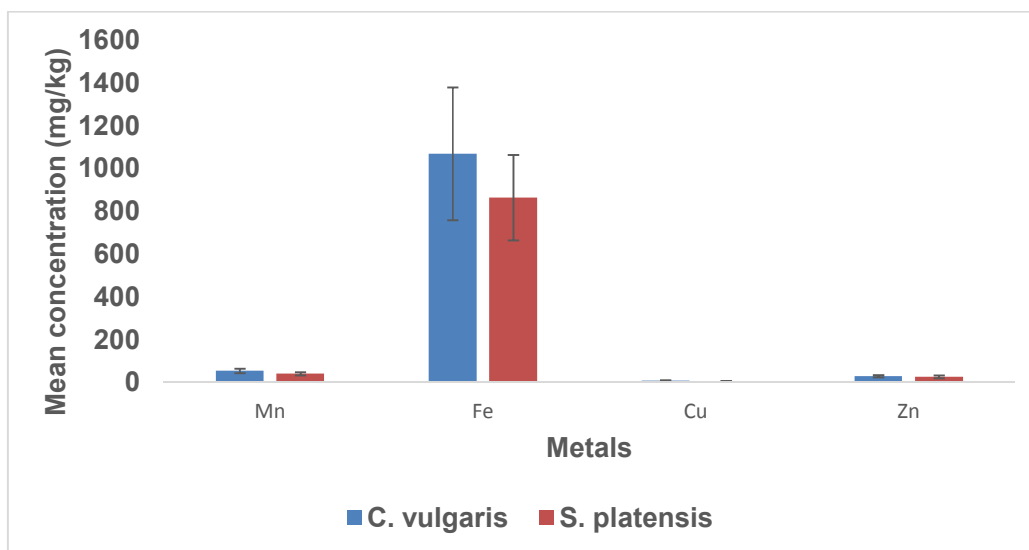


Fig. 1b. Metals mean concentrations of *C. vulgaris* and *S. platensis* slurry measured by TXRF, *cv* = *Chlorella. vulgaris*, *n*= 10 and 12 respectively standard error shown as error bars, elements from Mn – Zn

relative sensitivities a standard solution is spiked with known amount of internal standard and measured.

The mean concentrations \pm (SE) of both metals and some toxic metals for samples *C. vulgaris* were reported. The emphasis in this work was to determine metals concentration including iron, zinc, copper, manganese and magnesium in the algal samples. The mean concentration of metals

found in *C. vulgaris* increases as reported in Table 1 and 2. The findings in this study was compared to other published TXRF data that investigated metals concentrations algal strains and plants and our finding indicated certain amount heavy metals such Arsenic (As), Strontium (Sr) and Yttrium (Y) can also be detected. Some variable concentration of toxic metals was found in the investigated species. The concentration of Fe, Mn and Zn are

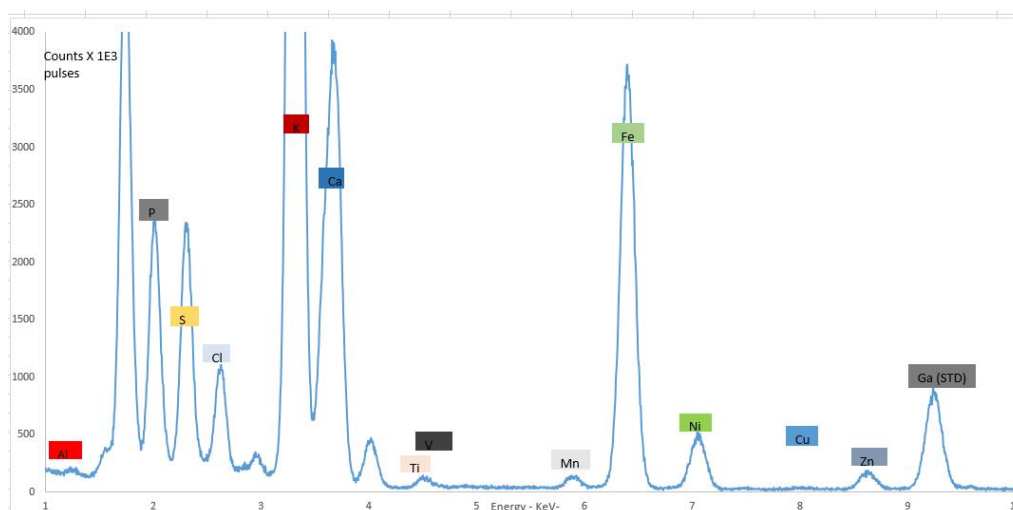


Fig. 2. An example of Spirulina platensis TXRF spectrum

higher in *C. vulgaris* compared to *S. platensis* and Cu was exclusively only detected in *C. vulgaris*. It can also be seen that *C. vulgaris* is rich in important metals that are necessary for both plants and animals' nutrition. Relatively low quantities of heavy metals were detected in the species. A similar study investigated the total macro and micronutrients profile of *Chlorella vulgaris* [19]. In that published paper, values for metals were reported as follows; Zn (213 mg/kg), Mn (6.37 mg/kg), Mg (96.2 mg/kg), Cu (54.4 mg/kg) and Fe (4.99 mg/kg) [19]. While for *S. platensis* the concentration of metals was found to be as follows: Mg > Fe > Mn > Zn as reported in Table 1 and 2. In *S. platensis* samples Cu could not be determined but other heavy metals such as Pb and As were detected. Similarly, in both the algal samples the mean concentration of Silicon was higher, and this was expected due to the composition of siliconized disc used. *S. platensis* have been widely reported to be among the most consumed microalgae by the public due to its good quality of proteins, antioxidant, vitamins, balanced fatty acid and minerals [20]. The species when taken is well known in preventing many health challenges such as anaemia, cancer, diabetes and arthritis among others [20]. The study involving 25 commercially purchased spirulina products cited some lower values for metals when compared to the present study [20]. It can also be seen that there was a significant variation of metals among the 2 species for Mg, Fe and Mn while Zn values are similar. In comparison to previously published research on plant samples of *Lemna gibba*, *Myriophyllum heterophyllum*, *Arenaria paludicola*,

Hydrocotyle ranunculoides and *Eichhornia crassipes* that were obtained from aquatic environment to monitor the impact of Zn, Cu, Cr, Pb and Ni using TXRF [21]. That study revealed that the maximum level of Pb (7.92 mg/kg) was found in the root of *E. crassipes*, Cr (7.54 mg/kg), Ni (5.96 mg/kg) in roots and Zn (64.10 mg/kg) and Cu (60.55 mg/kg) in shoots of *H. ranunculoides* [21]. In another study that utilized the use of TXRF on a soda float glass reference sample, it has been noted that metals including Mn (299 mg/kg), Fe (1850 mg/kg), Zn (112 mg/kg) and Cu (29.5 mg/kg) were detected at reasonable concentrations [22]. Further studies also revealed similar values for the investigation of *Chlorella* (unicellular, green), *Synechococcus* (unicellular, blue) and *Cylindrospermopsis* (unicellular, blue) reported by Imre et al. [7] for algae slurry samples where *Chlorella* shows an appreciative metals mean concentrations for K, Ca, Mn, Fe, Cu, Fe, Cu, Zn and Pb. The concentration of the metals was found to be in the range of 5.0 – 40000 mg/kg and slurry sample was prepared from 5.0 – 25 mg of freeze-dried algae. Similar work by other researchers that involves marine freshwater algae for TXRF analysis of specimens made from certified reference material (RRM) plankton slurries also reported values that agree with the results herein [22,23].

Further statistical analysis of each strain was conducted as *P* values reported in Table 1 and 2. T-test was used to test for the null hypothesis between the elements. The result shows only Ba has significant difference between *Chlorella*

vulgaris and *Spirulina platensis* species ($P = .05$). A t-test was used to compare literature values to those obtained in this work. Result show no significant difference ($P = 0.05$) suggest the applicability of TXRF for multi element analysis.

4. CONCLUSION

Total reflection x-ray fluorescence (TXRF) has been successfully applied to investigate and analyse elemental concentrations from the slurry frieze dried *Chlorella vulgaris* and *Spirulina platensis* species. It can be reliably report that, based on this study TXRF is a suitable analytical technique to analyse the elemental content of *Chlorella vulgaris* and *Spirulina platensis*. The two species investigated both shows a measurable concentration using TXRF of trace elements including manganese, and magnesium iron, copper and zinc. TXRF shows good accuracy and precision with ability to determine sample in small quantity (μl). The sensitivity of TXRF technique can also be seen in determining the concentrations of many elements including those with lower atomic number such as Ca, P, Al and K. However, there are limited studies in investigating the elemental composition of microalgae using TXRF. Therefore, further studies are needed to determine why the variation in the number of metals of these microalgae strains.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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