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Resistance, accumulation and allocation of zinc in two ecotypes of the green alga *Stigeoclonium tenue* Kütz. coming from habitats of different heavy metal concentrations

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Abstract

Response to elevated Zn concentrations of two various ecotypes of the periphytic green alga *Stigeoclonium tenue* Kütz. was studied. An ecotype, classified as *S. tenue* (T), isolated from ditches containing mining water, was Zn-tolerant. It was able to grow and reproduce by zoospores at external Zn concentrations $\geq 15 \mu\text{M}$. Another ecotype of this species, classified as *S. tenue* (S), isolated from unpolluted lake water was Zn-sensitive; its exposure to $15 \mu\text{M}$ and higher Zn concentration caused a significant decrease of chlorophyll content, inhibition of zoospore settling and, finally, death. The morphology of mature thalli of these two ecotypes cultivated under the same conditions also differed, i.e. the erect system of *S. tenue* (T) consisted of filaments with long, narrow cells, while the erect system of *S. tenue* (S) consisted of filaments with shorter, barrel-shaped cells. Therefore, *S. tenue* (S) thalli were more compact than thalli of *S. tenue* (T). *S. tenue* (T) exposed to $30 \mu\text{M}$ Zn for 3 weeks was able to release zoospores, while *S. tenue* (S) was not. The two algal ecotypes differed in the amount of intracellularly accumulated Zn and Pb. The Zn-tolerant ecotype *S. tenue* (T) accumulated (independently of the exposure conditions like pH and orthophosphate concentration) significantly more Zn and Pb than the Zn-sensitive ecotype. A new cytochemical method was developed to visualise intracellular Zn in algal cells. In the long-term, Zn-exposed cells of both ecotypes, pink-orange Zn–dithizone complexes were observed in peripheral vacuoles, while in cells not exposed to Zn, no deposits were present. The data obtained suggest that the Zn-tolerant ecotype *S. tenue* (T) is able to detoxify the excess of accumulated zinc more effectively than the sensitive ecotype, however, vacuolar Zn compartmentalisation does not seem to be the main tolerance mechanism.

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1. Introduction

Enrichment of heavy metals in the bed and water of streams and rivers selects for a particular micro- and macroflora over a long period of time (Genter et al., 1988; Takamura et al., 1989). Periphytic filamentous green algae belonging to the genus *Stigeoclonium* (Chaetophorales) are organisms of high plasticity, living at a broad range of environmental conditions differing in pH, salinity, and nutrient and pollutant concentrations (McLean and Benson-Evans, 1974; Francke and Rhebergen, 1982; Francke and Ten Cate, 1980; Kinross et al., 2000). *Stigeoclonium tenue* is frequently reported as an alga inhabiting waters polluted by heavy metals, and alga populations from sites with high zinc levels are more resistant to zinc than those from sites with low zinc levels (Harding and Whitton, 1976; Kelly and Whitton, 1989; Takamura et al., 1990). However, the mechanisms underlying the Zn-resistance and development of metal-resistant populations of algae are not recognised. In this paper, Zn-resistance, intracellular zinc and lead accumulation and Zn localisation in two different ecotypes of *S. tenue* Kütz., isolated from habitats differing in heavy metal content, were compared to look for possible mechanisms of Zn-adaptation. A new cytochemical method was developed, which enabled visualisation of intracellularly accumulated Zn in the studied algae.

2. Materials and methods

2.1. Isolation and cultivation of algae

S. tenue Kütz. grew abundantly in a drainage stream flowing from a zinc and lead ore mine in southern Poland. It was isolated at the end of April 1998. The mining water contained mainly Zn ($15\ \mu\text{M}$); its chemical characteristics were reported earlier (Pawlik-Skowrońska, 2001). The alga was cultivated in modified Woods Hole medium, pH 7.2 (Simons et al., 1986) under laboratory conditions ($21 \pm 1\ ^\circ\text{C}$, in a 12:12 light–dark regime, light $35\ \mu\text{mol m}^{-2}\text{s}^{-1}$). Taxonomic investigation of this strain, based on the type of zoospore germination and thallus propagation, was carried out according to Simons et al. (1986). This ecotype of alga was classified as *S. tenue* (T). Another ecotype, classified as *S. tenue* (S), was isolated from unpolluted lake water in the Reserve Area Botshol in the Netherlands and cultivated under the same laboratory conditions as *S. tenue* (T). Morphological observations were made using a light microscope.

2.2. Zn-sensitivity

Mature thalli of each ecotype (50 mg fresh weight) were exposed to $15\ \mu\text{M}$ zinc (as nitrate) in Woods Hole medium, pH 7, for 8 days under the temperature and illumination conditions mentioned in Section 2.1. Both algae without Zn addition were also cultivated (control cultures). Three biomass samples were withdrawn from each culture for chlorophyll determinations (made in two repetitions). Chlorophyll ($a + b$) content was determined in algal biomass after extraction in 80% acetone and calculations were made according to McKinney (1941). The dry weight (DW) was determined by drying the algae at $90\ ^\circ\text{C}$.

2.3. Long-term Zn effect on algal growth and zoospore settling

Thalli of 60 mg fresh weight (FW) of each ecotype were inoculated in Woods Hole medium (of various orthophosphate concentrations), pH 7, spiked with 30 μM Zn and cultivated under the above conditions for 3 weeks. Algal cultures without Zn addition were regarded as controls. Macroscopic and microscopic observations of thallus morphology and zoospore germination were carried out. Chlorophyll ($a + b$) content was also determined.

2.4. Zn and Pb accumulation

In short-term accumulation experiments, 30 mg FW of algal biomass was placed for 17 h in a non-complexing solution of 5 mM Hepes buffer (pH 6.8 or 8.2) containing 2.2 mM $\text{Ca}(\text{NO}_3)_2$ and 15 μM $\text{Zn}(\text{NO}_3)_2$ or $\text{Pb}(\text{NO}_3)_2$. In long-term accumulation experiments, 60 mg FW was placed for 3 weeks in the modified Woods Hole medium of lowered orthophosphate concentrations (0.01 or 0.3 mg P l^{-1}) containing 30 μM $\text{Zn}(\text{NO}_3)_2$. Four independent algal samples were then withdrawn from each culture, washed three times with 5 mM EDTA and deionised water to remove superficially bound metals and mineralised. Wet mineralisation of the dry biomass was carried out using HNO_3 and H_2O_2 (2:1, v/v) and ultrasonication. The Zn and Pb contents in the mineralised biomass samples were determined by means of atomic absorption spectrophotometry (AAS).

2.5. Ultrastructural Zn localisation

Algae were cultivated in Woods Hole medium spiked with 15 μM $\text{Zn}(\text{NO}_3)_2$. Then algae were separated from the medium by filtration and washed with 5 mM EDTA and deionised water. A rapid cytochemical method was developed to detect Zn in algal cells. Cytochemical staining of zinc in cells was achieved by placing *Stigeoclonium* filaments in 0.005% dithizone made up in 50:50 ethyl alcohol and deionised water. Cells were treated with this solution for 15 min at room temperature. The dithizone solution did not cause cell plasmolysis. The analysis of intracellular localisation of zinc was carried out with the light microscope by looking for pink-orange complexes of dithizone with heavy metals (Marczenko and Balcerzak, 1998). Controls consisted of filaments not subjected to Zn, but otherwise treated exactly as the tests.

3. Results

The *S. tenue* ecotypes isolated from the freshwaters of different zinc content were compared for their morphology, asexual reproduction, Zn-resistance, intracellular Zn and Pb accumulation, as well as Zn compartmentalisation. The morphology of mature thalli of the ecotypes studied differed from each other, concerning their erect systems, while the prostrate systems of the algal thalli were similar. The erect system of *S. tenue* (T) consisted of long filaments with long, narrow cells, while *S. tenue* (S) filaments consisted of shorter, barrel-shaped cells (Fig. 1). As a consequence, thalli of *S. tenue* (T) were less compact than the thalli of *S. tenue* (S). High Zn concentrations exerted different impacts on the ecotypes.

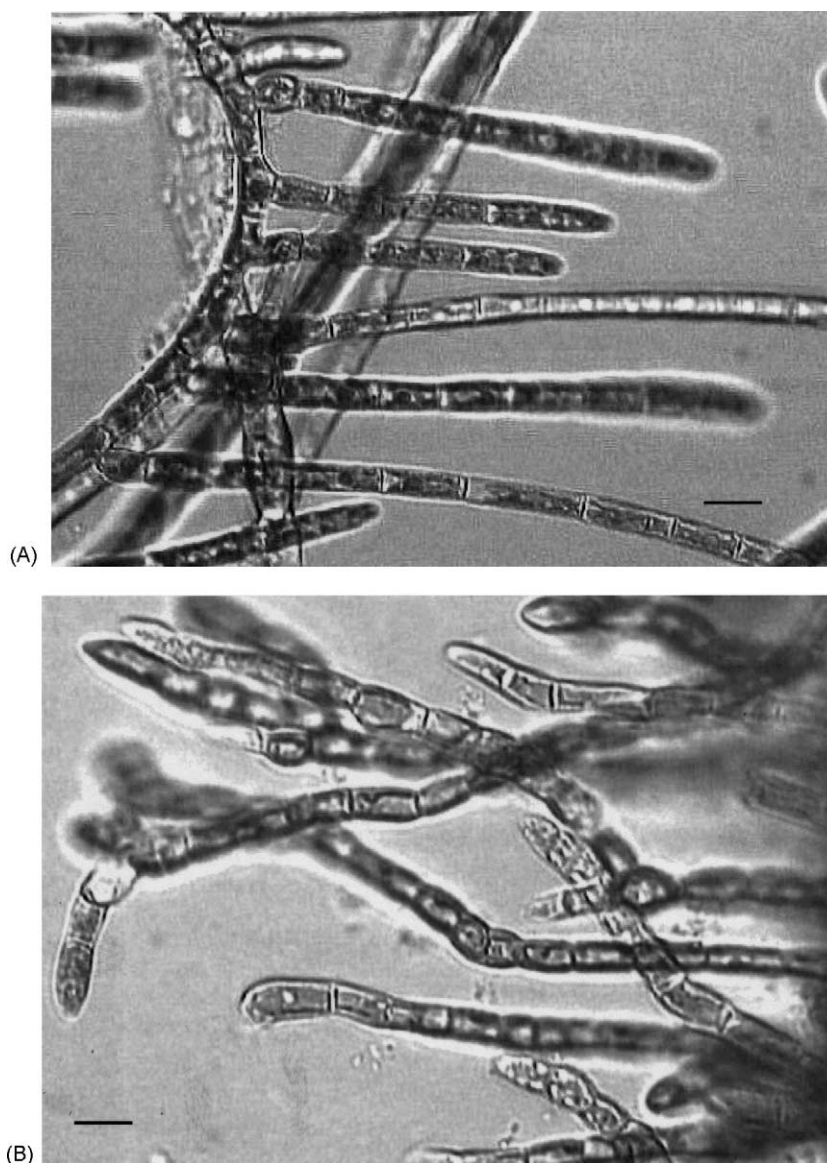


Fig. 1. Light micrographs of filaments of erect systems: (A) *S. tenue* (T); (B) *S. tenue* (S), unfixed. The bars indicate 10 μm .

During long-term exposure to 30 μM Zn, *S. tenue* (T) released zoospores which developed to young thalli, while the Zn-sensitive ecotype did not. As shown in Table 1, 15 μM Zn was toxic for *S. tenue* (S) isolated from unpolluted water. During an 8-day exposure, its thalli bleached compared with non-exposed control thalli; chlorophyll ($a + b$) content decreased

Table 1

Effect of Zn on the chlorophyll (*a* + *b*) content in thalli of two ecotypes of *S. tenue* exposed under the same conditions

Algal ecotype	Zn exposure	Chlorophyll content ($\mu\text{g mg}^{-1}$ DW)		
		0 day	5 days	8 days
<i>S. tenue</i> (T)	Control	15.5 ± 1.29	48.0 ± 2.36	52.1 ± 1.27
	15 μM Zn	15.5 ± 1.29	42.0 ± 3.09	46.7 ± 4.54
<i>S. tenue</i> (S)	Control	32.2 ± 2.57	38.5 ± 0.91	48.7 ± 0.73
	15 μM Zn	32.2 ± 2.57	$32.5 \pm 1.09^*$	$25.2 \pm 1.82^*$

Results are verified with Student's *t*-test.

* Difference significant at $P = 0.05$.

by 48%. In *S. tenue* (T), isolated from mining water with an elevated Zn concentration, such symptoms of metal toxicity were not observed. During the 3-week Zn exposure (Table 3), thalli of the Zn-sensitive *S. tenue* (S) became yellowish and their chlorophyll content was lower by 60% compared with the green thalli of the Zn-resistant ecotype *S. tenue* (T).

Independent of the conditions of metal exposure (time, pH of surrounding medium and orthophosphate concentration), *S. tenue* (T) accumulated intracellularly significantly more Zn and Pb than *S. tenue* (S) (Tables 2 and 3). At alkaline pH (8.2), only Pb accumulation was lower than at a near-neutral pH (6.8). After long-term exposure (Table 3) to 30 μM Zn in a nutrient solution, the green thalli of *S. tenue* (T) contained approximately 13-fold more Zn than the bleached thalli of *S. tenue* (S). Elevated orthophosphate concentration (0.3 mg P l^{-1}) in solution did not decrease significantly the Zn accumulation in both ecotypes compared with exposure at a low P concentration (0.01 mg l^{-1}).

To study intracellular localisation of accumulated zinc in *S. tenue*, a new simple cytochemical method using dithizone was developed. When the dark green solution of dithizone contacted fresh filaments of the long-term Zn-exposed *Stigeoclonium*, pink-orange deposits

Table 2

Intracellular metal accumulation in thalli of two ecotypes of *S. tenue* after 17-h exposure to Zn and Pb in non-complexing solutions

Metal exposure	Algal ecotype	pH	Metal content ($\mu\text{g mg}^{-1}$ DW)
Zn (15 μM)	<i>S. tenue</i> (T)	6.8	0.88 ± 0.13
	<i>S. tenue</i> (S)	6.8	$0.18 \pm 0.03^*$
	<i>S. tenue</i> (T)	8.2	0.77 ± 0.16
	<i>S. tenue</i> (S)	8.2	$0.37 \pm 0.06^*$
Pb (10 μM)	<i>S. tenue</i> (T)	6.8	0.86 ± 0.06
	<i>S. tenue</i> (S)	6.8	$0.54 \pm 0.08^*$
	<i>S. tenue</i> (T)	8.2	0.38 ± 0.06
	<i>S. tenue</i> (S)	8.2	$0.16 \pm 0.03^{**}$

The Zn content in the algal biomass before Zn exposure was $0.14 \pm 0.03 \mu\text{g mg}^{-1}$ DW. Results were verified with Student's *t*-test.

* Difference significant at $P = 0.05$.

** Difference significant at $P = 0.1$.

Table 3

Intracellular Zn accumulation and chlorophyll (*a* + *b*) contents in thalli of two ecotypes of *S. tenue* exposed for 3 weeks to 30 μM Zn in a modified nutrient medium of different orthophosphate contents

Algal ecotype	$\text{PO}_4\text{-P}$ (mg l^{-1})	Zn content ($\mu\text{g mg}^{-1}$ DW)	Chlorophyll content ($\mu\text{g mg}^{-1}$ DW)
<i>S. tenue</i> (T)	0.01	8.13 ± 0.34	31.0 ± 3.82
	0.30	7.78 ± 0.39	34.1 ± 3.27
<i>S. tenue</i> (S)	0.01	$0.62 \pm 0.02^*$	$11.7 \pm 1.09^*$
	0.30	$0.61 \pm 0.03^*$	$15.4 \pm 2.18^*$

Results verified with Student's *t*-test.

* Difference significant at $P = 0.05$.

of zinc–dithizone complexes were observed in peripheral vacuoles (Fig. 2C and D). Such Zn localisation was characteristic for both ecotypes. However, in the Zn-sensitive ecotype, only filaments which survived, revealed the Zn compartmentalisation in vacuoles. In short-term exposed thalli (2 days), there were no colourful deposits in vacuoles, but the entire cytoplasm was homogeneously coloured, indicating that Zn had not been sequestered

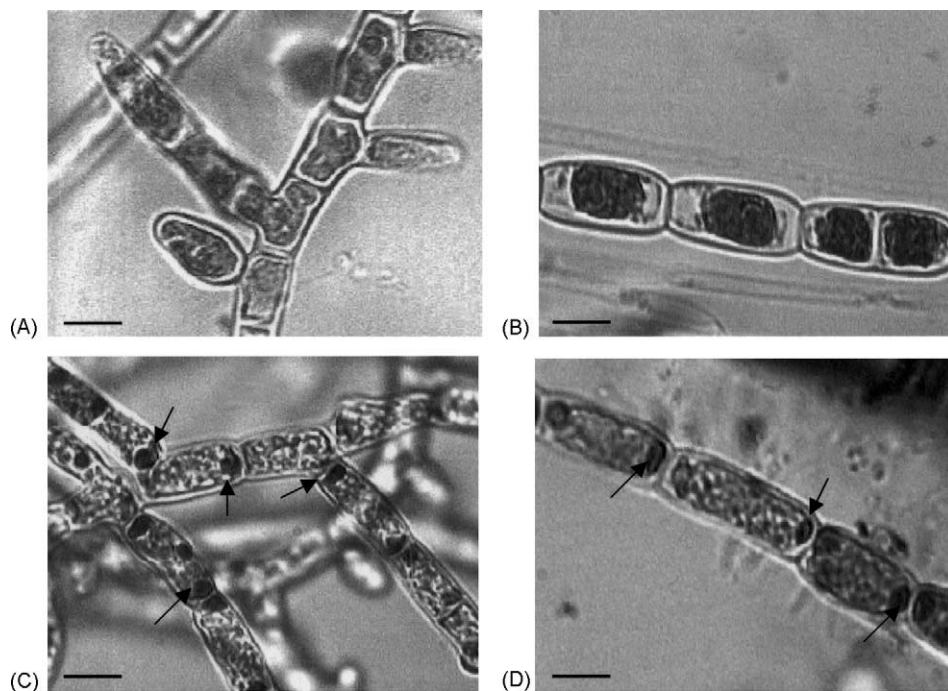


Fig. 2. Dithizone treatment of fresh *Stigeoclonium* shows deposits of intracellular Zn in algal cells. (A) *S. tenue* (T) control (not exposed to Zn); (B) thalli of *S. tenue* (T) exposed to 15 μM Zn for 2 days; (C) thalli of *S. tenue* (T) exposed to 15 μM Zn for 6 weeks; (D) filaments of *S. tenue* (S) exposed to 15 μM Zn for 6 weeks. Arrows show deposits of Zn–dithizone complex in peripheral vacuoles. The bars in (A) and (C) indicate 6.25 μm ; in (B) and (D) 4.55 μm .

in vacuoles (Fig. 2B). In control preparations, not exposed to Zn but treated with dithizone, neither colourful deposits in vacuoles nor stained cytoplasm were observed.

4. Discussion

Stigeoclonium tenue may be considered one of the more successful algae in zinc-polluted waters. The alga sampled from waters of high Zn concentrations contained more Zn in biomass than the alga sampled from waters of low Zn concentrations, however, the sampling sites differed in chemical composition (Kelly and Whitton, 1989). Data presented in this paper on the intracellular Zn and Pb accumulation in two different ecotypes of *S. tenue* are in agreement with the reported observations.

It is well documented that heavy metal availability, accumulation and toxicity in aquatic biota depends essentially on many environmental variables (Campbell and Stokes, 1985; Skowroński, 1986; Skowroński et al., 1991; Pawlik-Skowrońska and Skowroński, 2001; Pawlik-Skowrońska, 2002). As reported previously (Pawlik-Skowrońska, 2001), abiotic factors like high pH and the content of suspended matter in water play an essential role in modulating heavy metal bioavailability to *S. tenue* by changing metal speciation and lowering the level of labile metal forms. Environmental factors may also influence Zn accumulation and toxicity to algae (Harding and Whitton, 1977; Vymazal, 1986). Usually, in algae found in the field, the amount of accumulated metals reflects both the population-specific accumulation capability and various metal bioavailabilities depending on the environmental conditions.

The data presented in this paper show, however, that two *S. tenue* ecotypes accumulated, intracellularly, various amounts of heavy metals under the same exposure conditions. The ecotype, isolated from Zn-polluted mining water, independent of exposure conditions (time, pH and PO₄-P contents), always took up more Zn and Pb than the ecotype from unpolluted water. The data presented strongly suggest that the observed differences in metal accumulation between *S. tenue* (T) and *S. tenue* (S) can be due to the ecotype properties more than to the exposure conditions. *S. tenue* (T), the ecotype from mining water, grew well (developing long filaments) and reproduced by zoospores at high Zn concentrations in its body (8 µg mg⁻¹ DW). According to Levitt's definition (1980), this ecotype is truly Zn-tolerant. *S. tenue* (S), the ecotype from unpolluted water, was Zn-sensitive. Its chlorophyll content decreased considerably compared with that of the tolerant ecotype and it did not survive at 15 µM and higher Zn concentrations, in spite of much lower internal Zn concentration (<1 µg mg⁻¹ DW). Chlorophyll damage and photosynthesis breakdown in heavy metal stressed aquatic plants and algae is well documented (Küpper et al., 1996, 2002). As reported by Knauer et al. (1997), Zn concentrations higher than 1 µM inhibited the growth rate of freshwater green microalgae *Scenedesmus subspicatus* and *Chlamydomonas reinhardtii*. These toxic effects occurred in microalgae when the intracellular Zn concentration was higher than 3×10^{-5} mol g⁻¹ algae (i.e. 1.95 µg Zn mg⁻¹ algae). In *S. tenue* (S), toxic effects were observed at half the intracellular Zn concentration than that mentioned above, while *S. tenue* (T) was tolerant of a four-fold higher intracellular Zn content.

The ecotypes of *S. tenue* also revealed differences in thallus morphology. Such morphological plasticity has been reported for other macroalgae, and for populations of

S. tenue inhabiting sites of different salinity (Francke and Rhebergen, 1982). As reported by Harding and Whitton (1976), tolerance to Zn might be lower during a particular phase in the life cycle of algae and in *S. tenue*; increasing Zn levels brought about a reduction in the erect part of the thallus. Microscopic observations revealed that in *S. tenue* (T), both filaments of the erect part and zoospores were Zn-tolerant and germinated to young thalli, while Zn-exposed *S. tenue* (S) did not release settling zoospores and its erect filaments were reduced.

Mechanisms underlying metal tolerance in algae are not fully recognised and may rely on several mechanisms operating simultaneously. Silverberg (1975) suggested that intravacuolar compartmentalisation of Pb can be a mechanism of detoxification of the metal excess in *S. tenue*. It was also estimated (Reid et al., 1996) that in the giant cells of *Chara corallina*, most of the intracellular zinc was stored in vacuoles. In this paper, a new method for Zn localisation in algal cells, using dithizone, has been presented. In 2-day exposed algal thalli, pink-orange Zn–dithizone complexes were homogeneously spread in the cytoplasm, however, in 6-week exposed algae, colourful Zn complexes were visible in peripheral vacuoles. They were observed in cells of both ecotypes of *S. tenue*. This simple and rapid method gives, similar to the rhodizonate staining of Pb (Silverberg, 1975), only qualitative information on Zn localisation in algal cells. The results obtained suggest that vacuoles in *S. tenue* are involved in Zn immobilisation and detoxification, however, it seems insufficient for the observed Zn-tolerance of *S. tenue* (T). Other adaptive (present only in tolerant phenotypes) mechanisms for coping with elevated metal concentrations in cells have to exist and operate in the Zn-tolerant alga. Such mechanism (Pawlik-Skowrońska, *in press*) seems to be connected with effective intracellular Zn complexation by sulphur-rich peptides.

5. Conclusions

Populations of the filamentous green alga *S. tenue* Kütz. from different habitats appear to be different ecotypes with different erect system morphologies and Zn-tolerance. *S. tenue* (T) isolated from mining water, has a higher capability to accumulate Zn and Pb intracellularly than *S. tenue* (S), the ecotype from unpolluted water. *S. tenue* (T) is Zn-tolerant; it was able to grow and reproduce by zoospores at high internal Zn concentrations. *S. tenue* (S) is a Zn-sensitive ecotype; it was poisoned in spite of a much lower internal Zn content. Lower metal accumulation in the Zn-sensitive ecotype was not a consequence of limited heavy metal availability. As shown by a new cytochemical method, intravacuolar Zn sequestration takes place in both ecotypes. However, it is not sufficient to protect the Zn-sensitive ecotype against metal toxicity. Another adaptive mechanism must be involved in the observed Zn-tolerance of the ecotype grown in mining water.

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