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TECHNICAL ARTICLE

Accumulation of Arsenic and Copper by Bryophytes Growing in an Aquatic Environment near Copper Mine Tailings

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Abstract Bryophytes with high As accumulation affinity were identified in the aquatic environment. We surveyed a stream near copper mine tailings and then conducted laboratory experiments to confirm As accumulation in the bryophytes with high As affinity. We found that a moss, *Scopelophila cataractae,* accumulates As in addition to Cu in aquatic environments and confirmed it in laboratory experiments. The highest value for As in *S. cataractae* from the field survey was 1300 mg/kg dry weight at relatively low As concentrations in the stream water (0.005 mg/L). In addition, *Brachythecium plumosum* and *Rhynchostegium riparioides* may also be useful bryophytes for accumulation of Cu and As, though the mechanisms of As accumulation might differ between these two bryophytes and *S. cataractae.*

Keywords *Brachythecium plumosum* · Phosphorus · Phytoremediation · *Scopelophila cataractae* · *Rhynchostegium riparioides*

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Introduction

About 7000 abandoned or closed mines exist in Japan (Koide et al. 2012). In the past, these mines caused serious environmental and human health problems (Arao et al. 2010). Treatment of mine drainage and pollution prevention improved after the establishment of the Mining Safety Act of 1949. However, even now, some streams that flow near mine tailings and old mines contain contaminants such as arsenic (As) and lead (Pb) at concentrations above the Japanese environmental standards for public health (Japanese Ministry of Environment 2013). Since most of these mines are located in the upper regions of watersheds, the effects of harmful elements in the outflow of mine tailings on agricultural fields and human health are a concern. For example, low-grade copper (Cu) ores sometimes contain As; therefore, Cu tailings could be a source of As contamination. Arsenic can cause cancer and other As-related diseases (Bhattacharya et al. 2007; Tripathi et al. 2007). Therefore, various remediation techniques for As-contaminated water have been developed (Litter et al. 2010; Mohan and Pittman 2007; Sen Gupta et al. 2009; Vithanage et al. 2012).

Phytoremediation (using plants that accumulate a specific element at high concentrations) is an effective technique to decrease concentration levels in soils or waters contaminated with As and metals (Prasad and Freitas 2003). Various plants, including trees, herbs, aquatic plants, algae, and bryophytes have been evaluated for their phytoremediation potential (Brooks 1998; Pulford and Watson 2003; Vithanage et al. 2012). In the case of As contaminated soils, the fern *Pteris vittata* L. is a well-known As accumulator (Ma et al. 2001). To apply As phytoremediation to the aquatic environment, such as outflow from mine tailings, it is necessary to identify

candidate plants that would potentially be useful for removal of As from water. Favas et al. (2012) studied As accumulation by various aquatic plants, such as submerged species, free-floating species, bryophyte species, and emergent species, in a region of uranium deposits in Portugal, and the highest concentration of As was observed in the submerged species *Callitriche lusitanica* at 2346 mg/kg dry weight (DW). Guimaraes et al. (2012) experimentally evaluated the potential of As removal using three aquatic macrophytes, and found that *Lemna gibba,* which is a free-floating plant, could accumulate 1397 mg/kg DW of As when exposed to 5.0 mg/L of As solution. In applying phytoremediation to flowing conditions such as outflow currents from mountainous mining areas in Japan, bryophyte species grown on rocks were considered more suitable than submerged species or free-floating species because they should be easy to cultivate and harvest. Therefore, we focused on As accumulation by indigenous bryophytes growing on rocks in such aquatic environments.

The purposes of this study were to identify bryophytes with high As accumulation affinity in aquatic environments through field surveys, and to confirm this As accumulation through laboratory experiments. We presumed the following two processes for high As concentrations in bryophytes: adsorption on the surface of the bryophyte tissues and absorption into the interior of bryophyte tissues (Sardans and Peñuelas 2006; Satake et al. 1988), corresponding to physicochemical processes and biological processes, respectively. Therefore, two experimental methods, batch and flowing experiments, were applied and compared. The physicochemical process was confirmed by a 1 h batch experiment and the biological process by a flowing experiment under simulated natural conditions for 1 week.

Materials and Methods

Field Survey

Bryophytes growing around an old Cu mine in Fukui Prefecture, central Japan, were sampled in October and November 2009. The old Cu mine was discovered during the eleventh or fourteenth century, depending on the information source and closed in 1922. Chalcopyrite was mainly mined and refined on site. It was known that the chalcopyrite from this mine contained As (Fukui pref. 1994). A large amount of tailings remained after mine closure and a stream with a number of small tributaries flows through the area. Several types of bryophytes were collected from rocks at 11 different sites in the main stream and its tributaries. Water samples were collected at the same sites five times during the sampling term, and the pH and electrical conductivity (EC) of the water were measured in situ. Water samples were stored in the laboratory at 4 °C until chemical analysis. Identification of bryophytes was conducted morphologically.

Laboratory Experiments

Three species of bryophytes that were found to have relatively high concentrations of As during the field survey were used in both the batch and flowing method experiments. In the batch method, a 10 g sample of shoots packed in a polyethylene mesh bag (1 mm mesh) was soaked in 800 mL of treatment solution in a 1 L polypropylene bottle. The bottle was shaken (Recipro Shaker SR-1, TAITEC) for 1 h and the plant sample was used for chemical analysis after washing with distilled water. Each flowing method-based experiment was conducted using a flowing system as shown in Fig. 1. Each sample (1 g) was placed in a 50 mL polypropylene centrifuge tube with a hole covered by a polyethylene mesh (1 mm mesh) sheet at its bottom. A circulation pump (New Space Power FIT S, Suisaku Co.) connected to nine sample tubes was immersed into 800 mL of treatment solution in a 1 L polypropylene bottle (Fig. 1). The treatment solution flowed into the centrifuge tube containing each sample and returned to the bottle through a funnel. The flow rate was 220 mL/min in each tube. The flowing system was placed in a greenhouse under natural light conditions. The flowing process continued for 1 week with additional deionized water supplied to maintain the water level in the bottle. The averages for temperature and humidity in the greenhouse during the flowing treatment were 16.2 °C and 44.8 %, respectively.

Three kinds of treatment solutions were used: 0.1 mg/L of As and 1 mg/L of As solutions were prepared by adding As standard solution (1000 mg/L As, Wako Pure Chemical Industries Ltd., Osaka) to tap water; the control was tap water with no addition. The initial values of pH and EC of

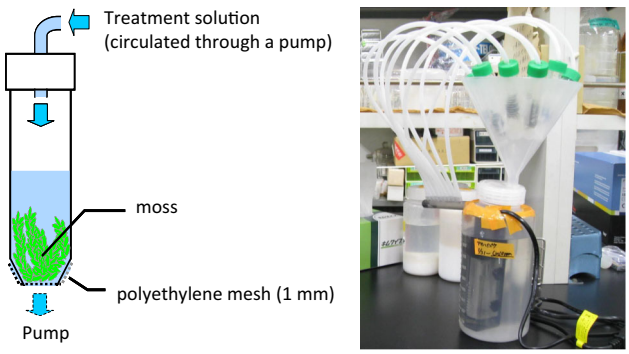

Fig. 1 Apparatus for the flowing experiment

the treatment solutions were 6.5 ± 0.1 and 5.6 ± 0.1 mS/m, respectively. Five replicates of each treatment were conducted in both experiments.

Chemical Analysis

Bryophyte samples were washed carefully with distilled water and dried at 80 °C for 48 h. Whole samples were digested with nitric acid using a microwave digester (ETHOS 1600, Milestone, Sorisole). Water samples were filtered with a 0.45 μm membrane filter. Concentrations of As, Ca, Cd, Cu, Fe, Mn, P, Pb, and Zn in the samples were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES; IRIS ICARP, Jarrel Ash Nippon Co., Yokohama). Bryophytes and water samples with As concentrations below the detection limit of ICP-AES were analyzed by inductively coupled plasma mass spectrometry (ELEMENT 2, Thermo Fisher Scientific, Massachusetts), with internal standardization using ^{103}Rh and ^{185}Re.

Subcellular Fractionation

The subcellular distribution of elements in plant samples from the laboratory experiments (one sample per treatment) was determined using a protocol described by Carrier et al. (2003) with some modifications. A 10 g sample of bryophytes was homogenized in a buffer solution (50 mM HEPES with pH 7.5, 500 mM sucrose, 1 mM DTT) and sieved through a nylon cloth (250 and 11 μm mesh). The solution was then centrifuged at 500*g* for 5 min to obtain the cell wall fraction. The supernatant was centrifuged again at 10,000*g* for 30 min to isolate cell organelles and the soluble fraction containing membranes. All homogenization and centrifugation procedures were performed at 4 °C. Each fraction was digested with HNO_3 and its elemental concentrations determined by ICP-AES and ICP-MS.

Statistical Analysis

Significance differences in the data obtained by chemical analysis among bryophytes species were determined by one-way ANOVA using Tukey's test. Significance differences in As concentration data among the treatments in the laboratory experiments were determined by one-way ANOVA using Scheffe's test.

Results

Nine species of mosses, namely *Anisothecium palustre* (Dicks) I. Hagen [=*Dicranella palustris* (Dicks) A. C. Crundwell], *Brachythecium plumosum* (Hedw) Schimp, *Brachythecium rutabulum* (Hedw) Schimp, *Campylium squarrosulum* (Besch) Kanda, *Niphotrichum barbuloides* (Cardot) Bednarek-Ochyra and Ochyra [=*Racomitrium barbuloides* Cardot], *Plagiomnium vesicatum* (Besch) T. J. Kop, *Rhynchostegium riparioides* (Hedw.) Cardot, *Rosulabryum capillare* (Hedw.) J. R. Spence [=*Bryum capillare* Hedw.], and *Scopelophila cataractae* (Mitt) Broth, and one liverwort *Pellia endiviifolia* (Dicks) Dumort, were found in the stream and tributaries of the old Cu mine area. The concentrations of As, Ca, Cd, Cu, Fe, Mn, P, Pb, and Zn in the dominant five species and stream water are summarized in Table 1. The stream water was not acidic and the average concentrations of As and Pb of the stream water complied with Japan's environmental standards (As: <0.01 mg/L, Pb: <0.01 mg/L, Ministry of Environment), whereas the concentration of Cd exceeded the standard (Cd: <0.003 mg/L). Among the five species, concentrations of As, Cu, Fe, Mn, and Pb were significantly the greatest in *S. cataractae* ($p < 0.01$), while the Ca content of *S. cataractae* was the least ($p < 0.01$). *B. plumosum* and *R. riparioides* also accumulated Cu (8.38 and 1.84 mg/g DW, respectively), but the values were less than those of *S. cataractae*. The As concentration in *B. plumosum* (100 mg/kg DW) followed that in *S. cataractae* (450 mg/kg DW). The concentration of P in *P. vesicatum* was significantly the greatest among the five species, and there was no distinction in the element concentrations of *P. endiviifolia*.

During the laboratory experiments, no visible symptoms of As treatment were observed. The pH values of the treatment solutions did not change in either experiment. Arsenic concentrations in bryophyte samples in the batch and flowing experiments for *S. cataractae*, *B. plumosum*, and *R. riparioides* are shown in Fig. 2. For *S. cataractae* samples, the As concentration significantly decreased in the control samples (67 %), but no changes in As concentrations were observed in the samples exposed to treatment with 0.1 or 1 mg/L As during batch experiments. In contrast, in the flowing experiments, there were increases in the As concentration in the samples exposed to 0.1 and 1 mg/L of As (15 and 16 % increases, respectively), whereas there was a slight decrease in the As concentration in the control (8 % decrease). In the case of *B. plumosum,* there was no significant increase in the concentration of As under the control or 0.1 mg/L of As batch treatment, whereas the 1.0 mg/L of As treatment increased As concentrations by 61 %. Within the corresponding flowing experiment, a significant increase in As concentration was observed with the 1.0 mg/L As treatment (a 174 % increase), and a small increase in As concentration was observed with the 0.1 mg/L As treatment (a 26 % increase). The batch treatment for *R. riparioides* had no effect on As concentration in the samples; however, a significant increase in As concentrations was observed with the 1.0 mg/

Table 1 Concentrations of elements in bryophytes taken from mining stream (mg/gDW) and in stream water (mg/L)

Species	n	As Mean ± SE	Cd Mean ± SE	Cu Mean ± SE	Fe Mean ± SE	Mn Mean ± SE
Scopelophila cataractae	24	0.45 ± 0.04aa	0.031 ± 0.001bb	14.9 ± 0.47aa	45.3 ± 6.65aa	0.89 ± 0.06aa
Brachythecium plumosum	22	0.10 ± 0.03bb	0.045 ± 0.003aa	8.38 ± 0.54bb	12.3 ± 2.75bb	0.21 ± 0.03bb
Rhynchostegium riparioides	38	0.021 ± 0.002bb	0.024 ± 0.002bb, cc	1.84 ± 0.26cc	9.90 ± 1.10bb	0.34 ± 0.03bb
Pellia endiviifolia	4	0.054 ± 0.003bb	0.013 ± 0.002cc	0.19 ± 0.11cc	6.70 ± 2.29bb	0.32 ± 0.06bb
Plagiomnium vesicatum	4	0.005 ± 0.002bb	0.011 ± 0.003cc	0.82 ± 0.28cc	2.41 ± 1.38bb	0.16 ± 0.03bb
Stream water	55	0.005 ± 0.001	0.008 ± 0.002	0.023 ± 0.005	0.001 ± 0.000	0.006 ± 0.002

Species	n	Pb Mean ± SE	Zn Mean ± SE	Ca Mean ± SE	P Mean ± SE	PH Mean ± SE	EC (mS/m) Mean ± SE
Scopelophila cataractae	24	2.39 ± 0.15aa	3.69 ± 0.22b	3.07 ± 0.10aa	0.64 ± 0.03aa, a		
Brachythecium plumosum	22	1.13 ± 0.14bb	4.62 ± 0.33aa, a	7.10 ± 0.23bb, a	1.05 ± 0.11aa, b		
Rhynchostegium riparioides	38	0.80 ± 0.11bb	1.91 ± 0.16bb	8.07 ± 0.21bb, b	1.40 ± 0.08bb, c		
Pellia endiviifolia	4	0.15 ± 0.04bb	1.03 ± 0.15bb	6.30 ± 0.81bb, a	1.20 ± 0.23aa		
Plagiomnium vesicatum	4	0.08 ± 0.04bb	0.89 ± 0.36bb	8.79 ± 0.48bb, b	2.29 ± 0.36cc		
Stream water	55	0.004 ± 0.000	0.880 ± 0.212	6.13 ± 0.72	0.004 ± 0.000	6.67 ± 0.05	4.94 ± 0.25

The different characters of as aa, bb, cc indicated significant differences between the species at $p < 0.01$ by the Tukey test

The different characters of a, b, c indicate significant differences between the species at $p < 0.05$ by the Tukey test

L As treatment in the flowing experiment (a 173 % increase).

The results of subcellular fractionation for As, Cu, and P in *S. cataractae, B. plumosum*, and *R. riparioides* for the laboratory experiments are summarized in Table 2 as ranges of fraction values (%), as only one sample per treatment was analyzed. In *S. cataractae,* most of the As in these samples was distinctly distributed in the cell wall fraction. In contrast, Cu and P were distributed in soluble fractions as well as in the cell wall, followed by the cell organelles fraction in *S. cataractae.* For *B. plumosum* and *R. riparioides,* considerable amounts of As, Cu, and P seemed to be in the soluble fractions.

Discussion

Among the bryophyte species situated in the streams flowing through the Cu mine area, *S. cataractae* is a well-known copper-accumulating species that accumulates Cu at high concentrations (8.44–17.9 g/kg; Satake et al. 1990) from soils. This species has a broad geographic distribution that ranges across North, Central, and South America, Europe, and Asia (Shaw 1995). To date, several studies have examined the ecological and genetic aspects of Cu accumulation in *S. cataractae* as well as its possible accumulation mechanisms (Konno et al. 2010; Shaw 1995). We found that As concentrations in *S. cataractae* was significantly higher than in the other bryophytes studied (Table 1). The highest concentration of As in *S. cataractae* was 1300 mg/kg DW, and its BCF (Bioconcentration Factor = concentration in plant/concentration in water) was calculated as 2.6×10^5, comparable to the highest value (7.6×10^5 in *Oenanthe crocata*, emergent species) reported by Favas et al. (2012). Bakar et al. (2013) reported the potential of three submerged aquatic plant species (*Cabomba piauhyensis, Egeria densa*, and *Hydrilla verticillata*) to be used for As, Al, and Zn phytoremediation in a gold mining area in Malaysia, and the highest As accumulation (196 mg/kg DW, BCF: 1.96×10^5) was observed in *E. densa* after 14 days of exposure. Ruiz-Chancho et al. (2008) reported analytical data for several kinds of bryophytes grown in soils of old mine areas and revealed a high concentration of As (1750 mg/kg) in *Brachythecium* cf. *reflexum* (F. Weber and D. Mohr) Schimp from a site with high levels of As in the soil (21.2 g/kg). Considering the difference in habitats (edaphic compared with aquatic) and relatively low As concentrations in the stream water (0.005 mg/L in Table 1), it is suggested that *S. cataractae* obtained from the Cu mine area has a high affinity for As accumulation in the aquatic environment.

Although accumulation of elements other than Cu, such as Co, Fe, Pb, and Zn in *S. cataractae* is known (Aikawa et al. 1999; Itouga et al. 2005), no evidence of As accumulation in this species has previously been obtained. When investigating the accumulation of various elements including As by *S. cataractae*, Itouga et al. (2005) treated gametophytes of *S. cataractae* with various solutions

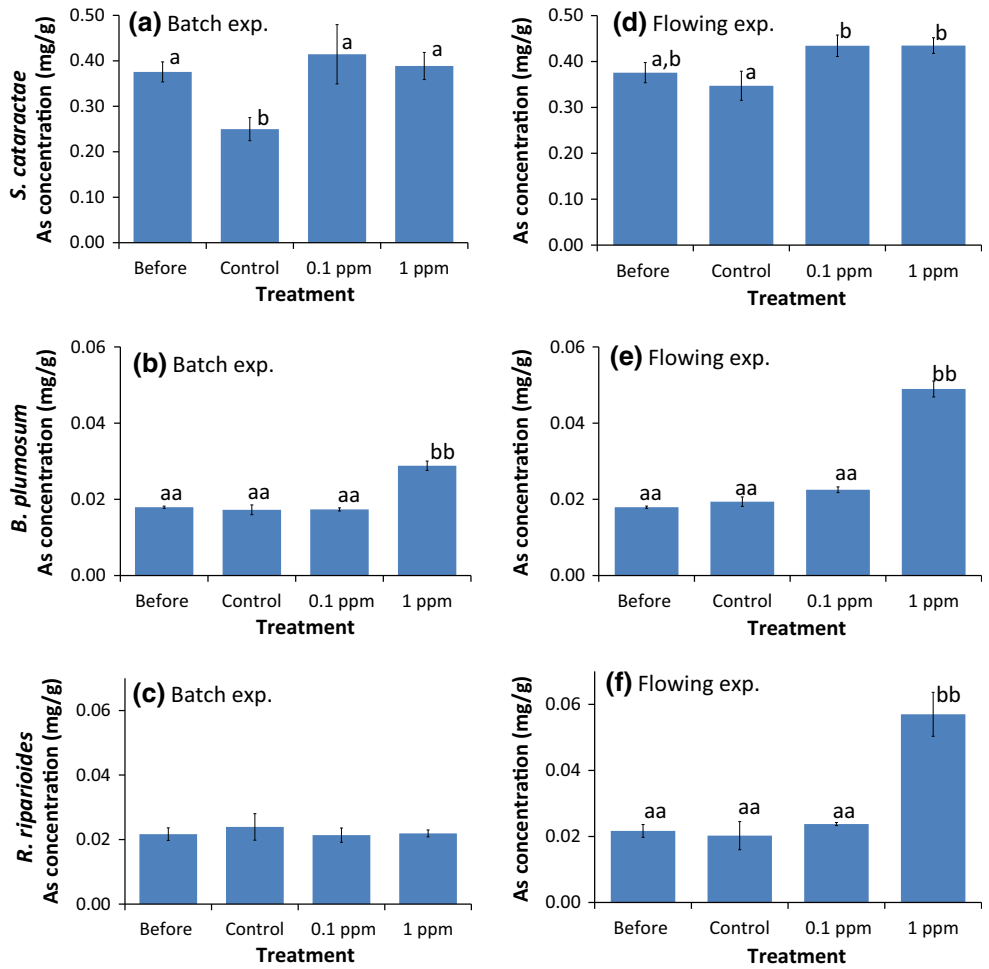

Fig. 2 Arsenic concentrations in bryophyte samples analyzed by batch experiment and flowing experiment. The results for *S. cataractae, B. plumosum* and *R. riparioides* by batch experiment are shown in (**a**), (**b**), and (**c**), respectively. Results for *S. cataractae, B. plumosum* and *R. riparioides* by flowing experiment are shown in (**d**), (**e**), and (**f**), respectively. The *error bar* shows standard error (n = 5). The different letters such as "*aa*" and "*bb*" indicate a significant difference between the treatments with $p < 0.01$, and letters such as "*a*" and "*b*" indicate the significant difference between the treatments with $p < 0.05$

including 15 mM sodium hydrogen arsenate and 15 mM Cu sulfate among many, for two hours, and subsequently performed analysis by analytical X-ray microscopy. No mapping image indicating As accumulation was obtained, but accumulation of Cu was clearly observed. Consequently, *S. cataractae* was concluded to be ineffective for As remediation. However, the results of the present study indicate a high concentration of As in *S. cataractae* from aquatic environments. One reason for the discrepancy between the results of Itouga et al. (2005) and the present study may be the relatively short exposure to As solution (2 h) in the experiment by Itouga et al. (2005) compared with the field observation. The other reasons for disagreement with the results of Itouga et al. (2005) might be attributable to the genetic variation among *S.cataractae* specimens from different habitats, such as the precincts of Toshogu or rocks in the outflow through Cu mines, or to the difference of analytical sensitivity for As between X-ray microscopy and ICP-MS. Further research is necessary.

In batch experiments, As concentration in *S. cataractae* in the control treatment decreased, compared with the original samples. This means that As in the moss was desorbed or dissolved into the tap water. The results from subcellular fractionation analysis for *S. cataractae* showed that 75–87 % of As is distributed in the cell wall. Therefore, a part of As adsorbed onto the cell wall might be easily desorbed through batch extraction. However, As concentration in the control sample did not decrease during the flowing experiment. Given this information, it is hypothesized that the desorption of As by the batch experiment might be caused by a physical process, perhaps by the shaking. Robinson et al. (2006) reported that high-As aquatic macrophytes released As when placed in

Table 2 Distribution of As, Cu and P in cell structure of three bryophytes used in the laboratory experiments

Species	Method	Element	Fraction[a] (%)		
			Cell wall	Cell organelles	Soluble
S. cataractae	Batch	As	75–87	4–11	6–14
		Cu	34–55	11–16	34–53
		P	28–49	16–26	32–52
	Flowing	As	61–82	8–14	10–25
		Cu	33–41	16–18	40–49
		P	21–35	22–30	39–50
B. plumosum	Batch	As	0–30	0–20	70–100
		Cu	3–6	6–9	87–92
		P	3–6	16–22	74–82
	Flowing	As	0–52	0–20	48–100
		Cu	3–5	7–9	87–90
		P	3–5	14–22	74–83
R. riparioides	Batch	As	0–53	0–12	35–100
		Cu	8–13	10–15	72–82
		P	5–8	11–15	78–84
	Flowing	As	0–50	0–12	0–77
		Cu	12–16	15–18	68–73
		P	5–8	12–15	78–83

[a] Data show the range of each fraction for four kinds of samples in each species and method

uncontaminated water, and then hypothesized that As accumulation by aquatic plants occurs via physicochemical adsorption to the plant surface. Our batch experiments results support their hypothesis. In *S. cataractae,* 35–55 % of Cu is distributed in the cell wall. In general, the cell wall is known to play an important role in reducing toxicity of toxic metals by sequestering them into the cell wall matrix (Carginale et al. 2004; Krzeslowska et al. 2009). The results of Cu in *S. cataractae* reported here are consistent with those reported by Konno et al. (2010), indicating that Cu accumulates in cell wall pectin in *S. cataractae*. Zheng et al. (2008) reported that concomitant exposure to Cu sulfate and sodium hydrogen arsenate solutions affects Cu and As concentrations in plant tissues in ferns (pteridophytes). They showed that accumulation of As reduces Cu phytotoxicity in gametophytes of *P. vittata*. In their results, Cu localized to the cell wall and As was distributed in the soluble fraction (mainly cytoplasm) in *P. vittata*. The difference in As distribution between *P. vittata* and *S. cataractae* suggests a different mechanism of As accumulation in each species. The accumulation mechanism for As, probably as arsenite or arsenate, into cell walls in *S. cataractae* has not been clarified. Arsenate, a phosphate analogue, has been reported to be taken up by the phosphate transport system in a wide range of plants (Meharg and Macnair 1994; Ullrich-Eberius et al. 1989). Subcellular fractionation analysis of *S.cataractae* showed that 28–49 % of P was localized in the cell wall. This value was much higher than the levels of P found in cell wall fractions of *B. plumosum* (3.3–6.0 %) or *R. riparioides* (5.2–7.5 %) in Table 2. It is evident that the P concentration in *S.-cataractae* was the lowest among the five species (Table 1). Even if we consider the low P concentration, the amount of P in the cell wall (0.18–0.31 mg/g DW) seemed to be higher than that of the other species (0.07–0.11 mg/g DW for *R. riparioides*). These results suggest that the P accumulation property in cell walls of *S. cataractae* might be related to the higher affinity of this species for As accumulation.

Field observations in the present study indicated that *B. plumosum* is an accumulator for Cu, with a higher affinity for As accumulation, and that *R. riparioides* has a higher affinity for Cu. The results of the laboratory experiment suggested that *B. plumosum* could take up As when treated with a 1 mg/L solution in both batch and flowing experiments. This means that physicochemical adsorption accompanied by biological uptake might be a possible mechanism for As accumulation in *B. plumosum*. In the case of *R. riparioides*, no increase in As concentration accompanied batch treatment, but a significant accumulation was observed by the flowing experiment. These results suggest that only biological uptake was employed for As accumulation. The distribution of As in these two species differ from those of *S. cataractae*. In the case of *B. plumosum,* 70–100 % of the As was located in the soluble fraction (mainly cytoplasm), together with Cu (87–92 %)

and P (74–81 %). Similar fractionation results were obtained in *R. riparioides*. These results indicate that *B. plumosum* and *R. riparioides* might not have a Cu and As tolerance mechanism based on sequestration of these elements in the cell wall, although there were no visible symptoms of toxicity after treatment with 1 mg/L of As. More precise experiments related to the chemical form of As in cell walls and the interaction of As with the P transport system should be conducted to understand the As accumulation mechanism accompanied with Cu accumulation in mosses such as *S. cataractae* and *B. plumosum.*

Conclusion

The field survey and laboratory experiments confirmed that *S. cataractae* grown near Cu mine areas accumulates As in addition to Cu in an aquatic environment. Therefore, *S. cataractae* is a candidate moss for phytoremediation in water contaminated with low levels of As and Cu. In addition, *B. plumosum* and *R. riparioides* may be useful bryophytes for accumulation of Cu and As, but probably by a different accumulation mechanism from that of *S. cataractae*.

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