

To recognize unrecognized – the study about cultivable microbiome of the aquatic fern *Azolla filiculoides* L. as new source of beneficial microorganisms

Abstract: The aim of the study was to determine unrecognized till now the microbiome associated with aquatic fern *Azolla filiculoides*. During experiment 58 isolates (43 epiphytes and 15 endophytes) of different morphology were obtained. We successfully identified 85% of microorganisms by assigning them to 9 bacterial genera: *Achromobacter*, *Bacillus*, *Microbacterium*, *Delftia*, *Agrobacterium*, *Alcaligenes* (epiphytes) and *Bacillus*, *Staphylococcus*, *Micrococcus* and *Acinetobacter* (endophytes). We also studied *A. filiculoides* cyanobiont originally classified as *Anabaena azollae*, however, the analysis of its morphological traits suggest that this should be renamed as *Trichormus azollae*. Finally, the potential of identified microbial genera representatives to synthesize plant growth promoting substances such as: indole-3-acetic acid (IAA), cellulase and protease enzymes, siderophores and phosphorus (P) utilization potential were checked. From all of identified genera, *Delftia* sp. AzoEpi7 was the only one displaying all studied growth promoters, thus was recommended as the most beneficial bacteria in the studied microbiome. The other three potentially advantageous isolates (*Micrococcus* sp. AzoEndo14, *Agrobacterium* sp. AzoEpi25 and *Bacillus* sp. AzoEndo3) displayed 5 parameters: IAA (excluding *Bacillus* sp. AzoEndo3), cellulase, protease, siderophores (excluding *Micrococcus* sp. AzoEndo14), P mineralization and P solubilization (excluding *Agrobacterium* sp. AzoEpi25).

Keywords: *Azolla*, *Delftia* sp., endophytes, plant growth promoting potential, *Trichormus azollae*

1. Introduction

Plants and microorganisms form complex associations displaying diverse interactions ranging from mutualism to pathogenicity. The habitat for microorganisms could be both the interior (endosphere occupied by endophytes) and the surroundings (phyllosphere, rhizoplane and rhizosphere, occupied by epiphytes) of the host plant [1,2]. Microbial genomes, termed as microbiome or plants' second genome [2,3] together with plant genome constitute the specific plant microbiome. Additionally, a co-evolution process between plants and their associated microbiome, resulting in a strong genomic interdependency, leads to considering plants and their microbiome as metaorganism or holobiont [4].

Plant-associated microbes, especially endophytes play a crucial role in plants growth and development allowing them to survive harsh conditions [1] what is important for food production (increased crops, biocontrol of plant diseases) as well as for coping with contaminants (phytoremediation). These beneficial microorganisms are termed as Plant Growth-Promoting Bacteria (PGPB) [3,5]. Their beneficial function is the improvement of plant fitness and the protection against biotic and abiotic stresses by facilitation nutrient acquisition and providing plant hormones and other metabolites [3,6]. The presence of pollutants, such as heavy metals may also pose a threat for both plants and some microorganisms (e.g. metal tolerant species), however, they could be able to immobilize or

decompose pollutants protecting plants or improving their defense mechanisms. This issue is crucial for bioremediation and phytoremediation processes [7,8].

Despite of enormous microbial abundance in different environments and the substantial progress in their cultivation methods, still only 1% of them may be cultured [9]. In addition, some endophytes are commensals of yet unknown function in plants. It is also very common to study the function of microbiome for a specific group of species and focus mostly on terrestrial plants than on broader taxonomical spectrum of plant species [3]. Thus is why it is worth to discover microbiomes associated with plants providing new, potentially valuable for humans plant microbiomes. One of valuable plants in the aspect of potential microbiome hosts are ferns belonging to the genus *Azolla* playing important roles in some branches of industry.

Azolla filiculoides L. (*Salviniaceae*) is a small (2.5 cm), heterosporous, floating aquatic or semiaquatic pteridophyte occurring on the surface of eutrophic waters in temperate and tropical climate around the world (Figure 1a). It can exist either individually or in mats, which can reach a thickness of up to 20 cm. Fern has bilobed leaves, a dorsal lobe has an ovoid cavity inhabited by the community of cyanobacteria *Anabaena azollae* (Starrb.) (Figure 1b) capable to atmospheric nitrogen fixing using nitrogenase enzyme (EC 1.18.6.1) in specialized, thick-walled cells called heterocysts (Figure 1c).

Figure 1. (a) The culture of *A. filiculoides*; (b) filaments of *A. azollae* in a leaf cavity; (c) close up of *A. azollae*, both pictures taken from light microscope. Photo: A. Banach.

This trait makes *Azolla* sp. independent on other external nitrogen sources allowing its fast grow and production of high-protein biomass. This feature allows the usage of *Azolla* sp. as a green manure on rice fields and animal feed [10,11]. Another important feature of the fern is its ability for heavy metals accumulation [11,12].

Over the years, the cyanobiont has been named *Nostoc azollae*, *Anabaena azollae* and *Trichormus azollae*, but, to date, no definitive classification exists. Studies by Plazinski et al. [13] suggested that the endosymbiont is rather *Nostoc* sp. than *A. azollae*. Gebhardt and Nierzwicki-Bauer [14] stated that cyanobacteria classification depends on host plant. In 2003 Baker and co-authors [15] using comparisons of the sequences of the phycocyanin intergenic spacer and a fragment of the 16S rRNA, found that the cyanobiont from an *Azolla* belongs to neither of these genera. In 2014, Pereira and Vasconcelos [16] made another attempt to solve this dilemma but their results were also unclear. Consequently, till now the problem remains unsolved. There are some information about bacteria as a third partner in the symbiosis. First presence of bacteria in the *Azolla* leaves was reported by Grilli in 1964 [17] and their microscopic observations were done by Nierzwicki-Bauer and Aulfinger [18] and Carrapiço [19]. Study of Serrano et al. [20] determined bacterial species accompanying *Azolla* sp. being recognized in XX century. However, no more detailed analysis or identification of these bacteria has been performed.

All of above mentioned issues make a huge gap in knowledge on microorganisms inhabiting *Azolla* sp., so our intention was to fill it. Thus, the novelty and main goal of the study was to isolate, identify and describe an unrecognized bacteria constituting the core microbiome of *A.*

filiculoides. As the fern is used in agriculture and water treatment it would be useful to discover its microbiome which may help to know its role in symbiotic system Azolla-microorganisms and indicate its possible applications in mentioned branches of industry.

2. Results

2.1. Azolla cyanobiont

Isolation of the cyanobiont allowed us to observe the presence of dense agglomerations of cyanobiont filaments together with plant debris. After one week of cyanobiont culture we collected enough living material for further studies; the example of living culture is presented in Figure 2.

Figure 2. The UV microphotograph of cyanobiont culture. Photo: A. Banach.

After conducting PCR reaction using *nifDf* and *nifDr* starters specific products of 600 bp were obtained. This observation confirms the presence of *nif* gene encoding enzymes important in atmospheric nitrogen fixing, a feature typical for cyanobacteria. In the case of starters targeting fragment of 16S rRNA gene specific for cyanobacteria we got two products of 1500 and 1700 bp. After PCR products purification the latter one has been further analyzed.

The analysis of similarity the cyanobacterium-specific 16S rRNA gene fragments to homologous gene revealed similarities to previously described *Azolla* sp. cyanobionts (Table 1). We found four hits with similarity of 90-94% what indicate that it is likely that studied DNA fragments belong to *Anabaena* sp.

Table 1. Microorganisms similar to homologous 16S rRNA gene specific for cyanobacteria.

2.2. The cultured microbiome of *A. filiculoides*

Isolation yielded in 58 microorganisms among which 15 were obtained from the interior of the plant. We noticed differences in colonies morphology which suggest affiliation of isolates to different taxonomic groups. The morphology of obtained isolates were summarized in Table 2.

Table 2. The morphological traits of the obtained isolates.

Obtained isolates displayed wide range of colonies sizes ranging from <1 mm to ca. 0.5 mm. More than 40% had both punctiform and small size, above 20% were moderate in size and the biggest constituted about 14%. Epiphytes showed the same pattern, whilst endophytes were in 46% the smallest, 20% were small, 27% moderate and only ca. 7% had large size.

Circular shape was dominant in the studied pool of microorganisms (60%), among them much higher counts were recorder for endophytes (87%) than epiphytes (51%). Oval and irregular shapes accounted 19% for each of these shapes. Only one epiphytic isolate formed filamentous colonies (2%). Epiphytic microorganisms formed also oval and irregular shapes in 26% and 21% cases. Endophytes did not form oval and filamentous colonies and irregular form was recorded in 14% of these microorganisms.

In the case of surfaces we distinguished dull-glistening and rough-smooth combinations. The most abundant was glistening/smooth surface accounting for 50% of all isolates (35% and 93% of epiphytes and endophytes, respectively). The next two abundant surface were glistening-rough (22%, 28% and 6% for the total microbiome, epiphytes and endophytes, respectively) and dull/smooth (19% of all microbes) surfaces. The latter was present in 26% of epiphytes whilst endophytes displayed no such surface type. Dull/rough surface was the least common (8.6%); it was not observed for endophytes and only 12% of epiphytes were characterized by such surface.

We observed three types of colony texture: butyrous (BUT), mucoid (MUC) and brittle (BRIT). First (BUT) was the most common in all microorganisms (67%) followed by MUC (19%) and BRIT (14%). Similar number were recorded for epiphytes whilst 73% of endophytes had BUT and 27% MUC texture.

In terms of colony transparency we divided microorganisms into opaque (OPQ), translucent (TRANS) and iridescent (IRID). More than half or all microbes, both epi- and endophytes produced non-transparent (OPQ) colonies whilst 40% were transparent (44% of epiphytes and 27% of endophytes). Opalescent color was observed in 9% of isolates (4.7% epi- and 20% of endophytes).

Isolated microorganisms displayed two types of pigmentation – white-cream-beige (76%) (Figure 3a) and yellow-orange (24%) (Figure 3b). Similar number were recorded for epiphytes whilst 2/3 of endophytes were included in “white group” and 1/3 in “yellow group”. Deeper analysis within each group revealed cream pigmentation as the most abundant (36%, 40% and 27% for all, epiphytes and endophytes, respectively). Beige and white-cream colonies were observed at 34% isolates (equally distributed between these two pigmentations) and only 5% of colonies were white. Epiphytes were in 21% cream, in 12% white-cream and in 5% white. Endophytes had in 33% cases white-cream pigmentation, in 6.7% cream with no white colonies. The second, “yellow” group was decomposed into yellow-cream, yellow and yellow-orange sub-groups. Among them pure yellow color was observed in 14% of all microbes – 7% of epiphytes and 33% of endophytes. The latter did not make colonies in other hue of yellow. Bright yellow pigmentation was recorded for 7% of all isolates (9% of epiphytes) whilst more orange colonies were in 3.5% of the total number of microorganisms (4.7% of epiphytes).

Next studied trait was colony elevation: flat (F), raised (R), convex (C) and umbonate (U). We found raised colonies as the most abundant (52%). As much as 80% of endophytes and 42% of epiphytes were characterized by such colony elevation. 26% of isolates were flat, 17% umbonate and only 5% convex. The two microbial groups, 30% of epiphytes had flat colonies, 21% umbonate and 7% convex. For endophytes no convex colonies were recorded, 13% were flat and 7% – umbonate.

Margin was the last morphological trait – we observed colonies with entire, undulate and filiform margins. First type of margin was found to be the most common (71%) – 67% of epiphytes and as much as 80% of endophytes displayed such type of margin. Undulate margin was in 30% counts (30% for epiphytes and 20% for endophytes). Filiform margin was

observed only in one case, the same epiphyte having filamentous form of colony (2%) (Figure 3c).

For additional characterization we determined type of cell wall using Gram staining. We found Gram positive bacteria as dominant group – 66% counts (56% of epiphytes and 93% of endophytes).

Figure 3. Examples of the most common isolates: (a) cream epiphyte, (b) yellow endophyte and (c) white-cream, filamentous form of epiphyte no. 37. Photo: A. Banach.

2.3. Detection of the obtained isolates

On the basis of 16S rRNA (hypervariable fragments V2-V4) analysis we showed the similarity of obtained sequences on the level 99% (Table A1). Analysis of 16S rDNA gene fragment evidenced the three identical epiphytic isolates number 22, 30 and 32, only no. 22 was entered into GeneBank database. In addition we did not obtain sufficiently good results for epiphytes no. 8, 15, 26, 27, 31, 33, 39 and 40 as well as one endophyte number 15. As a result, successfully identified and entered to the database were 35 epiphytes and 14 endophytes (85% of isolated microorganisms). Their names and the accession numbers are presented in Table 3.

Table 3. List of identified microorganisms. Column ‘No.’ represents number of isolate corresponding to these in Table 2. Note some missing numbers due to unsuccessful identification.

The identification procedure revealed that isolates belong to 9 genera (6 for epiphytes, 4 for endophytes; note that some epi- and endophytic microorganisms belong to the same genus). Basing on their numbers it could be stated that the dominant epiphytic phylum was Firmicutes (60%) followed by Proteobacteria (34%) and Actinobacteria (6%). For endophytes Firmicutes (86%) were also dominants, whereas Proteobacteria and Actinobacteria constituted equally 7%. Within Firmicutes *Bacillus* was the only representative genus in a case of epiphytes (21 isolates). For endophytes this phylum was equally represented by *Bacillus* (6 isolates, 43%) and *Staphylococcus* (4 isolates, 29%) genera. Epiphytes belonging to Proteobacteria were classified to order Rhizobiales (Alphaproteobacteria) represented by genus *Agrobacterium* – 11% (4 isolates) and Burkholderiales (Betaproteobacteria) represented by *Alcaligenes* (1), *Achromobacter* (6) and *Delftia* (1 isolate) genera (23%). *Acinetobacter* was the only genus representative for endophytic Proteobacteria (1 isolate), *Microbacterium* (2) for epiphytic Actinobacteria whilst *Micrococcus* (3 isolates) for endophytes from this phylum.

2.4. The synthesis of plant growth promoters

In our study we would like to present the potential of isolated microorganisms in synthesizing substances promoting plant growth what in the case of *A. filiculoides* is very poor recognized. Consequently, the levels of IAA, cellulase and protease activities, P utilization and siderophores production were evidenced. For this purpose we selected one representative of each identified genera resulting in 6 epiphytes and 4 endophytes.

The quantification of IAA synthesis revealed 3 promising strains: *Micrococcus* sp. AzoEndo14, *Delftia* sp. AzoEpi7 and *Agrobacterium* sp. AzoEpi25. First mentioned produced the highest amounts of auxin (17.9 $\mu\text{g ml}^{-1}$); meanwhile the other two yielded in 3.575 and 6.39 $\mu\text{g ml}^{-1}$, respectively (Table 4). For studying the ability of microorganisms to the lysis of pathogens cell wall we assayed the cellulase and protease activities. Importantly, positive reaction for all studied isolates was recorded (Table 4).

Table 4. The levels of IAA, cellulase and protease activities of studied isolates (means $\pm$ SD).

1 EN – endophyte, EP – epiphyte; 2 h – halo size; 3 h:c – halo-to-colony size ratio; 4 n/a – no positive reaction.

The diameter of halo after cellulose decomposition ranged from 0.23 – 1.5 cm. Endophytes were characterized by stronger cellulase activity – we observed zones of 0.98 (*Staphylococcus* sp. AzoEndo11) to 1.5 cm (*Micrococcus* sp. AzoEndo14). It could be assumed that only for *Acinetobacter* sp. AzoEndo8 the halo was lower by 20% in comparison to colony diameter. Other 3 isolates had by 5-19% higher zone when compared to the size of developed colonies. In the case of epiphytes the lowest activity was noted for *Delftia* sp. AzoEpi7 (0.23 cm) and the highest of 1.03 cm for *Achromobacter* sp. AzoEpi1. We noticed that *Achromobacter* sp. AzoEpi1, *Bacillus* sp. AzoEpi2 and *Delftia* AzoEpi7 developed smaller halos in relation to their colonies – h:c ratios of 0.67, 0.47 and 0.27, respectively. Other three, i.e.: *Alcaligenes* sp. AzoEpi21, *Microbacterium* sp. AzoEpi23 and *Agrobacterium* sp. AzoEpi25 formed halos higher by 20-33% (Table 4). Protein substrate consumption was indicated by halos of more than 1 cm in all samples. Endophytic *Micrococcus* sp. AzoEndo14 generates the smallest zone (1 cm) being also 69% smaller than colonies. *Bacillus* sp. AzoEndo3, in contrast, developed the biggest zone of 1.76 cm (1% bigger than colonies). However, epiphytic *Bacillus* sp. AzoEpi2 showed the lowest protease activity of 1.1 cm halo (72% smaller zone than colonies). *Agrobacterium* sp. AzoEpi25 produced the biggest zones 2.95 cm with the highest h:c ratio of 1.1 (Table 4).

Importantly, all tested isolates demonstrated potential for organic P mineralization; endophytes had halos ranging from 0.26 cm (*Staphylococcus* sp. AzoEndo11) with h:c ratio of 0.23, to 0.62 cm (*Bacillus* sp. AzoEndo3) and 0.47 h:c ratio. Epiphytes mineralized more phosphate on the average: the halos ranged between 0.35 (*Alcaligenes* sp. AzoEpi21) – 0.52 cm (*Bacillus* sp. AzoEpi2) with h:c ratios of 0.33-0.43, respectively. Inorganic P appeared to be more difficult to solubilize – endophytic *Acinetobacter* sp. AzoEndo8 was unable to utilize it and only epiphytic *Delftia* sp. AzoEpi7 was able to develop halo of 0.79 cm being 10% bigger than its colonies. The rate of P solubilization by endophytes ranged between 0.29 cm (*Micrococcus* sp. AzoEndo14), 64% of colonies sizes and 1.95 cm (*Staphylococcus* sp. AzoEndo11), 91% bigger than colonies (Table 5). This study allowed us to qualify all isolates as PMB and only *Staphylococcus* sp. AzoEndo11, *Micrococcus* sp. AzoEndo14, *Bacillus* sp. AzoEndo3 (endophytes) were qualified as PSM, meanwhile *Delftia* sp. AzoEpi7 as the only PMPSB.

Production of siderophores was visualized only in some samples: one endophyte, *Bacillus* sp. AzoEndo3 (huge halo of 3.56 cm with low colony growth (10 times lower colony size), and 3

epiphytes. Among them *Delftia* sp. AzoEpi7 made the biggest halo (0.98 cm) being 64% of colony size. Next *Achromobacter* sp. AzoEpi1 followed by *Alcaligenes* sp. AzoEpi21 (0.1 and 0.56 cm, respectively).

Table 4. The levels of IAA, cellulase and protease activities of studied isolates (means $\pm$ SD).

3. Discussion

One of our goals was to answer the question what cyanobiont does co-exist with *A. filiculoides*? Due to various information from the literature it is still not completely clear. In the study by Pereira and Vasconcelos [16] deep screening of the classification and phylogeny of the cyanobiont was made. The existing controversy of its classification depends on the method applied, in addition, a co-evolution between the cyanobiont and the *Azolla* host is possible as well as the existence of more than one genus or more than one species strain. This could explain our different classification originating from molecular and botanical analysis. Despite the fact that many publications traditionally name this cyanobacteria as *Anabaena azollae* or *Nostoc azollae* [10] and this also occurs in new publications [25], Komárek and Anagnostidis [26] renamed it to *Trichormus azollae*. Results of Baker and colleagues [15] are consistent with the latter study. In addition, most of the non-planktonic species of *Anabaena* without gas vacuoles are now included in *Trichormus*. All the planktonic species with gas vacuoles shown earlier remain in *Anabaena*. The difference between the two genera, *Trichormus* and *Anabaena*, as present understood, depends on the developmental relationships between the heterocysts and spores [27]. Performed by us botanical observation revealed these traits allowing to conclude that the cyanobiont is *T. azollae*. It is also convincing that the AlgaeBase states that *A. azollae* is currently regarded as a synonym of *T. azollae* [28].

In our study cultured microbiome of *A. filiculoides* detection and identification was the main goal. Previous works mentioned the presence of bacterial endosymbionts within fern's cavities, yet, their identification is missing. This could be attributed to insufficient identification tools available this time, now many modern tools are available. Studies by Serrano et al. and Carrapiço [19,20] typed some bacterial genera: *Pseudomonas*, *Alcaligenes*, *Caulobacter* and *Arthrobacter*. Nierzwicki-Bauer and Aulfinger [18] presented description of 5 different microorganisms inhabiting leaf cavities of *A. caroliniana* being both G⁺ and G⁻ bacteria. All these studies based on the use of biochemical and microbiological test for describing these microorganisms. Anyhow, none of them use any molecular analysis for bacteria identification. However, recently an interesting paper of Dijkhuizen and colleagues [25] was published who performed metagenomic study of *A. filiculoides* genome. They found Burkholderiales, Caulobacteriales and Rhizobiales as the most abundant microbial groups accompanying the fern. Deeper analysis revealed microorganisms belonging to *Microbacterium*, *Hypomicrobium*, *Shinella*, *Ralstonia*, *Rhizobium* and *Hydrocarboniphaga* genera [25]. In our study Burkholderiales constituted 23% and Rhizobiales 11% of epiphytic microbiome what is one-third of the whole identified microbiome. In addition, we obtained the two isolates belonging to *Microbacterium*. As there is no more similar studies we consider our study as next, very important which is going to deep knowledge on *A. filiculoides* microbiome.

For phenotyping of the microbiome we found only one work describing ability of *A. filiculoides* and *A. pinnata* endosymbiotic *Arthrobacter* sp. for IAA production, where auxin concentration remained on the level of 1.5-10 $\mu\text{g ml}^{-1}$ at L-tryptophan dosage of 100-600 $\mu\text{g ml}^{-1}$ [29]. Other studies demonstrated different effectivity for IAA production by various microorganisms. In the study of Ghodsalavi et al. [30] the highest production of IAA ($>20 \mu\text{g ml}^{-1}$) was recorded for *Pseudomonas* sp., whereas in respect to *Bacillus* sp. and *Agrobacterium* sp. amounted 3-7 and 16 $\mu\text{g ml}^{-1}$, respectively. Dutta et al. [31] showed IAA production of 87.9 $\mu\text{g ml}^{-1}$ for *Bacillus* sp. Meanwhile, Morel et al. [32] proved that *Delftia* sp. JD2 is able to synthesize IAA up to 80 $\mu\text{g mg}^{-1}$ dw when exposed to Cr(VI) ions. The strains belonged to genus *Bacillus* was reported as displaying both cellulase and protease activity [31,33]. Ghodsalavi et al. [30] reported about high ability of *Bacillus* sp. for protease activity which made halos 2-3 cm diameter what is about twice bigger than in our study. *Agrobacterium* sp. made 2.3 cm halos whilst in our study it made 2.95 cm zones. Opposing to our study Cho et al. [33] indicated no ability to decompose the pathogen cell wall by *Microbacterium* sp. An ability for P solubilization by *Bacillus* sp. was reported by Dutta et al. [31]. In the study of Jorquera and colleagues [34] PSB, PSM and PMPSB microorganisms were reported, however, authors mentioned only the strains belonged to genera: *Pseudomonas*, *Enterobacter*, and *Pantoea*, whereas Chen et al. [35] presented for the first time *Delftia* sp. as PSB. The production of siderophores by *Bacillus* sp. was reported by Ghodsalavi et al. [30] and Dutta et al. [31]. Morel et al. [32] reported *Delftia* sp. JD2 to produce siderophores under Cr(VI) stress conditions.

It is worth to emphasize that other authors presented ability for plant growth promotion of microorganisms originating from soil or isolated from terrestrial vegetation. However, any data regarding to aquatic microorganisms are poorly recognized. Most of described microorganisms were not found in *A. filiculoides* microbiome and only *Bacillus* sp. was often pointed.

Delftia sp. AzoEpi7, by displaying the highest potential in growth promotion among all isolates, particularly attracted our attention. Members of the genus *Delftia* are aerobic, non-endospore forming, Gram-negative rods that inhabit diverse ecological niches. Taxonomically, this genus belongs to Comamonadaceae family within the Burkholderiales order of the Betaproteobacteria class. Currently, it comprises five species: *D. acidovorans*, *D. tsuruhatensis*, *D. lacustris*, *D. litopenaei* and *D. deserti* [36]. *Delftia* sp. is known as halotolerant bacteria possessing the ability for organic biodegradation [37]. It has been also reported that *Delftia* sp. have potential roles in bioremediation of organic and inorganic pollutants and production of industrially valuable compounds resulting in some patents [38]. In addition, as an effect of *Delftia* sp. genome sequencing (6-6.7 Mb, GC content of approx. 66%) it was proved that particular genetic elements are involved in diverse biodegradation pathways, and heavy metals resistance [36,39], phytohormone and siderophore production [32] and antimicrobial compound production [40]. In 2013, Johnston et al. [41] reported that *D. acidovorans* displayed resistance against gold by producing a secondary metabolite allowing biomineralization of metal from liquid. Recently, Li and colleagues [42] proved that *D. tsuruhatensis* GX-3 is able to bioaccumulate gold forming nanoparticles outside its cell. This makes *Delftia* sp. extremely interesting bacteria from biotechnological and engineering

point of view as any ways for reclaiming precious metals are tempting and wanted. In work of Jangir and colleagues [43] another interesting potential of *Delftia* sp. was demonstrated. It appeared to use extracellular electron transfer (EET) strategy for energy harvesting generating anodic current using acetate as electron donor. This may indicate on *Delftia* sp. potential in electricity generation via Microbial Fuel Cells (MFC). All these information testify about high importance of this bacteria. Another feature of *Delftia* sp. is the production of nanopods, extracellular structures important in cell-to-cell interactions, when grown on phenanthrene [44].

It would be worth to study the role of microbiome in metal remediation as there are studies on microbially-assisted phytoremediation [7,9,45] what would be beneficial for designing better metal-removing biological systems. Moreover, our previous studies [11] showed high potential of *Azolla* sp. in lowering metals levels in waters what encourages us to study newly discovered microbiome and *Delftia* sp. AzoEpi7 in particular.

4. Materials and Methods

4.1. Plant material

A. filiculoides originated from our laboratory culture established in 2010 using material obtained from Warsaw Botanical Garden (Poland). Plants were grown according to the recommendation of International Rice Research Institute [46] (Appendix A). After 3 weeks obtained biomass was used for the microbial isolation.

All reagents were dedicated for microbiological analyzes and purchased from Sigma-Aldrich, also water was deionized and sterilized prior the use (sdH₂O).

4.2. *Azolla* cyanobiont

The isolation of the cyanobiont was performed by crushing plant material (sterilized three times in 0.12% NaClO, 0.01% Triton X-100 for 10 min, next in 70% ethanol and sdH₂O) between two sterile microscopic slides and washing with nitrogen-free BG110 medium [46]. Next material was incubated in batch culture using 15 ml BG110 medium (1:6, culture : medium ratio) at 23°C and 63 μ mol quantum photosynthetically active radiation (PAR) per m² s at 24/0h photoperiod (n=5). After 7 days cells were passaged by inoculating 20 ml of fresh BG110 medium with 2.5 ml inoculum (n=4). One week later next subculture was performed (6.5 ml of inoculum + 40 ml of both media, 7 days). Importantly, after each step the samples have been taken for microscopic observations and the end of last passage the material was used for isolation of cyanobacterial DNA.

4.3. Isolation of microorganisms

Prior the isolation of endophytic microorganisms plant material was sterilized under laminar chamber. For this operation 3 healthy plants were randomly chosen from the culture in order to provide repeatability. The material was washed in sdH₂O and separated into shoots and roots. Next, plants parts were immersed for a given time in the subsequent reagents: (1) 0.1% Tween 80 for 30 s, (2) 1% NaClO for 5 min, (3) 70% ethanol – 5 min and (4) sdH₂O – 5 min. The efficiency of sterilization was assessed by inoculating Petri's dishes with the water from

last washing. Each sterilized plant part was grinded in a mortar using 1 ml of phosphate buffer (pH 6.7). Next, three 250 μ l samples of each grinded material were transferred into Eppendorf tubes. All samples were subjected to series dilutions up to 10^{-4} . The samples were grown on nutrient agar medium as described below.

The microorganisms present on the surface of *A. filiculoides* were isolated in two ways. First involved placing few randomly chosen plants into beaker with 10 ml of phosphate buffer and after carefully stirring plants were discarded. Three 250 μ l samples of the solution were diluted to 10^{-4} and sowed on nutrient agar medium. Second variant of isolation aimed on placing randomly picked plants onto the agar ($n=3$) setting their top part on the agar.

4.4. Cultivation and description of isolated microorganisms

All samples (250 μ l) were spread on sterile nutrient agar supplemented with nystatin (50 mg ml⁻¹) to avoid fungi grow (which were not the subject of this study) and incubated in dark at 30°C for 7 days (Hereus B20, Thermo Fisher Scientific, USA). Then microbial colonies were inoculated into fresh medium and cultivated as above and procedure was repeated until pure cultures were obtained.

The morphology of colonies were described in terms of their: shape (surface, elevation, margin, texture, size), pigmentation and opacity. Their counts were made and referred to the total number of isolates (58) and total counts of both epi- (43) and endophytes (15). These numbers were further discussed as percentages but in the table they are presented as individual counts. In order to describe cells shape of the isolates and the type of their cellular wall Gram staining method was applied. The resulting slides were examined under Nikon Eclipse 80i microscope and photographs were taken using digital camera with NIS-Elements software (Japan). Cells of purple color were considered as Gram-positive, whilst red color indicated Gram-negative microorganisms.

The isolates was cultured on a liquid nutrient broth for 7 days at 30°C (New Brunswick™ Innova® 42R, Eppendorf AG, Germany). During incubation optical density (OD₆₀₀) was determined spectrophotometrically (Shimadzu UV-1800, Japan) to construct growth curves for the microorganisms (Table A2). For long-term storage 700 μ l of each inoculum was mixed with 300 μ l of glycerol (3 replicates for endophytes and 2 replicates for epiphytes) and frozen at -80°C (ZLN-UT 300, Pol-Eko-Aparatura, Poland). Remaining samples were used for the subsequent analyses.

4.5. Identification of cyanobiont

Only living material from cultures was applied for taxonomic identification. Microscopic observations in a light field and using a UV lamp were carried out to observe and measure colonies, cells and heterocysts of the studied cyanobacteria. The taxonomic designation was based on Komárek [47] and Hindak [48].

4.6. Molecular techniques

Total genomic DNA was isolated according to Stepniewska et al. [49] (Appendix A) followed by PCR reaction. PCR mixture contained 1X Phusion Flash High-Fidelity PCR Master Mix

(Thermo Scientific, USA), 1 µl of template DNA (1100 µg/ml on the average, Table A1) and sterile double-distilled water (free DNase) in a total volume of 25 µl. Universal eubacterial primers (each 1.0 µM): 27F and 518R (Table A2) were used. The reaction was carried out under the following conditions: 98°C for 10 s; 30 cycles of 95°C for 5 s, 56°C for 5 s, and 72°C for 40 s (LABCYCLER, SensoQuest GmbH, Germany). For cyanobiont DNA amplification four following primers were used: cyanobacterium-specific 23S30R and CYA359F whilst for targeting *nif* gene: *nif*-Df and *nif*-Dr (Table A2). PCR reactions were carried out as follows: 98°C for 10 min; 30 cycles of 98°C for 5 s, 55°C for 5 s, and 72°C for 60 s (*nif* starters) and 98°C for 5 min; 30 cycles of 98°C for 35 s, 54°C for 45 s, and 72°C for 60 s (16S rRNA starters). PCR products were run on agarose gel (1%) and visualized with use SimplySafe™ (EURx, Poland). What is more, the control reactions were performed: negative – contained only sterile double-distilled water (free DNase) without a DNA template, as well as a positive, in which a template was DNA isolated from *E. coli* DH5α™. Then, all PCR products were purified and sent to sequencing (Genomed S.A., Poland). Obtained sequences were analyzed by BLASTN algorithm (NCBI, USA) for identification of the isolates. Identified sequences have been deposited in GeneBank under following accession numbers: MG859252-7, MG881884-915, MG881917-9, MH605441-3 and MH605510-14.

4.7. Phenotypic characterization

Bacterial strains in an exponential phase were applied for testing the ability of microbiome for synthesizing plant growth promoters. The production of indole-3-acetic acid (IAA) was initiated by inoculating the liquid nutrient broth, supplemented with 1 g l⁻¹ of L-tryptophan. Quantification of IAA was performed using Salkowski's reagent (35% HClO₄ + 0.5 M FeCl₃·6H₂O) and colorimetric analysis at 530 nm in reference to calibration curve. Samples of a pink color were considered positive for the production of IAA [50] (Appendix A). Results were presented as means±SD (standard deviation).

The ability for synthesis of cellulolytic enzymes was assayed by growing microorganisms (30°C for 24 h) on nutrient agar supplemented with 1% carboxymethylcellulose (CMC) sodium salt (cellulase activity indicator medium). For the visualization of cellulose activity Lugol's solutions was applied. The positive reaction was observed when colonies of isolates were surrounded by a yellow halo against dark background [51]. Protease activity was determined by culturing of selected isolates on nutrient agar supplemented with 5% skim milk at 30°C in darkness (protease activity indicator medium). Development of clear zones around colonies during revealed protease activity.

Phosphate utilization by microorganisms was determined using two P sources: organic, sodium phytate, C₆H₁₈P₆O₂₄·12Na·xH₂O (PSM medium) and inorganic calcium phosphate (Ca₃(PO₄)₂) (NBRIP medium). First was used for identification of P-mineralizing bacteria (PMB) and the second for P-solubilizing bacteria (PSB) [34]. Inoculated media were incubated in 30°C for 4 days. The presence of clear zones around the colonies was taken as an indicator of phytate mineralization and phosphate solubilization. Basing on this observations we divided microorganisms into PMB, PSM and those using both P-sources (phosphate mineralizing, phosphate solubilizing bacteria, PMPSB).

Siderophore production was quantified using CAS-agar assay of Alexander and Zuberer [52] (Appendix A). Positive results were indicated by the formation of a clear halo around the colonies, showing a visual change in color from dark blue to yellow.

For all plate tests, Petri's dishes were inoculated with 15 µl of cell suspension in 4 points. To quantify the enzymatic activities, the sizes of colonies, halos and halo-to-colony size ratios (n=3) were calculated and data are presented as means±SD.

5. Conclusions

Our experiment proved that *A. fliucluides* is inhabited by not only its cyanobiont but also by bacteria present both on its surface (epiphytes) and inside the plant (endophytes).

In general, the isolates belonged to Gram positive bacteria being mostly punctiform size (epiphytes also small size) of a circular shape, raised of glistening and smooth surface (epiphytes also rough) with butyrous texture, opaque and cream pigmentation (endophytes also yellow and white-cream) having entire margin.

Phylogenetic analysis allowed us to classify the isolates to 9 bacterial genera. For epiphytes they belonged to: *Achromobacter*, *Bacillus*, *Microbacterium*, *Delftia*, *Agrobacterium*, *Alcaligenes*, while endophytes were classified as: *Bacillus*, *Staphylococcus*, *Micrococcus* and *Acinetobacter*.

The tests applied for plant growth promotion features determination revealed high importance and benefits of tested microbiome for plants. All isolates were able to synthesize the enzymes responsible for cell wall lysis (cellulase and protease). In addition, all of them showed P mineralization potential and some P solubilization. Three bacterial strains (*Micrococcus* sp. AzoEndo14, *Delftia* sp. AzoEpi7, *Agrobacterium* sp. AzoEpi25) synthesized IAA. Siderophores were only produced by endophytic *Bacillus* sp. AzoEndo3 and epiphytic: *Achromobacter* sp. AzoEpi1, *Delftia* sp. AzoEpi7, *Alcaligenes* sp. AzoEpi21 and *Agrobacterium* sp. AzoEpi25. *Delftia* sp. AzoEpi7 seemed to be the only one showing ability to synthesize all studied growth promoters, thus is why we recommend it as the most beneficial for host plant.

Author Contributions: The concept of the study was made by A.B. and A.K., the methodology was selected and applied by A.B. and A.K. (isolation of microorganisms, cultivation, description, molecular studies, phenotyping); cyanobiont was identified by R.F. Results were analyzed and described by A.B., A.K., R.F. and A.W. Manuscript was written by A.B. and reviewed by A.K., A.W. and R.F.

Appendix A

Cultivation of *A. fliucluides*

Plants were grown in glass aquaria (20×30×15 cm) on recommended IRRI medium [46] without nitrogen supply supplemented in 0.1 ml l⁻¹ anti-algal agent, Algin (Topical®, Poland), containing CuSO₄·5H₂O as the active substance. Fluorescent Philips lamps Master TL-D 36W/830 were used to provide 3500 lux light energy (corresponding to 14 W m⁻² or 6.3 µmol quantum of photosynthetically active radiation, PAR per m² s) at 16/8h

photoperiod, temperature of $20.69 \pm 1.55^\circ\text{C}$ and relative humidity of $84.5 \pm 5.16\%$ (H-881t hygrometer, Zootechnika, Poland).

Isolation of bacterial DNA

The method bases on original Sambrook et al method [53]. Cells from 10 ml samples of late exponential cultures were collected by centrifugation. The pellet was suspended in 250 μl of TE buffer containing 50 mM Tris-HCl (pH=8,0) and 50 mM EDTA (pH=8,0). To achieve complete lysis of the cells, 1 ml of GES buffer (pH=8.0) containing 5 M guanidine thiocyanate, 100 mM EDTA, and 0,5% sarkosyl was added. The mixture was incubated at room temperature for 10 minutes and then “crude lysates” were cooled on ice. After addition of 125 μl of ammonium acetate (7,5 M), the samples were mixed and further incubated on ice. The DNA obtained was purified with 250 μl of a chloroform-isoamyl alcohol (24:1) mixture, precipitated with isopropanol, washed with cold ethanol, and dissolved in 50 μl of sterile distilled water.

Phenotypic analysis

IAA production. Cultures (n=3) were incubated in 30°C for 5 days in darkness on a rotary shaker (125 rpm) on liquid nutrient broth supplemented with 1 g l^{-1} of L-tryptophan. Next, samples were centrifuged at 10,000 rpm for 10 minutes and 2 ml of supernatant was mixed with 4 ml of Salkowski's reagent (50 ml 35% HClO_4 , 1 ml 0.5 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) [54]. After letting the mixture in 30°C for 30 minutes in darkness concentration of IAA was measured colorimetrically at 530 nm (Shimadzu UV/VIS-1800, Japan) using calibration curve ranging up to $100\text{ }\mu\text{g ml}^{-1}$. The calibration was prepared by processing IAA solution in the same manner as samples.

Siderophore production. 4 solutions were made: Fe-CAS indicator (1.21 mg ml^{-1} of CAS in 1 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10 mM HCl), HDTMA (1.82 mg ml^{-1}), buffer (30.24 g of PIPES, 0.3 g KH_2PO_4 , 0.5 g NaCl, and 1.0 g NH_4Cl , pH 6.8), medium (493 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 11 mg CaCl_2 , 1.17 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.4 mg H_3BO_3 , 0.04 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.2 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.0 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) and 10% (w:v) casamino acids. All solutions were autoclaved separately, cooled to 50°C and mixed together.

Appendix B

Table A1. Similarity of sequences of the isolates to the sequences in GeneBank and their DNA concentration.

Table. A2. Primer sequences used for PCR.

Figure A2. Growth curves for cultured microorganisms.