Polish J. of Environ. Stud. Vol. 19, No. 2 (2010), 413-417

Original Research

Estimation of Carbonate and Element Content in Charophytes – Methods of Determination

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Received: 3 March 2009
Accepted: 23 November 2009

Abstract

Charophytes possess the capacity to store large amounts of various elements, but until today no studies about known methods of determining $CaCO_3$ and elements in charophytes have been conducted. Because of the large and varying amounts of $CaCO_3$, it is difficult to estimate the actual element content in dry mass. Simple comparisons of elements in charophyte plant tissue or between charophytes and higher plants are impossible. The main aim of this study was to investigate through laboratory experiments content of carbonates and elements in charophyte tissue using different methods of decomposition (mineralization). The proposed protocol: determination of calcium carbonate content in plants, determination of elements present in the plant dry mass, and calculation of the element content in the plant dry mass as described.

Keywords: *Chara*, *Nitella*, *Charophytes*, determination, carbonates, calcium, element, content, ash

Introduction

Charophytes are a submerged macrophyte group of green algae that dominate submerged calcium-rich vegetation in oligotrophic and moderately eutrophic lakes [1-6]. They are a common component of various water bodies. As the use of HCO_3^- is an alternative for CO_2, charophytes could withdraw exceptionally large amounts of HCO_3^- and could contain up to 75% carbonate biominerals in dry mass. Among the methods of biomineralizing charophyte are biologically induced internal incrustation and external encrustations on the cell walls of the stems and, in some species, organic-matrix-mediated calcification within the walls of the oogonium [7, 8]. The biomineralizing process is limited to certain charophyte groups; some species calcify regularly, several rarely, and some never. External calcifications appear on stems and represent annular bands in some ecorticate species, like *Chara corallina* or *Chara braunii,* or manifest as apparently non-banded encrustations on stems of some ecorticate- and many corticate species [8, 9] like *Chara vulgaris* or *Chara hispida*. The cellular physiology of some ecorticate species of *Chara* (*C. corallina*, *C. braunii*) as well as the photosynthetic uses of calcification have been intensively investigated [8, 10, 11]. Yet little attention has been paid to methods of determining carbonates and elements in charophytes, which reveal intra-specific and species-to-species variations within the element content [8]. This phenomenon seems especially important, because charophytes storing large amounts of various elements, especially nutrients, behave as a phosphorus sink in lakes by the process of co-precipitating P with calcite [3, 12, 13]. A simple comparison of element content in the plant tissue between charophytes or between charophytes and higher plants seems impossible and can give false results [14].

As stated above, the main aim of this study was to investigate, through laboratory experiments, the contents of carbonates and elements in charophyte tissue using different methods of decomposition. The proposed protocol was to determine the carbonate content in the plants, determine the elements present in the plant dry mass, and calculate the element content in the plant dry mass as described.

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Material

The general purpose of pretreating biological samples for element analysis is the complete decomposition of the sample [15]. It is an important but highly time-consuming stage in the determination of elements in plants. Decomposition methods commonly used involve different mixtures of acids (HNO_3-HCl, HNO_3-H_2SO_4, HNO_3-$HCLO_4$, etc.). For instance, very often metals are extracted from plants by heating their samples with HNO_3 and $HClO_4$ mixtures in long glass tubes or with reflux condensers. These methods, however, have several drawbacks. If an open system is used, there is a risk of loss of elements [16]. To avoid such problems, the wet digestion technique is often carried out in PTFE (Teflon) bombs using microwave ovens. Microwave extraction has previously been tested for organic-rich plant material with good results [16]. The analytical procedure used in this experiment was as follows.

Methods

Sample Preparation and Decomposition

Samples of five different charophyte species were used in the experiments: *Chara hispida* L, *Chara intermedia* A. Braun 1836, *Chara filiformis* Hertzsch 1855, *Chara baltica* Bruzelius 1824 and *Nitellopsis obtusa* (Desv in Loisel.-Deslong.) J. Groves 1919. The plants were collected by hand: *C. baltica* in the Baltic Sea; *C. intermedia* in central Poland (Małopolska upland); and *C. hispida*, *C. filiformis* and *N. obtusa* in northern Poland (Suwalsko Augustowskie Lakeland). The samples were placed between paper sheets and dried to a constant weight on a surface at 105ºC. The plants were not homogenized before digestion- and carbonate determination procedures; whole plant parts – namely tips of 10 cm length – were used for these experiments to estimate the carbonate and element content directly in the plant biomass. The plant material from each species was divided into 40 subsamples. Ten subsamples of each species were used in different methods of sample digestion.

All the chemicals used were analytical grade and the use of glass tubes or Teflon bomb blanks containing only acid mixtures prevented contamination during digestion of the samples. Finally, samples were analyzed for all the elements (Ca, Mg, and K) investigated using AAS (Varian SpectrAA 200). The reliability of the digestion methods was assessed using a reference material consisting of *Populus* leaves. The agreement with certified values was mostly good, except for method A, which was lower by 15-25%. Comparisons were made with the reference material; Table 2 contains the results of the recovery rates obtained for the reference material with methods A, B and C; ten replicate samples were tested and the average is presented.

The differences between various methods of digestion were tested by ANOVA - (*test of least significant difference* - LSD) using the Statistica 8.0 package [17].

Method A: Carbonate- and Element Content (Analyzed after Carbonate Determination)

Ten of the 40 samples of each species were used to determine carbonate content. A number of methods have been described in the literature [18-20], but most of them are based on the reaction between carbonates and strong acids, which results in carbonate dissolution and CO_2 generation [18]. The three simple ways to measure the amount of the carbonates are:

1) to measure the volume of gas under isobarometric conditions using equipment such as the Scheibler apparatus;
2) volumetrically (with methyl orange indicator), using a characteristic reaction of carbonates with acids;
3) using the gravimetric method. The last method lacks enough accuracy and should not be used for estimating carbonate content.

In the present study, Scheibler's method was used (accuracy 0.1 ml) [21]. This method is relatively easy and no other substances such as NaOH or indicators contaminate the sample, which is important when analyzing for Ca, Mg-, and K content in samples.

The carbonates and bicarbonates present in the sample as ($CaCO_3$) and magnesium ($MgCO_3$) or mixed carbonates such as $CaMg(CO_3)_2$, and are converted by the reaction with acid, and expressed as equivalent calcium carbonate.

The determinations were done in 100-ml Pyrex glass tubes. During the investigation, 10 ml HCl was added to the glass tubes containing the plant sample and the volume of CO_2 measured. All procedures and calculations adopted conformed to International Standard Methods [22]. After completing carbonate determination, the same samples were digested with 5 ml conc. HNO_3 and $HClO_4$ (4:1). Preoxidation with H_2O_2 was used to prevent the risk of explosion when organic matter reacted directly with $HClO_4$. The major advantage of this method is that $HClO_4$ appears to be a good matrix for AAS (weak background) [23]. All the test tubes were left overnight at room temperature. All 100-ml test tubes were placed in an aluminum heating block with temperature control (accuracy ± 5ºC) at 150ºC. The samples were digested for 150 min. or until the emission of brown fumes of nitrogen dioxide ceased. The digest, a clear liquid, was allowed to cool, filtered, and the volume made up to 25 ml with deionized water.

Method B Determination of Element Content in Glass Tubes (without Previous $CaCO_3$ Determination)

The ten other samples of investigated species without previous determination of carbonates were digested with a mixture of HNO_3^- and HCO_4 acids (including H_2O_2) in glass Pyrex tubes, left overnight, and heated following the procedure described above.

Table 1. Content (%) of carbonates and elements in dry weight of investigated species. Elements content analyzed after carbonate determination in glass tubes (method A) and without carbonate determination in glass tubes (method B) or in teflon bombs (method C).

Chara baltica											
Sample (n=10)	Ash	Carbonates	Ca			Mg			K		
			A	B	C	A	B	C	A	B	C
Min. – Max.	37.8-43.3	25-30	20-30	26-41	31-42	1.42-1.69	1.59-2.1	1.62-2	1.67-2.1	1.97-2.5	2.0-2.7
Mean ± S. D.	40.4±1.9	26.8±1.7	24.8±3.7	34.4±4.6	36.9±4.1	1.53±0.08	1.82±0.16	1.83±0.14	1.94±0.15	2.26±0.18	2.28±0.23
LSD - test	*p<0.001*		A - B *p<0.001*			A - B *p<0.001*			A - B *p<0.001*		
			A - C *p<0.001*			A - C *p<0.001*			A - C *p<0.001*		
			B - C *p=0.189*			B - C *p=0.878*			B - C *p=0.786*		
Chara filiformis											
Sample (n=10)	Ash	Carbonates	Ca			Mg			K		
			A	B	C	A	B	C	A	B	C
Min. – Max.	76-80.3	71-77	33-46	42-47	51-64	1.34-1.51	1.52-1.89	1.58-1.96	1.5-1.76	1.89-2.2	1.86-2.3
Mean ± S. D.	78.2±1.6	73.7±2.6	41.9±4.5	43.7±4.1	46±4.3	1.43±0.06	1.70±0.13	1.75±0.12	1.65±0.08	2.05±0.10	2.05±0.13
LSD - test	*p<0.001*		A - B *p<0.001*			A - B *p<0.001*			A - B *p<0.001*		
			A - C *p<0.001*			A - C *p<0.001*			A - C *p<0.001*		
			B - C *p= 0.241*			B - C *p=0.274*			B - C *p=0.786*		
Chara hispida											
Sample (n=10)	Ash	Carbonates	Ca			Mg			K		
			A	B	C	A	B	C	A	B	C
Min. – Max.	54.7-57.2	52-61	34-42	39-49	38-46	1.4-1.66	1.61-1.92	1.56-1.9	1.5-1.86	1.99-2.3	1.83-2.5
Mean ± S. D.	56.1±0.9	56±2.8	39±2.5	43.3±3.0	42.6±2.9	1.48±0.08	1.76±0.11	1.72±0.13	1.70±0.11	2.10±0.11	2.11±0.19
LSD - test	*p=0.917*		A-B *p<0.001*			A-B *p<0.001*			A-B *p<0.001*		
			A-C *p<0.001*			A-C *p<0.001*			A-C *p<0.001*		
			B-C *p=0.873*			B-C *p=0.446*			B-C *p=0.864*		
Chara intermedia											
Sample (n=10)	Ash	Carbonates	Ca			Mg			K		
			A	B	C	A	B	C	A	B	C
Min. – Max.	62.9-70.5	64-67	31-52	37-47	35-44	1.42-1.72	1.39-1.67	1.41-1.71	1.6-2.0	1.71-2.3	1.66-2.3
Mean ± S. D.	68.2±2.2	65.4±1.0	40.9±7.3	43.9±3.3	42.0±3.4	1.56±0.10	1.48±0.08	1.53±0.10	1.77±0.14	1.96±0.19	2.01±0.18
LSD - test	*p<0.001*		A-B *p<0.001*			A-B *p=0.061*			A-B *p<0.001*		
			A-C *p<0.001*			A-C *p=0.430*			A-C *p<0.005*		
			B-C *p=0.7*			B-C *p=0.259*			B-C *p=0.530*		
Nitellopsis obtusa											
Sample (n=10)	Ash	Carbonates	Ca			Mg			K		
			A	B	C	A	B	C	A	B	C
Min. – Max.	55.3-65.0	55-58	19-34	36-42	32-41	1.48-1.63	1.63-1.95	1.64-1.93	1.72-2.0	1.91-2.3	1.93-2.4
Mean ± S. D.	60.6±1.1	56.7±1.1	24.6±5.1	39±2.0	36.6±2.1	1.56±0.06	1.78±0.10	1.78±0.10	1.94±0.10	2.17±0.14	2.14±0.14
LSD - test	*p<0.001*		A-B *p<0.001*			A-B *p<0.001*			A-B *p<0.001*		
			A-C *p<0.001*			*A-C p<0.001*			A-C *p<0.001*		
			B-C *p=0.896*			B-C *p=0.959*			B-C *p=0.728*		

Method C
Determination of Element Content in PTFE Bombs (without Previous $CaCO_3$ Determination)

The next ten samples were digested using a mixture of HNO_3, $HCLO_4$, and H_2O_2 (following the procedure described above) in PTFE (Teflon) bombs in a CEM microwave oven.

Ash Content

Ash content in the plant biomass was determined by incinerating samples in a muffle furnace at 575ºC for 5 h using the ramping program and the calculation described in the LAP report [24].

Results and Discussion

It is evident from Table 1 that there is a large difference in the contents of carbonates, ash, and elements in charophyte species investigated. This contradicts the findings of high content of carbonates in the charophytes reported in previous studies [2, 14]. Plants collected from freshwater had higher carbonate content in the biomass than *C. baltica* collected in the Baltic Sea. In *N. obtusa* the mean content of carbonates was found to be 56.7% (Table 1) and is consistent with another analysis [25]. This was easily confirmed during field investigation as the content of carbonates, especially of calcium carbonate, is often visible to the naked eye (more so, when the plants are dry).

Similarly, data presented in Table 1 show that ash content in charophytes could vary considerably among the species. Such variations reflect probable differences in bicarbonate uptake during intensive growth of plants and their abundant carbonate content [26, 27]. Ash is sometimes treated wholly as mineral (carbonates) content in plant biomass, but both ash and carbonates statistically differed in the species of *C. baltica*, *C. filiformis*, *C. intermedia* and *N. obtusa* analyzed. No differences were found in the case of *C. hispida*. In most cases, differences between carbonate content and ash were not large and could well reflect the content of carbonate encrustation on the stem; however, the actual carbonate content should be tested separately. These differences, although sometimes not large, could give wrong results when detailed recalculation of elements content, e.g. nitrogen or phosphorus, become necessary.

As described above, three different methods were used for the determination of Ca-, Mg-, and K contents in charophytes to decide the one that would give the best results. Comparisons between the digestion methods using glass Pyrex tubes and Teflon bombs revealed no significant differences (LSD test) (between methods B and C, Table 1). Similarly, no differences were observed between methods A, B, and C for determination of Mg content in *Chara intermedia* (Table 1). Significant differences were, however, observed in the digestion method in glass tubes after carbonate determination (method A) and that in glass tubes or in Teflon bombs without previous carbonate determination (methods B, C). It is likely that the digestion in method A (Table 1) was not fully effective, because of the additional dilution of the HNO_3 and $HClO_4$ mixture with 10 ml HCl (added during calcium carbonate determination), and resulted in showing lower content of elements for all the plants investigated.

Table 2. Reference data by flame AAS obtained during the experiments (percentage - plus/minus SD).

Samples	Ca (%)	Mg (%)	K (%)
Method A - wet digestion after $CaCO_3$ determination			
Populus leaves (10 replicates)	(1.44 ± 0.08)	(0.51 ± 0.08)	(1.19 ± 0.05)
Reference material value	(1.81 ± 0.07)	(0.65 ± 0.03)	(1.38 ± 0.04)
Method B - wet digestion without $CaCO_3$ determination			
Populus leaves (10 replicates)	(1.77 ± 0.04)	(0.69 ± 0.09)	(1.46 ± 0.08)
Reference material value	(1.81 ± 0.07)	(0.65 ± 0.03)	(1.38 ± 0.04)
Method C - wet digestion without $CaCO_3$ determination			
Populus leaves (10 replicates)	(1.85 ± 0.07)	(0.60 ± 0.08)	(1.34 0.07)
Reference material value	(1.81 ± 0.07)	(0.65 ± 0.03)	(1.38 ± 0.04)

Before the analysis was done, it would appear that method A was the easiest for determining element content in charophytes (involving the initial determination of calcium carbonate content, and later digesting the same samples with HNO_3 and $HClO_4$ and, finally, determining the elements) that could reduce laboratory work. This methodology could normally be used for routine identification of carbonates. Unfortunately, in similarly prepared samples (method A), the contents of the elements investigated (Ca, Mg, and K) were lower (compare Table 1) and significantly different than in the other methods of digestion (methods B, C). This indicates that carbonates and elements should be determined separately. Methods B or C produce good results in the determining elements present in charophytes and estimating element content (e. g. Ca, Mg, and K) in biomass.

As mentioned before, without detailed determination of the total amount of carbonates present, comparison between element contents in the samples of charophytes collected from various sites or between charophytes and higher plants is not possible. Furthermore, not only carbonate content, but charophyte carbonate mineralogy and elements found in plants from various ecosystems (e. g. *C. aspera* in fresh and brackish water) could differ significantly. Observations of [8] various marine- and non-marine aquatic environments with varying water chemistries and salinities reveal significant mineralogical variations in both gyrogonite- and stem calcifications. Calcite, high-magne-

sian calcite (HMC), and aragonite might be present in both oogonium and stem calcifications. Some *Chara* species always produce the same biominerals (i.e. only calcite in *C. vulgaris*), whereas other taxa, like *C. globularis*, may form calcite encrustations, HMC, or even aragonite on the stem and HMC within the same salinity range in which *C. vulgaris* mineralizes only calcite. No correlation exists between the charophyte carbonate mineralogy and the salinity of the water where they formed [8], which could be a reason for the intraspecific differences present between the species growing in similar environments. This absence of correlation indicates sharp specific differences in the biological mechanisms of mineralization in this group of plants and before element determinations are attempted single carbonate analysis should be done. But the types of mineralization observed by [8] strongly undermine the importance of determining carbonate content before determining the actual presence of elements. This is especially important for charophytes, which can play the role of a phosphorus sink in various water bodies and could contain phosphorus coupled with calcite encrustation. Comparisons of P- and N content in plants from different habitats, without detailed determination about the carbonate content, are not possible.

Acknowledgements

This study was financially supported by the Polish Ministry of Science and Higher Education, project No. N N303 506238.

The author is grateful to Andrzej Leszek Rudecki and Andrzej Wasik for technical advice, and Ludwik Żołnierz and Bożena Lenicka for help in chemical analysis and sample preparation. I also wish to thank my wife and family for their patience and support during my work on this paper.

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