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Wastewater Treatment by *Azolla Filiculoides* (A Study on Color, Odor, COD, Nitrate, and Phosphate Removal)

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ABSTRACT: The aquatic fern Azolla, a small-leaf floating plant that lives in symbiosis with a nitrogen fixing cyanobacteria (Anabaena), is an outstanding plant, thanks to its high biomass productivity along with its tremendous rate per unit area for nitrogen-fixation. The present study investigates the potential growth of Azolla in secondary effluents for removal of COD, phosphorus, and nitrogen. Results have shown that N and P removal at 100 ppm of each component in separate medium turned out to be 36% and 44%, respectively, whereas in case of a mixed solution of these two compounds, N and P removal declined to 33% and 40.5%, respectively. Moreover, results have suggested that in the presence of phosphorus nitrogen absorption decreased. Furthermore, Azolla has revealed a high potential of COD removal by 98.8% in 28 days. Finally, Azolla may be one of the most promising agents to remove COD and treat nitrogen-free and phosphorus-rich wastewaters.

***Keywords*:** Azolla, wastewater treatment, COD removal, Nitrate and phosphate removal.

INTRODUCTION

Azolla (water fern) is a unique freshwater fern, being one of the fastest growing plants on the planet due to its symbiotic relation with a cyanobacterium called Anabaena (Fig. 1). *Azolla filiculoides* is a species of Azolla, native to warm climates and tropical regions of the Americas as well as most of the old world, including both Asia and Australia (Khosravi and Rakhshaee 2005; Sadeghi Pasvisheh 2016). During the last few years, there have been extensive studies to evaluate the potentials of Azolla to be applied as a green manure in rice fields, feed supplement for aquatic and terrestrial animals, human food, medicine, water purifier, biofertilizer, weeds and mosquitoes controlling agent, or remover of nitrogenous compounds from water (Wagner 1997; Lumpkin and Plucknett 1980; Sadeghi Pasvisheh et al. 2013; Costa et al. 2009). *Azolla Filiculoides* also grows in South America, western North America, Alaska, and Europe, and is also listed among US noxious weeds. Azolla has already covered about 50% of the 20,000 hectars of Anzali wetlands. This overgrowth of Azolla is currently considered a serious issue in this unique ecosystem (Arshadi et al. 2017; Salehzadeh and Naeemi 2017).

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Being a floating aquatic fern, it can be found in usual stagnant waters, wetlands, and rice paddies in warm temperate and tropical regions, which is spread over lake surfaces to cover the water entirely (Wagner 1997; Lumpkin and Plucknett 1980; Sadeghi Pasvisheh et al. 2013). Its species distribution corresponds to fresh water ecosystems of temperate and tropical regions all over the world (Fig. 2).

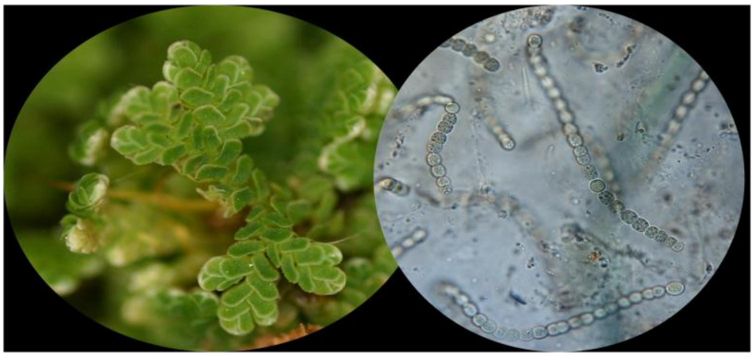

Fig. 1. Azolla (left) and its endosymbiont Anabaena (right).

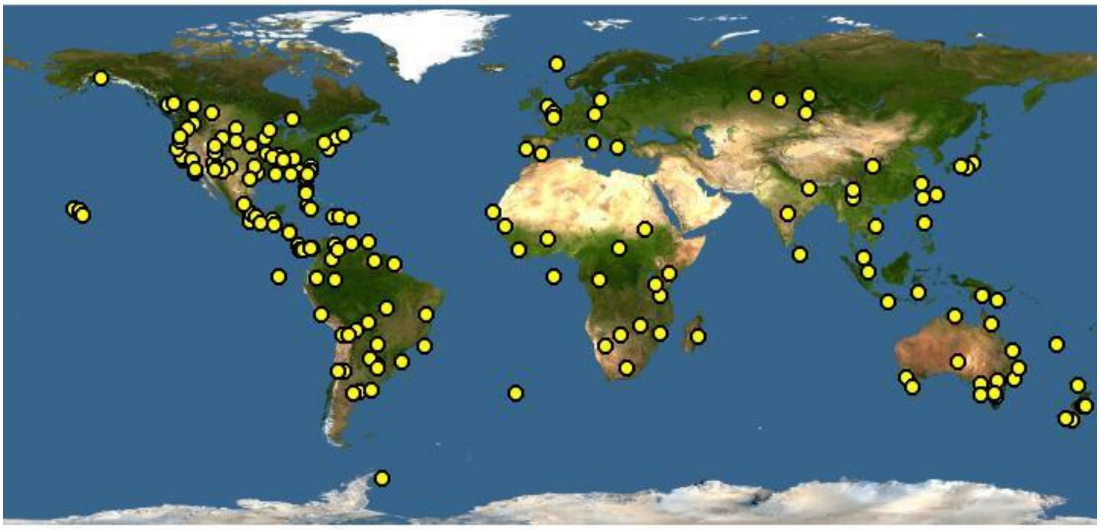

Fig. 2. Occurrence and distribution of Azolla species represented with yellow marks in the world. (http://renewablerevolution.createaforum.com/renewables/sustainable-food-production/)

Azolla leaves are delicate and sensitive. The plant's color depends on its maturity, changing as the plant grows: it is light green when it is germinated, thence to darken gradually, turning red and silver (Peters 1976; Newton and Cavins 1976; Van Hove 1989). The plant can double its size in two or three days. Azolla is sensitive to salt water, for it dies in sea water. Water plays a crucial role in the growth of Azolla and the fern is really sensitive to lack of it. Azolla can live in water with a pH of 3.5-10, though the optimum value is 4.5-7. Optimum temperature varies from one species to another, ranging within 20-30º C (Sadeghi et al. 2012a, b, 2013). The plant's productivity not only doubles that of conventional crops, but contains many commercially interesting compounds also, such as proteins, lipids, and polyphenols at high concentrations (Brouwer et al. 2016; Miranda et al. 2016; Golzary et al. 2016) (Fig. 3).

The present study has been conducted in two parts: cultivation and wastewater treatment. The cultivation step mainly at finding the optimum requirements in order to reach the highest growth rate of Azolla under laboratory conditions with the obtained results helping us manage the growth of this aquatic fern as well as conserving the wetland. The second step, however, tries to investigate the removal of nitrogen and phosphorus by Azolla from various solutions with of differing nitrogen and Phosphorus contents, COD, odors, and colors, subsequently being proposed as a model for the development of an Azolla biorefinery for future (Golzary et al. 2016) (Fig. 4).

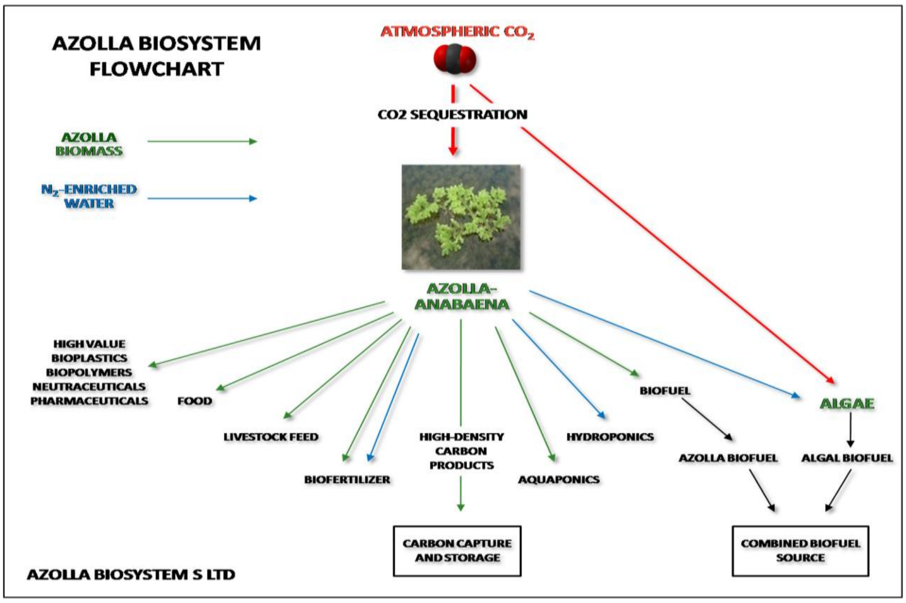

Fig. 3. Azolla biosystem flowchart

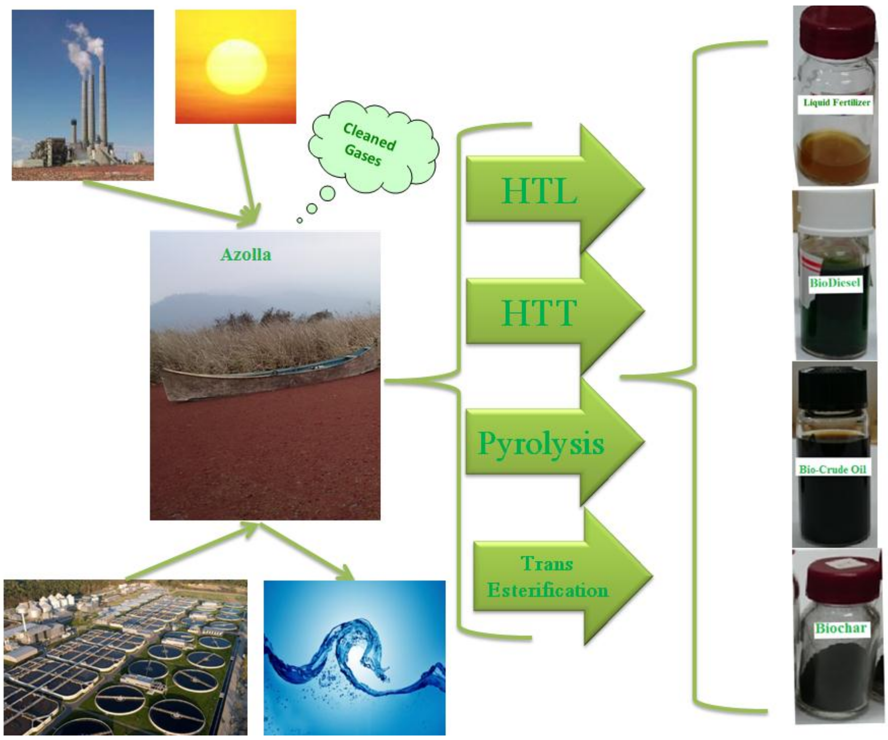

Fig. 4. Azolla biorefinery and resource recovery for future

MATERIALS AND METHOD

The research aims at examining the growth quality of Azolla in wastewater that affects the physical appearance of the wastewater, like its color, odor, and transparency. COD, N, and P of the wastewater have been also examined.

Petroleum refinery wastewater, used for the experiments, was obtained from Tehran Oil Refinery and *Azolla filiculoides* was collected from the Anzali wetland (Fig. 5a) and cultured in laboratory conditions in two 50 L laboratory-scale aerated Plexiglas bubble columns with a surface area of 7850 cm^2. The plant was grown in IRRI2 medium with the following composition: the macronutrients: 40 µM of $CaCl_2.2H_2O$, 40 µM of $MgSO_47H_2O$, 40 µM of K_2SO_4, 20 µM of $NaH_2PO_4.H_2O$; and the micronutrients: 0.01 µM of $CuSO_4.5H_2O$, 0.2 µM of H_3BO_3, 0.15 µM of $Na_2MoO_4.2H_2O$, 0.01 µM of $ZnSO_4.7H_2O$, 0.01 µM of $CoCl_2.6H_2O$, 0.5 µM of $MnCl_24H_2O$, and 0.5 µM of Fe-$EDTANa_2$. The medium got replaced every 6 days (Fig. 5b).

The volume of wastewater and Azolla were 1 L and 24 g, respectively. COD was measured on days 0, 7, 14, 21, and 28 with the temperature and pH being recorded every other day. The system was checked daily to control Azolla growth, wastewater transparency, and the number and color of the plant. Lightening remained optimal. In order to control light, the pond was covered with plastic bags and sun lamps were installed 30 cm above its surface. Once a decline was observed in the plant growth, the phosphorous checkup system was applied by injecting phosphor to the sample, if necessary (Azolla was nourished with phosphorus in wastewater). The designed experiment was conducted within 28 days. It was prepared by dividing the ponds into two parts, each 30 cm high. Equal amounts of petroleum refinery wastewater were added, one was examined as sample before biologic treatment, another as sample after refinery biological treatment.

Both flasks were aerated with a 50 L/min air pump (BOYU ACQ-003), split through a two-port manifold with a stainless steel needle valve for individual air flow control. For each needle valve, 100 cm of I.D. tubing, 0.48 cm wide, was attached to the air diffuser. CO_2 concentration in the growth room remained at ambient condition (i.e., 370-430 ppm). The lights that included fluorescent lamps (60 cm and 12 Watts) were positioned at the top of the vessel in the center, and the light intensity was measured at the surface of the vessels (Golzary et al. 2015). The temperature and pH of the wastewater at the beginning and the end of each experiment was measured, using a pH-meter (AZ Instruments) and a thermometer, respectively. To adjust the pH, NaOH and HCl solutions (both made by Merck, Germany) were used in their appropriate amounts, equal to 0.5 N and 5 N, respectively. During the cultivation phase, biomass growth was measured daily. Azolla was cultivated in three mediums with different (N and P) initial concentrations (10, 100, and 1000 ppm) as follow: (1) Distillation water with nitrate, (2) distillation water with phosphate, (3) distillation water with a mixture of nitrate and phosphate. Other environmental conditions remained constant. Temperature, pH, light, and relative humidity were adjusted at 25 ^oC, 6, 16 Klux, and 75%, respectively. Light and dark cycle was set 18 and 6 hours, respectively. Collected samples after centrifuge were analyzed with spectrophotometry of DR2000 at wavelengths of 410 and 690 nm.

RESULTS AND DISCUSSION

Table 1 shows the temperature and pH changes during the 28-day period, in which, there was an attempt to ensure the growth and proliferation of Azolla by examining such parameters. Values of pH changed within a range of 4.5-8.9, and temperature ranged from 24.4 to 32, with the optimum temperature being 20-25^o C. As shown in Table 2 and Figure 6, for the sample without the biologic treatment, the growth of Azolla in wastewater reduced the COD by 98.8% (from 4326 to 51 ppm) while this effect was 96.5% (from 1897 to 67 ppm) for the sample on which this treatment was applied. Results showed remarkable potentials of Azolla for wastewater treatment at the point of COD reduction.

Fig. 5. a) Collected Azolla from Anzali wetland, b) Cultivation of Azolla in laboratory-scale pond

Table 1. pH and temperature range of the sample (before and after biologic treatment) for a period of 28 days

#	Before biologic treatment		After biologic treatment	
Day	pH	Temperature	pH	Temperature
July 27	7.5	25.4	4.7	24.4
Aug 1	7.6	24.4	4.7	24.4
Aug 2	7.6	25.8	5.5	24.4
Aug 5	7.8	26.6	5.6	26.3
Aug 5	7.9	27.1	6.3	26.3
Aug 8	7.7	27.7	6.9	26.3
Aug 10	8.1	28.2	7.87	27.1
Aug 15	8.5	28.5	8.07	27.8
Aug 17	8.15	28.5	8.13	29
Aug 19	8.2	29	8.13	28.9
Aug 22	8.9	32	8.17	28

Table 2. COD removal from samples (before and after biologic treatment) for a period of 28 days

#	Before biologic treatment	After biologic treatment
Day	COD (ppm)	COD (ppm)
0	4326	1897
7	3042	1009
14	1324	693
21	135	124
28	51	67

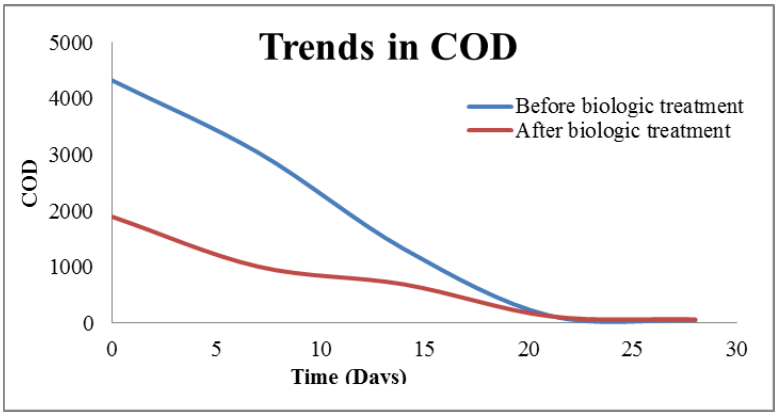

Fig. 6. COD removal from petroleum wastewater for two samples of before and after biological treatment during 28 days

It is noteworthy that at the end of 28-day experiment, the unpleasant odor of wastewater completely disappeared. In addition, water transparency was increased, letting light pass through, whereas in the beginning of the experiment the sample before and after the biological treatment was very and slightly turbid, respectively.

Figure 7-8 show the removal of nitrogen and phosphorous at different concentrations of 10 to 1000 ppm from aqueous solution (1) and (2) (mentioned at materials and methods section, above) by means of Azolla in 15 days. Figure 9 gives the data for nitrogen and phosphorous removal from aqueous phase, containing a mixture of N and P at different concentrations.

Results show that in solutions with different nitrogen concentrations, nitrogen removal ascended from 10 ppm during the first three days, but afterwards it started to descend, reaching its minimum value after eight days. In the first cultivation media, by increasing initial nitrogen concentration, the nitrogen removal rose as well, but with a low initial concentration, not only did the amount of nitrogen decrease, but nitrogen content increased in the solutions. The reason for this process comes from the fact that Azolla lives with a "blue-green algae", called *Aanabean*, symbiotically. Anabaena drew down the atmospheric nitrogen (N_2) directly, fulfilling all nitrogen requirements of Azolla for its growth, so it was not needed to use the nitrogen of these solutions. Consequently after three days, it began to slightly release nitrogen to the media (Fig. 7). In the presence of phosphorus, nitrogen absorption declined (Fig. 9). Phosphorous removal in aqueous phase (2) increased with time, but at third medium (mixture aqueous phase) phosphorous removal was uptrend for the first six days, then to start decreasing (Figs. 8-9).

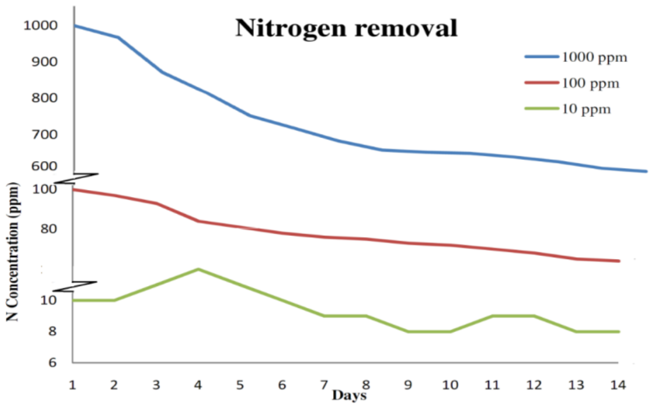

Fig. 7. Nitrogen removal from aqueous phase (1) by means of growing Azolla at 14 days

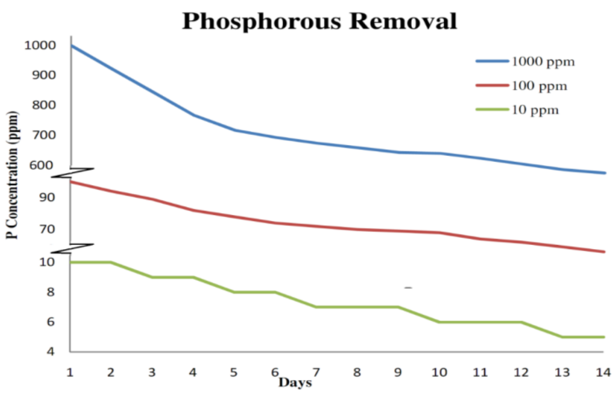

Fig. 8. Phosphorus removal from aqueous phase (2) by means of growing Azolla at 14 days

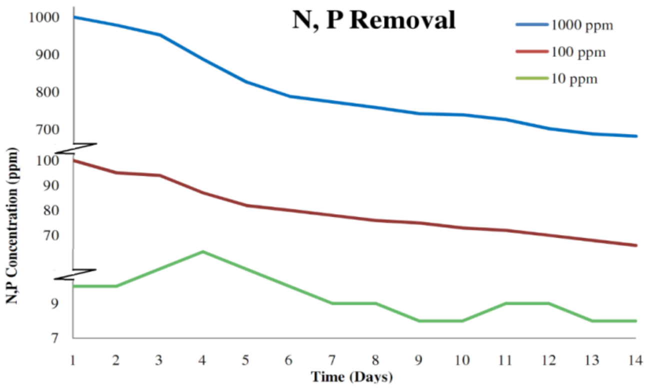

Fig. 9. Nitrogen and phosphorus removal from aqueous phase (3) by means of growing Azolla at 14 days

CONCLUSION

Results showed a high and remarkable potentiality of COD removal by 98.8% at 28 days for petroleum refinery wastewater. In case of removing N and P (100 ppm concentration) from aqueous phase in separate medium, efficiency of 36% and 44% was obtained, respectively. As for the mixed aqueous solution of these two compounds, N and P removal was decreased to 33% and 40.5%, respectively. Results also showed that nitrogen absorption was decreased in the presence of phosphorus. According to the data obtained from wastewater treatment with Azolla that can be found in North of Iran, industrialization of these laboratory findings, can be achieved with great success. In this regard, economic feasibility should be reviewed for the Azolla effectiveness time, providing the growth condition in wastewater and its cultivation before the treatment process. Azolla algae is capable of removing nutrients (phosphorus and nitrogen) from aqueous solutions; therefore, it can be used for advanced treatment of wastewater to improve the quality of effluent. However, it is not suitable for nitrogen removal at low nitrogen content wastewaters. Moreover, the laboratory studies showed that Azolla can double its biomass in 3.5 days and grow in nitrogen-free solution, since it does not need nitrogen nutrient medium for its biomass growing. In conclusion, Azolla is the most promising agent for treatment of nitrogen-free and phosphorus-rich wastewaters.

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