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# Responses of the nitrogen-fixing aquatic fern *Azolla* to water contaminated with ciprofloxacin: Impacts on biofertilization☆

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A B S T R A C T

We investigated the ability of the aquatic fern *Azolla* to take up ciprofloxacin (Cipro), as well as the effects of that antibiotic on the N-fixing process in plants grown in medium deprived (-N) or provided (+N) with nitrogen (N). *Azolla* was seen to accumulate Cipro at concentrations greater than 160 μg $g^{-1}$ dry weight when cultivated in 3.05 mg Cipro $l^{-1}$, indicating it as a candidate for Cipro recovery from water. Although Cipro was not seen to interfere with the heterocyst/vegetative cell ratios, the antibiotic promoted changes with carbon and nitrogen metabolism in plants. Decreased photosynthesis and nitrogenase activity, and altered plant's amino acid profile, with decreases in cell N concentrations, were observed. The removal of N from the growth medium accentuated the deleterious effects of Cipro, resulting in lower photosynthesis, N-fixation, and assimilation rates, and increased hydrogen peroxide accumulation. Our results shown that Cipro may constrain the use of *Azolla* as a biofertilizer species due to its interference with nitrogen fixation processes.

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## 1. Introduction

The endophytic N-fixing cyanobacteria *Anabaena azollae* Strasbuger exists in symbiotic complexes within the leaf cavities of the aquatic fern *Azolla* (Salviniaceae), with the cyanobacteria producing fixed nitrogen for itself and its host and the fern providing a protected environment and fixed carbon for the algae (Peters, 1978). Of the many uses of *Azolla*, the most outstanding is its application in agricultural production as a biofertilizer, due to its high growth and nitrogen fixing rates (Wagner, 1997). Under favorable conditions, *Azolla* can double its biomass every 3–5 days, while its nitrogen accumulation can vary between 70 and 110 kg $ha^{-1}$ (Ventura and Wantanbe, 1993). At the beginning of the 1990s, 2 to 3 million ha of rice fields throughout the world used *Azolla* as green manure (Giller and Wilson, 1991), and this area has drastically increased since then, mainly in Asia. The use of *Azolla* in rice fields promotes changes in the physical, chemical, and biological properties of the soil as well as the soil-water interface, favoring rice production (Cheng et al., 2015, 2010). *Azolla*, for example, has been shown to suppress aquatic weeds in flooded rice fields and increase rice yields (Cheng et al., 2015). Currently, however, mineral nutrition is not the biggest problem of agriculture. The use of water contaminated by toxic chemicals has limited plant production, and water contamination by antibiotics has become an emerging global problem.

The excessive and indiscriminate use of antibiotics in farm practices (mainly as feed additives) is the major source of these compounds to the surrounding environment, and aquatic systems are natural sinks for antibiotic runoffs from agricultural lands (Gomes et al., 2017). The potential environmental risks of antibiotics have attracted significant attention (Nie et al., 2013), and some of them are very resistant to abiotic and biotic degradation (Girardi

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et al., 2011) – which implies their persistence and accumulation in the environment. Many detrimental effects of antibiotics to non-target organisms have been described in the literature (Gomes et al., 2017; Lützhøft et al., 1999; Martins et al., 2012; Robinson et al., 2005), which has motivated investigations into their accumulation in the natural environment and their toxicity, as well as possible remediation techniques. Additionally, exposure to sub-inhibitory levels can promote antibiotic resistance in target organisms (Gomes et al., 2017).

*Azolla* has been shown to be useful in waste water treatment due to its ability to take up contaminants such as heavy metals (Antunes et al., 2001) and antibiotics (i.e., penicillin) (Malakootian et al., 2015). Among the antibiotics, fluoroquinolones such as ciprofloxacin [Cipro; 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dyhrydroquinoline-3-carboxylic acid] are widely used in both human and veterinary medicines (Migliore et al., 2003), and milligram levels of ciprofloxacin have been observed in the environment (Golet et al., 2003; Martínez-Carballo et al., 2007) – which is extremely worrisome, as at those levels, it has been found to be toxic to numerous organisms, including algae and plants (Gomes et al., 2017; Lützhøft et al., 1999; Martins et al., 2012; Robinson et al., 2005). Due to its structural similarity to quinones, Cipro (and other fluoroquinolones) can act as quinone site inhibitors, resulting in the impairment of photosynthesis (Aristilde et al., 2010) and respiration (Gomes et al., 2017). Additionally, by impairing the mitochondrial electron transport chain, Cipro has been observed inducing reactive oxygen species (ROS) formation, with related oxidative damage (Gomes et al., 2017) – which helps to explain its deleterious effects on non-target organisms such as animals and humans (Kozieł et al., 2006; Lawrence et al., 1996; Lowes et al., 2009).

Some antibiotics, such as erythromycin, were observed to be very toxic to *Anabaena*, killing that cyanobacteria – resulting in *Azolla* plants without their N-symbionts (Forni et al., 1991). Moreover, as was recently reported with the aquatic plant *Lemna minor*, Cipro concentrations higher than 1.05 mg $l^{-1}$ can be phytotoxic, impairing photosynthetic and respiratory pathways (Gomes et al., 2017). Any disruption of energetic metabolic pathways (photosynthesis and respiration) that produce ATP, sugars and reducing power for N-fixation will result in decreased N-fixation by the *Azolla/Anabaena* symbiotic complex. We therefore investigated the effects of Cipro exposure on *Azolla*, as that antibiotic would be expected to be toxic to *Anabaena* and detrimental to nitrogen fixation processes, inducing an inadequate nutritional status. We examined the effects of Cipro on *Azolla* plants deprived of N (with no N added to the culture medium) and those with N supplements in the growth media; in the latter case, the plants would not be fully deprived of N due to any possible effects of Cipro on *Anabaena*. More specifically, we sought to determine if that aquatic fern can take up Cipro and if it affects its symbiotic N-fixing processes. This study will be useful in the establishment of *Azolla* as a potential Cipro-phytoremediation species. Additionally, it is important to understand if water contamination by Cipro will result in N-fixing losses by *Azolla*, which would imply economic impacts in terms of its use as a biofertilizer (Ventura and Wantanbe, 1993; Wagner, 1997).

## 2. Materials and methods

*Azolla filiculoides* plants were acquired from the Fundação Zoo-Botânica de Belo Horizonte (Belo Horizonte, Minas Gerais state, Brazil). The effects of Cipro on *Azolla* plants were evaluated under two scenarios: in the presence (+N; 0.04 g $Ca[NO_3]_2$ $l^{-1}$) or absence (-N) of added nitrogen in the growth medium. To that end, plants were cultivated in sterile complete CHU 10 medium (Chu, 1942) (+N) or in sterile CHU 10 medium with no added N (-N). Prior to the initiation of the treatments, the plants were acclimated for 15 days in +N or −N CHU 10 medium under standardized growth conditions: temperature 20 ± 2 °C and under a 12-h photoperiod (45 μmol photons $m^{-2}$ $s^{-1}$, Philips T2 40 W/3lamps). Fifteen fronds of *Azolla* were then transferred to 250 ml Erlenmeyer flasks stoppered with cotton (to minimize evaporation and avoid contamination) with the appropriate medium (100 ml) with the addition of 5 different concentrations of ciprofloxacin (0, 0.75, 1.05, 2.25 and 3.05 mg $l^{-1}$). Analytical-grade ciprofloxacin (purity > 98%) was purchased from Sigma-Aldrich (Brazil) and was used in all of the experiments. The effects of Cipro on *Azolla*, plants were investigated after 5 days of exposure.

### 2.1. Ciprofloxacin concentrations in plants

High performance liquid chromatography (HPLC) coupled to a fluorescence detector was used for ciprofloxacin quantification in whole plants. After Cipro extraction following Palmada et al. (2000), with modifications by Migliore et al. (2003), the samples were dried in a SpeedVac (RC1010, Thermo) and the residue suspended in a mobile phase (0.4% aqueous triethylamine pH 3.0, acetonitrile and methanol – 75:10:15 v/v/v). Then, 10 μl was injected into a HPLC (Shimadzu) equipped with LC-10AD VP pump, SIL-10AF auto injector, using a SCL-10ASP system controller, and a Column Over CTO-10A VP. Evaluations were carried out following Zimmermann et al. (2016), using a fluorescence detector (RF-10A XL), C18 column (Discovery® HS C18 column 150 × 4.6 mm, particle size 5 μm), a flow rate of 1.0 ml/min, with excitation/emission detection at 278/453 nm. The calculated limit of detection and limit of quantification (Mocak et al., 1997) were 5 ng $l^{-1}$ and 10 ng $l^{-1}$, respectively. Calibration curves of six points showed good linearity for the analyte ($r^2 = 0.99$; $P < 0.0001$) within the domain of expected sample concentration. Each batch of samples included three blanks, three standards, and three fortified samples. Recovery rates were higher than 85%.

### 2.2. Numbers of heterocysts

The *Azolla* symbiont microorganism has been taxonomically examined using molecular approaches and has been treated as *Nostoc* or *Trichormus* in the literature. In the present work, the designation *Anabaena* was used as this term is frequently found in traditional studies. The heterocysts/vegetative cell ratio was evaluated by quantification of both cell types using an optical microscope at a magnification of 400×. Since absolute values could not be considered due to the difficulty of standardizing the wet masses of the *Azolla* leaves sampled, quantifications were focused only on comparisons of the relative abundances of heterocyts in *Anabaena* trichomes. For this, the leaves of three *Azolla* fronds per replicate were pooled together and lightly macerated; the material was then fixed with lugol acetic solution for later quantification. All mature heterocysts and vegetative cells were quantified in each field of a slide in at least 40 fields, for a total of at least 800 cells/slide (magnification 400×, using an Olympus CH30 microscope). We identified mature heterocysts by observing their evident micro-plasmodesmata and their more homogenous colors.

### 2.3. Nitrogenase activity and nitrogen metabolism

Nitrogenase assays were performed using the acetylene ($C_2H_2$) reduction technique, following Peters and Mayne (1974) with modifications. After the fifth day of antibiotic exposure, five fronds were incubated for 60 min in calibrated 15 ml flasks fitted with serum caps containing 5 ml of the test growth media and 10% (v/v)

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