Bull. Environ. Contam. Toxicol. (2003) 70:809–816

DOI: 10.1007/s00128-003-0054-9

Bulletin of Environmental Contamination and Toxicology

New Methodology for Extraction of Total Metals from Macroalgae and Its Application to Selected Samples Collected in Pristine Zones from Baja California, Mexico

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Received: 30 May 2002/Accepted: 11 December 2002

One of the main problems to adequately evaluate natural and anthropogenic contributions of trace metals to the oceans, is the inherent variability in their concentrations caused by the continuous change in the conditions of the marine environment, and by the dynamics of currents and upwelling processes. Mollusk bivalves, together with other filter-feeding organisms can be used as bioindicators of contamination, but they incorporate metals associated to the microscopic organisms that constitute their food, as well as from the ingestion of inorganic particulates (e.g., Struck et al. 1997, Páez-Osuna et al. 2000). An alternative that recently has been explored is the possibility of using macroalgae as bioindicators of metal concentrations in seawater (Leal et al., 1997; Haritonidis and Malea, 1999; Muse et al., 1999; Páez-Osuna et al., 2000; Sánchez-Rodríguez et al., 2001). One of the main advantages of analyzing metals in seaweed tissues is their capacity of accumulate trace metals in concentrations that, generally, are several orders of magnitude higher than their respective concentrations in seawater (e.g., Sánchez-Rodríguez et al. 2001). However, and contrary to filter-feeding organisms, metal concentrations in algal tissue are proportional only to the concentrations of dissolved metals in the water surrounding the macroalgae (Luoma et al. 1982).

Even though concentrations of trace metals associated to seaweeds have been studied in different parts of the world, their metal concentrations were obtained through a variety of extraction methods, since essentially every author used its own technique. However, what all these methodologies have in common is the digestion of algal tissue by strong oxidants and acids (e.g., H_2SO_4, HNO_3, aqua regia and H_2O_2), whose sole function is to oxidize and/or to degrade organic matter. The objective of this work was to develop a new methodology that, in addition to an acid digestion, will incorporate a basic solution to dissolve organic matter resistant to strong acids to ensure a complete digestion of the algal tissues. This technique was compared to three other methods described in the scientific literature using a certified standard. Once validated, the new methodology was applied to selected seaweed samples collected from pristine zones of Baja California, Mexico, and compared with measurements from other non-contaminated areas of the world described in the literature.

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MATERIALS AND METHODS

All the materials used in the sample collection and in the laboratory was thoroughly washed and acid-cleaned. Seaweed samples were collected manually and always trying to remove the complete macrophyte from the base. Once collected, each macroalgae was cleaned *in situ* from excess sand and epiphytes using seawater collected from the zone, stored in 250-mL high-density polyethylene bottles, and placed in ice chests. The samples were then transported to the laboratory where they were stored in a cold room at 4°C until they were analyzed. Before processing, the seaweeds were further cleaned with distilled water, pre-dried for three days at room temperature in an ultra-clean hood and, finally, oven-dried for 72 h at 70°C. The dry samples were ground and homogenized in a porcelain mortar and stored in high density polyethylene bottles before subjecting them to the different total digestion procedures.

Our extraction method was validated using the certified standard Citrus Leaves 1572 (National Institute of Standards and Technology, Gaithersburg, MD 20899-2322, USA) and subsequently applied to samples of the three main groups of seaweeds, represented by the species *Macrocystis pirifera* (brown algae), *Chondracanthus pectinatus* (red algae) and *Ulva lactuca* (green algae). The first two species were collected from Puerto Kennedy (Pacific coast of Baja California), whereas the third one was obtained from Bahia de los Angeles (occidental coast of the Gulf of California). All samples were analyzed by triplicate and their trace metal concentrations determined with either a flame atomic absorption spectrophotometer (FAAS) Thermo Jarrell Ash model Smith Hieftje 12 with background correction, or a graphite furnace atomic absorption spectrophotometer (GFAAS) Varian model Spectra 880Z with a GTA 100 Furnace Atomizer 188. This last instrument was used exclusively to measure the metal concentrations of the certified standard which were below their FAAS detection limits. Hollow cathode lamps were used for the detection of all analyzed metals at the following wavelengths (in nm): Cd (228.8), Cu (324.8), Mn (279.5), Pb (217.0), Co (240.7), Fe (248.3), Ni (232.0) and Zn (213.9). Their corresponding detection limits (in nmol g^{-1}, second value for GFAAS) were: Cd (14, 0.13), Co (21, 0.23), Cu (3.8), Fe (25), Mn (22), Ni (76, 0.79), Pb (7.9), and Zn (15). The reagents HNO_3 (Ultrex II), HCl (Baker Instra-Analyzed), H_2O_2 (Suprapure) and NH_4OH (Suprapure) used in the analyses were trace metal-free, as was confirmed by the blanks, which were always below their corresponding detection limits.

Selection of the most adequate method was based on the following criteria: (1) percentage of recovery of the analyzed metals from the certified standard; (2) cost of implementation; (3) degree of manipulation of the samples; (4) amount of time spent on the whole procedure; and (5) simplicity of the procedure. Essentially, we tried to develop, from the experimental point of view, a simple and practical extraction technique for trace metals associated to algal tissue. Preliminary tests made before the development of our proposed method showed that a sequential

digestion with nitric acid, perchloric acid and hydrogen peroxide at relatively high temperatures (80-100 °C) extracted approximately 70% of the Cu, Mn and Zn reported in the certified standard. Due to these results and taking into consideration the possible existence of residual organic matter resistant to the attack of oxidizing acids (e.g., Riget et al. 1997) we included a step in which a basic solution was added to the samples to obtain a complete digestion of the tissues. Following, is a brief description of the three chosen methodologies as well as the one developed in this work, which was compared with the first three to determine the best one suited for digestion of seaweed tissue:

In method #1, developed by Van Loon and Barefoot (1988), the sample is heated in a furnace, gradually increasing the temperature during 6 to 8 h until it reaches 450°C. The sample is then left at this temperature until it is reduced to ashes, which are later digested in aqua regia. The main disadvantage of this method is the high temperature used in the procedure, which most probably causes loss of volatile elements, like Pb, Zn and Cd, and the potential for sample contamination arising from the use of the furnace. The main advantage of the method is that samples of large size can be used without the utilization of perchloric acid, whose use requires special precautions. Developed by Lares-Reyes (1988), method #2 involves the digestion of the sample with HNO_3 at a temperature of 66°C and its subsequent carbonization at 350°C to remove lipids. The residue is finally oxidized with H_2O_2. The main limitation of this technique is the large volume of sample involved as well as the amount of time invested in its implementation. The methodology of Riget et al. (1997) consists in the incineration and reduction to ashes of the dry sample in a furnace at 450°C. The ashes are later dissolved in diluted nitric acid. This method presents the same advantages and disadvantages of Method 1. Our proposed methodology is partially based on the technique developed by Lares-Reyes (1988), since the samples are subjected to a digestive process with nitric acid, but with the difference that a basic digestion with concentrated ammonium hydroxide was introduced (Figure 1). This new method has several advantages over the previous ones in that it uses a small quantity of sample, loss of metals by volatilization is eliminated because extreme heating is absent, and, finally, there is a complete digestion of the seaweed tissue with the reagents used.

For the implementation of these methods, the analyses were standardized in terms of the quantities of sample required by each one of them, using 0.5 and 1.0 g of sample for the certified standard and collected seaweed samples, respectively. However, the reagent:sample proportions suggested by the author(s) of each method was preserved.

RESULTS AND DISCUSSION

Table 1 shows the percentages of recovery obtained from the certified standard for all the trace metals analyzed in this study. These results indicate that methods

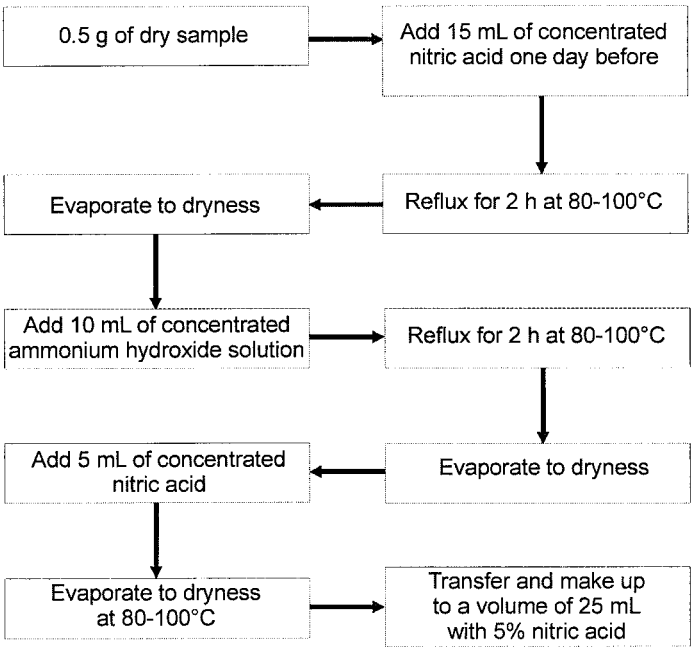

Figure 1. Flow diagram of method 4, proposed in this work, for the total extraction of metals in algal tissue.

1 and 3 were, in general, the ones which showed the lowest percentage of recovery (<62%) for Cd, Co, Ni and Zn (method 3, only), and overestimations for Pb (255%, method 3) and Co (277%, method 1). Random contamination of the samples by the furnace used during the reduction to ashes of the samples were probably responsible for the overestimations. However, methods 1 and 3 showed reasonable (97-111%) percentages of recovery for Cu, Fe and Mn, although other methods showed even better results. The low recovery percentages generally exhibited by these two techniques were probably caused by the utilization of high temperatures (450°C) during the processing of the samples, which in turn will tend to produce partial loss of relatively volatile elements like Pb, Zn and Cd.

Although the best recovery for Cd (86%) was obtained with method 2, it was not so successful with other elements (Co, Cu, Fe, Mn, Ni, Pb), for which their percentage recoveries were generally inferior to those obtained from the other three methods. These results are not surprising since this method was developed specifically for the analysis of Cd in algal tissue (Lares-Reyes 1988), possibly in detriment of the recovery of other elements. Method 4, developed in this work, generally presented the best results for Co, Cu, Fe, Mn, Ni and Pb, with recovery percentages generally in the range 98% to 106%, except in the case of Ni, for which a value of 84% was obtained (Table 1). We do not have an explanation for the overestimation obtained for Cd in method 4, since none of the reagents

Table 1. Average concentration (n = 3) ± one standard deviation and percentage recovery (PR) of metals obtained from each of the four methods (Mtd) assayed in this work using the Citrus Leaves Certified Standard (CS, underlined values). For the equivalence of method number, see text.

Mtd	Metal	Value (nmol g^{-1})	PR (%)	Metal	Value (nmol g^{-1})	PR (%)
1	Cd	0.02 ± 0.12	8	Co	0.94 ± 0.04	277
2	Cd	0.23 ± 0.12	86	Co	0.15 ± 0.00	45
3	Cd	0.06 ± 0.04	22	Co	0.14 ± 0.04	40
4	Cd	0.70 ± 0.06	264	Co	0.36 ± 0.05	105
CS	Cd	0.27 ± 0.09		Co	0.3*	
1	Cu	287 ± 4	111	Fe	1789 ± 121	111
2	Cu	211 ± 15	81	Fe	1302 ± 170	81
3	Cu	286 ± 13	110	Fe	1577 ± 106	98
4	Cu	275 ± 2	106	Fe	1602 ± 110	99
CS	Cu	260 ± 16		Fe	1612 ± 179	
1	Mn	412 ± 3	98	Ni	4.7 ± 2.0	46
2	Mn	335 ± 19	80	Ni	4.3 ± 3.4	42
3	Mn	405 ± 6	97	Ni	3.59 ± 0.84	35
4	Mn	424 ± 17	101	Ni	8.6 ± 6.2	84
CS	Mn	419 ± 36		Ni	10 ± 5	
1	Pb	57 ± 15	88	Zn	477 ± 18	108
2	Pb	87 ± 28	135	Zn	427 ± 13	96
3	Pb	163 ± 17	255	Zn	275 ± 22	62
4	Pb	64 ± 23	100	Zn	436 ± 8	98
CS	Pb	64 ± 12		Zn	443 ± 31	

*Non-certified value with unreported standard deviation.

involved in this digestion showed measurable concentrations of Cd; however, contamination during manipulation of the samples or interferences from other spectral wavelengths cannot be ruled out.

Comparison of our results with those reported for the certified standard showed absence of significant differences (Z test, $\alpha = 0.05$; Table 1) for Cd with method 2, for Cu, Mn, Ni and Zn with method 4, for Fe with methods 3 and 4, and for Pb with methods 1, 2 and 4. These statistical analyses indicate that method 4, developed in this work, recovered concentrations that were similar to those reported in the certified standard for Cu, Fe, Mn, Ni, Pb and Zn, although Cd concentrations were apparently overestimated by this method (Co was not included in the evaluation since its standard deviation is not included in the certified values).

Once validated, the precision of our method was determined based on

measurements made in the collected seaweeds. The calculated variability for the majority of the metals (Cd, Fe, Mn, Ni and Zn) was relatively low, since it ranged from 3 to 16%, 4 to 10%, and 8 to 16% in the brown, red and green algae, respectively, with a global variability range of 3 to 16% (Table 2). The high variability (over 100%) observed in the concentrations of Co and Cu associated to the brown and red macroalgae were probably produced by the proximity of their concentrations to their respective detection limits. For the green seaweeds, whose Cu concentrations were well above its detection limit, their variability was only 14% (Table 2). Besides Co and Cu, the element that showed the highest concentration variability (24 to 52%) was Pb.

Since the seaweed samples were collected in zones that are essentially free of anthropogenic contamination, the concentrations given in Table 2 represent background values for the coasts of Baja California. Figure 2 shows these same values, but plotted in combination with reported seaweed concentrations from different parts of the world that lack significant local contributions of trace metals. To take into consideration the diversity of environments and of species of algae involved, as well as to facilitate comparisons with the seaweed levels of metals analyzed in this work, the concentrations reported in the literature were plotted as box and whiskers diagrams. As can be observed in Figure 2, the literature values cover ranges that, in general, cover approximately three to six orders of magnitude, a wide enough range to accommodate the values obtained in this work. However, the concentration levels of Co, Pb, Ni, Zn (*C. pectinatus*) and Fe (*U. lactuca*) obtained in this work are located within the 25 and 75 percentiles of the values reported in the literature, whereas the concentrations of Cd (*M. pirifera* and *U. lactuca*), Zn (*U. lactuca*) and Fe (*M. pirifera* y *C. pectinatus*) are included within the percentiles 10 and 90. The rest of the values

Table 2. Average concentrations measured in brown, red and green seaweeds with method 4.

	Concentration (nmol g^{-1})					
Metal	**Brown* seaweed**	**RSD** (%)**	**Red* seaweed**	**RSD (%)**	**Green* seaweed**	**RSD (%)**
Cd	34.7 ± 2.6	7.6	**74.4 ± 3.3**	4.5	32.0 ± 2.6	8.2
Co	**53 ± 67**	127	33 ± 48	147	52 ± 44	85
Cu	1.8 ± 3.2	173	3.9 ± 4.8	124	**29.5 ± 4.0**	14
Fe	1072 ± 164	15	1196 ± 119	10	**5904 ± 939**	16
Mn	49.8 ± 5.5	11	41.7 ± 1.8	4.4	**179 ± 15**	8.4
Ni	321 ± 52	16	180 ± 17	9.2	**389 ± 50**	13
Pb	15.6 ± 8.1	52	19.9 ± 4.8	24	**26.8 ± 8.5**	32
Zn	117 ± 4	3.5	**474 ± 33**	6.9	155 ± 22	14

*Average concentration ± one standard deviation; **RSD = relative standard deviation. Values in bold indicate the highest values.

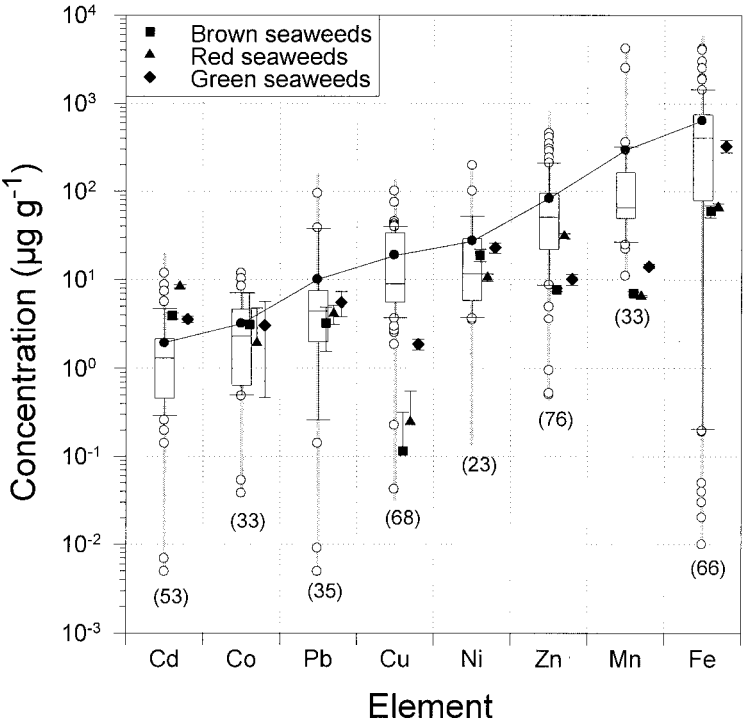

Figure 2. Average concentrations (± one standard deviation) of trace elements in brown, green and red seaweeds analyzed in this study compared with reported concentrations from different parts of the world lacking significant local anthropogenic inputs (box and whiskers diagrams). White circles represent values beyond the 90th and 10th percentiles, gray lines cover the ranges from minimum to maximum values, and black circles represent average concentrations. The numbers in parenthesis indicate the number of values included in the calculations of the average metal concentrations. Note the log scale in the Y axis. Data taken from Bryan and Hummerstone (1977), Rajendran et al. (1993), Riget et al. (1997), Struck et al. (1997), Leal et al. (1997), Haritonidis and Malea (1999), Muse et al. (1995, 1999), Páez-Osuna et al. (2000), and Sánchez-Rodríguez et al. (2001).

are located beyond these two last percentiles. Mn is a special case since all the values obtained for *M. pirifera* and *C. pectinatus* were consistently lower than the range obtained from the literature. Generally, *U. lactuca* was the seaweed showing the highest concentrations of Cu, Fe, Mn, Ni, and Pb, *C. pectinatus* the highest levels of Cd and Zn, and *M. pirifera* the highest concentrations of Co.

Acknowledgments. Thanks to I. Pacheco and A. González for their help in the collection of the different macroalgae, to A. Siqueiros for his help in the preparation and analysis of some of the samples, and an anonymous reviewer whose comments greatly improved the quality of this work.

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