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PHYTOREMEDIATION AS A PROMISING TECHNOLOGY FOR WATER AND SOIL PURIFICATION: *AZOLLA CAROLINIANA* WILLD. AS A CASE STUDY

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A b s t r a c t. The environmental pollution resulting from natural resources acquisition is one of the most severe problems nowadays. New environmental friendly and economically attractive techniques are proposed, using the ability of microorganisms (bioremediation) or plants (phytoremediation) for detoxification of their substrate. Depending on the type of pollutant and the mechanism of its immobilisation (accumulation or decomposition), several techniques are proposed. The specialised plant species are called hyperaccumulators, e.g. *Brassica juncia*, *Helianthus annuus*, *Nicotiana tabacum* or genetically modified *Arabidopsis thaliana*. *Azolla caroliniana* Willd. (*Azollaceae*) is an aquatic fern occurring in temperate and tropical climates. Recently, some natural stands of it were found in western Poland. The fern lives in symbiosis with cyanobacterium *Anabaena azollae* which is capable of fixing atmospheric nitrogen. Besides numerous application of *Azolla* in agriculture, e.g. as green manure, it was found that this plant possess a huge ability for phytoremediation. Our studies showed its ability for removal and accumulation of Hg, Cd, Pb, Cr, As, Ag, Pt and Au from waters (up to 100% of applied doses). These promising results open a new application of *Azolla* spp. for the purification of water polluted by heavy metals, for example as an additional step of wastewater purification.

Keywords: *Azolla*, heavy metals, phytoremediation, wastewater

INTRODUCTION

The environmental pollution is one of the most severe problems nowadays. Activities such as ore mining, crude oil extraction, their processing and use generate many wastes which are further transferred to waters, soils or emitted into the atmosphere, causing their pollution. Moreover, accumulated pollutants may have adverse effects to living organisms leading eventually to ecosystem degradation.

The observed progress in devastation of the environment highlights the need of measures which would help to restore the original state of each compartment of the environment providing, in other words, a remedy for the pollution.

Besides numerous physicochemical waste treatment methods (e.g. extraction, precipitation, ion exchange), there are also measures involving the use of living organisms possessing natural abilities of either the removal of pollutants or their degradation from organism substrate. This technique is called biotic remediation and it is broadly used where there is a need for water purification and soil reclamation. Depending on the organism used, three types of biotic remediation can be distinguished: bioremediation (microorganisms), mycoremediation (fungi) and phytoremediation (plants). The organisms are capable of inactivating both organic and inorganic compounds.

Most of the methods currently used focus on the use of different microbial organisms capable of reducing the concentration of certain pollutants due to their specific abilities, which is called bioremediation. The process can occur spontaneously as a part of microbial metabolism or be stimulated by providing optimal conditions for bacterial growth and metabolic activity. It is very common in wastewater treatment plants (2^{nd} step of purification) and is also used for soil reclamation from heavy metals, explosives, and organic wastes (Błaszczyk 2007). Fungi were also found to be efficient organisms in the degradation of many organic substances (Sing 2006), especially their mycelia are very effective in the decomposition of xenobiotics (Thomas 2000).

Plants have been successfully applied in soil reclamation in many remediation projects, both for organic and inorganic compounds. These organisms can filter out soil from accumulated heavy metals and immobilize them in their tissues. In addition, they can produce enzymes which allow decomposition of organic pollutants to non-toxic end-products such as carbon dioxide or water. Degradation can occur both in the plant tissue or in the rhizosphere outside of plant organism, which makes many different phytoremediation techniques available. For heavy metals and radionuclides, the process consists in the extraction and then transport, translocation (e.g. to aboveground parts) and hyperaccumulation. Organic compounds are mineralised after transport, depending on plant, and the end-products may be used by the plant, stored or volatilised. The following methods are used: phytoextraction, phytodegradation, rhizofiltration, phytostabilisation and phytovolatilisation (Salt *et al.* 1998, Meagher 2000).

There are many specialised plant species, called hyperaccumulators, suitable for phytoremediation. Indian mustard, *Brassica juncia* L., is a well known example of such a species; it is able to rapidly concentrate Cd(II), Ni(II), Pb(II), and Sr(II) into root tissues at levels 500-times greater than those in its liquid growing medium (Salt *et al.* 1995, Salt and Kramer 1999). Sunflowers were proved to concentrate uranium 30,000 fold from water (Dushenkof *et al.* 1997). Tobacco roots were able to reduce Hg(II) concentrations in water medium 100 times (the initial levels ranged 1-5 ppm, Heaton *et al.* 1998). There are also Cr accumulators such as *Dicoma niccolifera* Wild. (Wild 1974), *Sutera fodina* Wild. (Baker and Brooks 1989), *Pearsonia metallifera* Wild. (Wild 1974), *Berkheya coddii* Roessler (Morrey and Balkwill 1989) and *Solanum elaeagnifolium* (Rascon *et al.* 2000). There are attempts to use transgenic plants such as yellow poplar *Liriodendron tulipifera*, L. and *Arabidopsis thaliana* for the removal of Hg(II) (Rugh *at al.* 1996, 1998a,b). Also many aquatic species have a huge potential for the removal of heavy metals from waters (Dhir 2010).

Azolla caroliniana Willd. (*Azollaceae*) is a small (1-5 cm) floating aquatic fern occurring on the surface of warm, eutrophic, still waters in temperate and tropic climates. In Poland occasional stands of *Azolla filiculoides* L. have been reported (Wołkowycki 1999, Szczęśniak *et al.* 2009). The fern lives in symbiosis with cyanobacterium *Anabaena azollae* Strasb. (*Nostocaceae*) which fixes atmospheric nitrogen. This feature made *Azolla* a very useful plant in food production (e.g. on paddy fields) as green manure. In addition, it is used for biological control of weeds, mosquitoes, larvae and soil nematodes. The fern has also been applied for biogas and hydrogen fuel production (Wagner 1997).

Azolla has been tested for its ability for water purification. In numerous studies it was used for the filtration of water polluted with heavy metals, nutrients and organic compounds. It was shown that it could be an efficient accumulator of Cr (Zhao and Duncan 1997), Ni (Zhao and Duncan 1998), Au (Antunes *et al.* 2001), As (Zhang *et al.* 2008), Cd, Cu, Zn, Pb (Sela *et al.* 1989; Rakhshaee *et al.* 2006), Sr (Cohen-Shoel *et al.* 2002) and others (Sood *et al.* 2011). It can also remove sulpha drugs (Forni *et al.* 2001).

In our laboratory, we have conducted several studies to test the ability of *Azolla* to remove heavy metals, nutrients and organic compounds such as medicines, detergents and pesticides from waters. Unlike in some studies, we have tested the living organism. We would like to investigate the tolerance of *Azolla* to chosen water pollutants and if it could be used as a hyperaccumulator of them. This paper is an overview of our studies, the results of which have already been partially published.

MATERIAL AND METHODS

The *Azolla* plants originated from our collection which has been initiated by obtaining plants from the Warsaw Botanical Garden where it was classified as the species *Azolla caroliniana*.

The experiments were conducted in glass aquaria containing 3 dm^3 of liquid nutrient medium IRRI which is a mixture of micro- and macronutrients without nitrate form (Watanabe *et al.* 1992) in order to provide *Azolla* only symbiotic nitrogen. This solution was enriched in selected metals: Hg, Pb, Cr, Cd, Au, Ag, Pt at varying concentrations (0.1, 0.5 and 1.0 mg dm^{-3}) as $HgCl_2$, $PbCl_2$, $CrCl_3 \cdot 3H_2O$, $K_2Cr_2O_7$, $CdCl_2 \cdot 2½H_2O$, $H[AuCl_4]$, Ag_2SO_4, $H_2[PtCl_6]$. The control treatment contained only IRRI solution. A portion of 20 g of fresh plant was placed in each unit and the experiment was conducted for about 2 weeks under atmospheric temperature and humidity (25±2°C and 70±5%, respectively). *Azolla* plants were illuminated by artificial light for 16 h per day.

The ability of *Azolla* to remove and accumulate selected metals was estimated by the measurement of metal concentration in the medium over time (FAAS and GFAAS, Hitachi Z-2000, Japan) and at the end of the study in dry plant material after microwave destruction. In addition, biomass change was estimated. Additional parameters were estimated in order to characterise the growing conditions by the measurements of redox potential (Eh), pH and microdiffusion of oxygen (ODR), and to estimate plant defence mechanisms (activity of superoxide dismutase, SOD).

Obtained data were processed using ANOVA and correlation studies, the significance was accepted at $p<0.05$.

RESULT AND DISCUSSION

Heavy metals accumulation

During the experiment a continuous lowering of the concentrations of each tested metal in the medium was observed.

A day after the onset of the study, Hg(II) levels in all tested treatments were lower by about 50%, followed by a further decrease to values below 0.1 mg dm^{-3} ($p<0.001$). After 12 days the removal of Hg(II) amounted to 75%, 93% and 93% from the treatments containing 0.1, 0.5 and 1.0 mg dm^{-3} Hg(II), respectively (Bennicelli *et al.* 2004). A similar trend was observed for Pb(II), and the total decline of this metal was on the level of 82 and 90% for doses of 0.1 and 0.5 mg Pb(II) dm^{-3}, respectively, showing significantly lower concentrations ($p<0.001$). Only the highest concentration of Pb(II) 1 mg dm^{-3} was removed in 47.6%. During exposure to Cd(II) ions, the concentration of this metal in the medium did not change significantly at the lowest applied dose (0.1 mg dm^{-3}) and the decrease was only of 10%. Higher concentrations showed small (but significant, $p<0.001$) reduction of Cd(II) levels, resulting in final values lower by 18 and 22% for treatments with 0.5 and 1.0 mg Cd(II) dm^{-3}, respectively (Stępniewska *et al.* 2005). *Azolla* was also able to significantly reduce Cr concentrations in water. In the case of Cr(III) the final decrease constituted 91%, 90% and 74% of the initial concentration of Cr(III) of 0.1, 0.5 and 1 mg dm^{-3}, respectively. There was complete depletion of Cr(VI) ions (100%) in the treatment with 0.1 mg dm^{-3} where the concentration of tested metal on the fourth day of study was below detection limit. Higher doses of this metal were removed in 84 and 88% in treatments with increasing concentration, respectively (Bennicelli *et al.* 2004).

The response of *Azolla* to heavy metals presence was estimated by change of its biomass after the whole experiment. The fresh biomass of the fern exposed to different doses of Hg and Cr is shown in Figure 1.

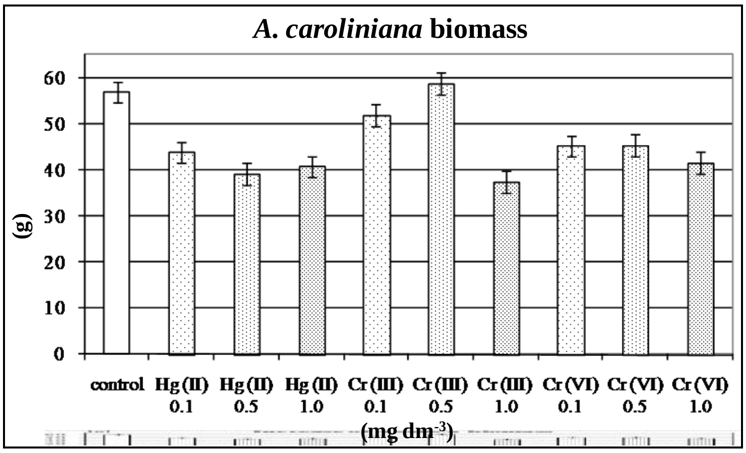

Fig. 1. Biomass of *Azolla caroliniana* (means±SEM) exposed to Hg and Cr (in doses of 0.1, 0.5 and 1.0 mg dm^{-3}) in relation to control (Bennicelli et al., 2004)

The cultivation of *Azolla* in IRRI medium only led to 3-fold higher biomass in relation to the initial situation; the presence of tested heavy metals interfered with the biomass growth significantly. The growth of the fern was distinctly limited in Hg(II) treatments, by about 23-31% in comparison to the control. This limitation was stronger at higher levels of Hg(II) ions. Cr(III) caused a much lesser growth reduction than Cr(VI) ions, this reduction being only 8% for 0.1 mg Cr(III) dm^{-3}, and even there was luxurious growth at 0.5 mg Cr(III) dm^{-3} (3%, Tab. 1). Only the highest applied dose of Cr(III) resulted in 34% lower biomass. The fern cultured in Cr(III) treatments was light green and had large, convex leaves. The presence of Cr(VI) ions caused the fern growth inhibition by about 20-27% (Bennicelli *et al.* 2004). This observation confirms the results of Mortvedt and Giordano (1975), Mukherji and Roy (1978) and Hossner *et al.* (1998) who observed greater toxicity of Cr(VI) than of Cr(III).

The *Azolla* response to exposure to Pb(II) and Cd(II) is shown in Figure 2. In this study, initial 20 g of fresh plant was 4.5 times higher after the end of the study (89.9 g). The presence of heavy metals caused significant growth reduction.

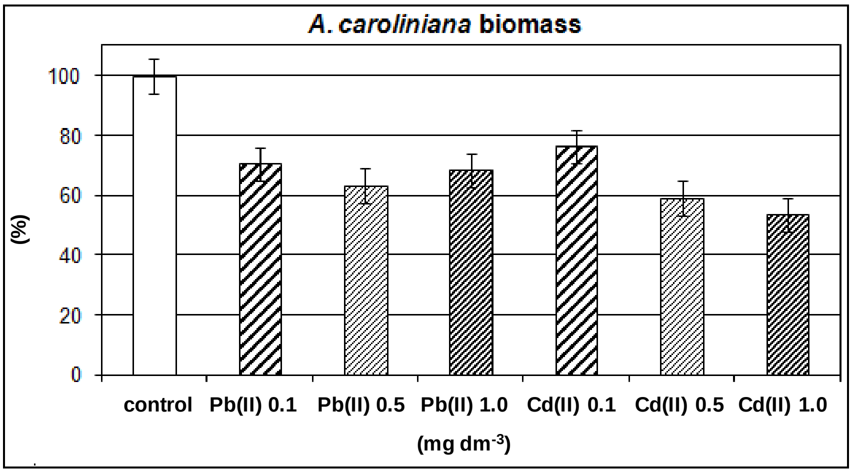

Fig. 2. Biomass of *Azolla caroliniana* (percentage, means±SEM) exposed to Pb and Cd (in doses of 0.1, 0.5 and 1.0 mg dm^{-3}) in relation to control (100%) (Stępniewska et al., 2005)

Growth of the fern in Pb(II) treatments was reduced by 29.5, 31.6 and 36.8% with increasing lead concentration, respectively. Cadmium presence had a linear effect on *Azolla* biomass; it decreased with increasing dose of Cd(II) ions. This inhibition was 23.6, 40.8 and 46.5%, respectively, and it was significantly lower at higher doses in comparison to 0.1 mg dm^{-3}. These data suggests higher toxicity of Cd to *A. caroliniana* than Pb, especially at higher selected concentrations.

The analysis of metal accumulation in *Azolla* biomass revealed its potential for the accumulation of tested pollutants (Tab. 1). There was linear positive relationship between doses of each metal and its concentration in biomass (R^2>0.9). Cr(III) was accumulated at much higher levels (up to 964 mg kg^{-1} dw) than Cr(VI) (up to 356 mg kg^{-1} dw). Cd was accumulated in very low amounts (up to 259 mg kg^{-1} dw), whilst Pb up to 416 mg kg^{-1} dw and Hg up to 578 mg kg^{-1} dw (Bennicelli *et al.* 2004, Stępniewska *et al.* 2005). These data confirm the fact that Cr(VI), Cd and Hg belong to toxic heavy metals. However, their accumulation and the survival of *A. caroliniana* suggest that this plant is capable of removing them at chosen doses from wastewaters.

Table 1. Biomass of *A. caroliniana* and levels of tested heavy metals at the end of experiment (after Bennicelli *et al*., 2004, and Stępniewska *et. al*., 2005, modified).

Treatment	Fresh weight (g)	Biomass change in reference to control (%)	Conc. in solution (mg dm^{-3})	Conc. in biomass (mg kg^{-1} dw)
Control	57.0	–	–	–
Hg(II) 0.1	43.9	–22.9	0.02	70.8
Hg(II) 0.5	39.3	–31.0	0.04	306
Hg(II) 1.0	40.8	–28.5	0.07	578
Cr(III) 0.1	52.0	–8.73	0.02	83.5
Cr(III) 0.5	58.7	+3.09	0.06	412
Cr(III) 1.0	37.4	–34.3	0.25	964
Cr(VI) 0.1	45.3	–20.5	0.00	91.1
Cr(VI) 0.5	45.4	–20.3	0.08	157
Cr(VI) 1.0	41.6	–27.0	0.12	356
Control	89.9	–	–	–
Pb(II) 0.1	63.4	–29.5	0.01	52.9
Pb(II) 0.5	56.8	–36.8	0.09	245
Pb(II) 1.0	61.5	–31.6	0.52	416
Cd(II) 0.1	68.7	–23.6	0.09	22.9
Cd(II) 0.5	53.2	–40.8	0.47	123
Cd(II) 1.0	48.1	–46.5	0.92	259

Precious metals accumulation

The application of precious metals led to their disappearance from the water. A day after setting up the experiment there was a rapid lowering the concentrations of Au(III) in all treatments, by about 67-91%, followed by fluctuations resulting in the final concentrations of 0.03, 0.1 and 0.35 mg Au(III) dm^{-3} for the initial concentrations of 0.1, 0.5 and 1.0 mg dm^{-3}, respectively. These constituted 65-79% of total metal removal after 5 days of the study (Tab. 2). Silver concentrations in the medium were also very low on the 2nd day of the study, however, only the lowest dose was efficiently removed (91%) whilst higher doses were reduced only in 12 (0.5 mg dm^{-3}) and 28% (1.0 mg dm^{-3}) at the end of the experiment (Tab. 2). In the case of Pt ions, a day after the beginning of *A. caroliniana* exposure to platinum ions the concentrations of tested metal were below detection limit till the end of the experiment.

The presence of tested precious metals affected the biomass of *Azolla*, most of the responses being growth inhibition (Fig. 3). Gold caused lower biomass growth by 13-24.5%, showing the strongest effect at the lowest applied dose, and each of them caused greater biomass by about 5% in relation to the previous Au(III) level. Under Ag(I) intoxication, *Azolla* showed an opposite response than that to Au; there was a distinct negative relationship between plant biomass and the applied dose of silver ions. The highest reduction of 38% was observed at the level of 1.0 mg Ag(I) dm^{-3} (Tab. 2). In the case of platinum there was a slight stimulation of *A. caroliniana* growth at higher doses, by about 4-6%. Only the lowest dosage led to a little lower biomass of the fern. However, these changes were not significant in relation to the control.

Table 2. Biomass of *A. caroliniana* and levels of tested precious metals at the end of experiment

Treatment	Fresh weight (g)	Biomass change in reference to control (%)	Conc. in solution (mg dm^{-3})	Conc. in biomass (mg kg^{-1} dw)
Control	35.2	–	–	–
Au(III) 0.1	26.6	–24.5	0.03	622
Au(III) 0.5	28.5	–19.1	0.10	1333
Au(III) 1.0	30.5	–13.3	0.35	4896
Ag(I) 0.1	39.4	–16.6	0.01	30.7
Ag(I) 0.5	24.0	–31.8	0.44	53.5
Ag(I) 1.0	22.0	–37.5	0.72	48.5
Pt(IV) 0.1	34.0	–3.4	0.00	18.1
Pt(IV) 0.5	37.4	+6.3	0.00	22.8
Pt(IV) 1.0	36.5	+3.7	0.00	41.8

The accumulation of the tested precious metals differed depending on the pollutant and was also related to plant biomass (Tab. 2). Au(III) showed a linear positive relationship between both metal doses, biomass and the amount of accumulated element. The highest level (about 5 g kg^{-1} dw) was noted for the dose of 1.0 mg Au(III) dm^{-3}. This high accumulation constituted up to 100% of the dosed gold. Similar findings were presented in the work of Antunes *et al.* (2001). Much lower accumulation of precious metals was observed for Ag and Pt, silver caused the strongest inhibition of *Azolla* growth, and the fern absorbed only up to 50 mg

Ag(I) kg^{-1} dw. The lowest accumulation was observed for Pt ions, ranging between 18 and 42 mg kg^{-1} dw. It is possible that there was precipitation of this metal, which could explain no Pt presence in water and its low levels in plant biomass.

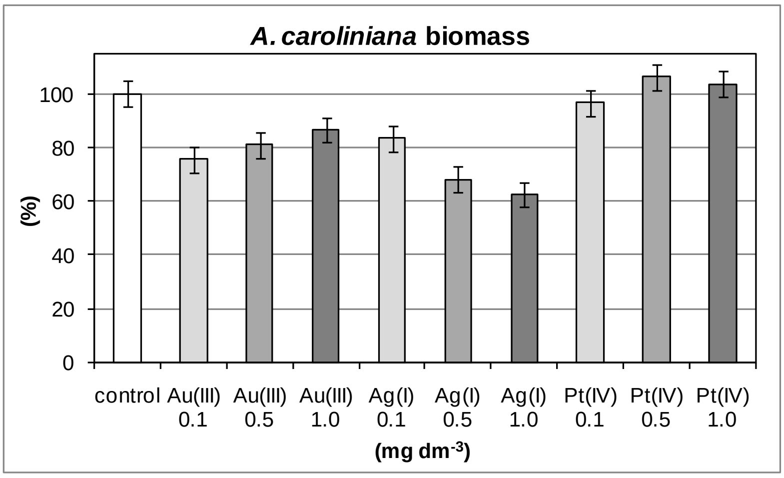

Fig. 3. Biomass of *Azolla caroliniana* (percentage, means±SEM) exposed to Au, Ag and Pt (in doses of 0.1, 0.5 and 1.0 mg dm^{-3}) in relation to control (100%)

Growth conditions

During the experiment pH of *A. caroliniana* growing medium fluctuated between around 4 and 5 in all experiments. Redox potential was above 400 mV (400-700 mV), indicating well aerated conditions. Moreover, we noticed a trend in increasing oxygen microdiffusion up to 60 µg O_2 m^{-2} s^{-1} from the levels of about 35 60 µg O_2 m^{-2} s^{-1} in all treatments (Bennicelli *et al.* 2003a,b). This fact indicates that despite heavy metal stress *Azolla* was able to minimise it by the release of oxygen to water layer. The elevated activity of SOD confirms the activation of the defensive mechanisms of the fern (Bennicelli *et al.* 2005). It is very beneficial for the water purification process. In addition, the active role of the fern in the aeration of its medium would be beneficial for the whole water ecosystem.

CONCLUSION

Presented data of our studies confirms other experiments concerning the ability of *Azolla* to remove heavy and precious metals from water solutions. The stress caused by the presence of these pollutants induces the defence mechanisms of the fern. On the enzymatic pathways and by releasing oxygen it is able to survive under these unfavourable conditions. This ability, together with fast biomass increase, make *Azolla* a promising plant in phytoremediation of waters. In addition, accumulated metals may be recovered and re-used, which would be beneficial nowadays when we struggle with a shortage of natural resources.

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FITOREMEDIACJA JAKO OBIECUJĄCA TECHNOLOGIA OCZYSZCZANIA WÓD I GLEB: *AZOLLA CAROLINIANA* WILLD. JAKO STUDIUM PRZYPADKU

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Streszczenie. Zanieczyszczenie środowiska, wynikające z pozyskiwania zasobów naturalnych jest obecnie jednym z najpoważniejszych problemów. Proponowane są nowe, przyjazne środowisku oraz ekonomicznie atrakcyjne techniki wykorzystujące zdolności mikroorganizmów (bioremediacja) lub roślin (fitoremediacja) do oczyszczania ich podłoża. Zależnie od rodzaju substancji zanieczyszczającej oraz mechanizmu jej unieszkodliwiania (akumulacja lub rozkład) zaproponowano kilka technik. Wyspecjalizowane gatunki roślin nazywane są hiperakumulatorami, np. *Brassica juncia*, *Helianthus annuus*, *Nicotiana tabacum* czy genetycznie modyfikowana *Arabidopsis thaliana*. *Azolla caroliniana* Willd. (*Azollaceae*) jest paprocią wodną występująca w klimacie umiarkowanym i tropikalnym. W ostatnich czasach odnaleziono kilka jej naturalnych stanowisk w Polsce. Paproć żyje w symbiozie z sinicą *Anabaena azollae*, która jest zdolna do wiązania azotu atmosferycznego. Oprócz licznych zastosowań *Azolla* w rolnictwie, np. jako zielony nawóz, odkryto, że roślina posiada wysoką zdolność do fitoremediacji. Nasze badania wykazały jej zdolność do usuwania i akumulacji Hg, Cd, Pb, Cr, As, Ag, Pt oraz Au z wód (do 100% wprowadzonych dawek). Te obiecujące wyniki otwierają nowe zastosowanie *Azolla* spp. w oczyszczaniu wód zanieczyszczonych metalami ciężkimi, np. jako dodatkowy etap oczyszczania ścieków.

Słowa kluczowe: *Azolla,* fitoremediacja, metale ciężkie, ścieki