



Research article

Long-term investigation of constructed wetland wastewater treatment and reuse: Selection of adapted plant species for metaremediation



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ABSTRACT

A highly diverse plant community in a constructed wetland was used to investigate an ecological treatment system for human wastewater in an arid climate. The eight-year operation of the system has allowed the identification of a highly adapted and effective plant consortium that is convenient for plant-assisted metaremediation of wastewater. This constructed wetland pilot station demonstrated effective performance over this extended period. Originally, there were twenty-five plant species. However, because of environmental constraints and pressure from interspecific competition, only seven species persisted. Interestingly, the molecular phylogenetic analyses and an investigation of the photosynthetic physiology showed that the naturally selected plants are predominately monocot species with C4 or C4-like photosynthetic pathways. Despite the loss of 72% of initially used species in the constructed wetland, the removal efficiencies of BOD, COD, TSS, total phosphorus, ammonia and nitrate were maintained at high levels, approximately 90%, 80%, 94%, 60% and 50%, respectively. Concomitantly, the microbiological water tests showed an extremely high reduction of total coliform bacteria and streptococci, about 99%, even without a specific disinfection step. Hence, the constructed wetland system produced water of high quality that can be used for agricultural purposes. In the present investigation, we provide a comprehensive set of plant species that might be used for long-term and large-scale wastewater treatment.

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1. Introduction

Because of increasing anthropogenic activity and poor wastewater management policies, humanity is now facing water shortages and water quality crises in many areas of the world. Several investigations have reported that only 20% of the wastewater worldwide receives adequate treatment (United Nations Educational, Scientific and Cultural Organization (UNESCO) UN-WATER, n.d.), and the treatment capacity is as low as 8% in low-income countries (Sato

et al., 2013). Wastewater from human dwellings and activities has been a primary target of many treatment technologies. In parallel, many strategies have been used in the attempt to solve this concern. Ecological and green strategies have emerged as low-cost, effective and socially accepted approaches. Concomitantly, recent 'omic' approaches, when combined with other methods, such as stable-isotope probing (SIP), yield information on community members that metabolize a particular substrate in complex ecosystems. Hence, it is possible to link taxonomic groups with organic pollutant degradation (Singleton et al., 2007, 2005). These approaches have highlighted the idea that complex organic pollutants are metabolized by microbial consortia and assemblages of green plants in the field (Terrence et al., 2013). These innovative approaches bring new insight on how organisms can function as a meta-organismic entity to both remediate polluted ecosystems and, by effectively treating

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human and industrial wastewater, prevent the contamination of water resources (El Amrani et al., 2015).

Indeed, plants have been shown to be the main ecological engineer in polluted environments, as plant roots release exudates that contain various nutritional and signalling molecules that influence bacterial and fungal populations. In wetlands, water-tolerant plants also pump air into their root systems, creating microzones where aerobic microbes can thrive. The complex interactions of these populations play a pivotal role in the biodegradation of complex organic xenobiotics. Studies on emerging integrative approaches, such as (meta-) genomics, (meta-) transcriptomics, (meta-) metabolomics, and (meta-) proteomics, illustrate how “omics” approaches can bring new insight to deciphering the molecular mechanisms of pollutants at both the single species and the community levels (El Amrani et al., 2015).

Accumulating data, considering the functioning of the whole biome involved in decontamination, show that plants that have adapted to contaminated ecosystems deeply influence the microbiome associated with the rhizosphere (defined as the area located in the vicinity of roots) and reprogram the microbiome in this area. Thereby, as distinct from phytoremediation, bioremediation or mycoremediation, which separately consider plants, bacteria or fungus, respectively, metaremediation was recently proposed as a new strategy to remediate polluted environments (El Amrani et al., 2015). Recent data have suggested that the joint action of plant-fungal-bacterial consortia results in the rapid and effective degradation of complex molecules (Boonchan et al., 2000). Consequently, in natural ecosystems, remediation must be evaluated at the meta-organismic level (Bell et al., 2014). Therefore, to reach a high catabolic potential, it is necessary to integrate the global meta-organism/holobiont scale to increase the efficiency of biologically based remediation.

The constructed Temacine wetland (Algeria) traces its origins to Biosphere 2 project systems based on high-biodiversity, subsurface-flow wetlands treatment/recycling systems for wastewater, which were developed by NASA and then further evolved into “Wastewater Gardens” (Nelson and Wolverton, 2011; Nelson, n.d.). The system studied was implemented by Dr. Mark Nelson and Florence Cattin of Wastewater Gardens International (www.wastewatergardens.com). The project was financed by the Algerian Government Ministry of Water Resources, Department of Sanitation and Environmental Protection (Ministère des Ressources en Eau (MRE) - Direction de l'Assainissement et de la Protection de l'Environnement (DAPE/MRE), and the town of Temacine with the support of the Belgian Technical Cooperation for the study and training part of the project. Covering 400 m² and shaped like a crescent moon, the Wastewater Gardens system was designed to handle 15 m³ of daily wastewater effluent from 100–150 people (Nelson and Wolverton, 2011).

Constructed wetlands are engineered systems designed to use natural wetland processes, associated with wetland hydrology, soils, microbes and plants, to treat wastewater (Vymazal, 2009). Recent investigations have confirmed that the rhizosphere in wetlands is especially effective at reducing contaminants and improving water quality (Kaplan et al., 2016).

This technological approach has been spreading around the world as thousands of wastewater-constructed wetlands were implemented in many countries in varying climates and ecological conditions over the last few decades (Kadlec RH and Wallace SD, n.d.; United Nations Educational, Scientific and Cultural Organization (UNESCO) UN-WATER, n.d.). This strategy has been shown to produce high water quality in a myriad of conditions; thus, the concept of using constructed wetlands has gained support in Africa. Firstly in South Africa then in the late 1990s, wetlands were piloted in Egypt in Alexandria, Abbu Attwa, Ismailia (Williams et al., 1995), and several

systems were implemented in Morocco (Salama et al., 2014). However, there is no information concerning the longevity of the functioning of these particular wetland ecosystems and how the initial high biodiversity evolved in this wetland and climatic environment.

In this work, we present the data collected during eight years, from 2008 to 2015, of a pilot station of a national program aiming to use biological treatment of wastewater in small agricultural communities and towns. The constructed wetland studied in this paper was installed in the town of Temacine located in Wilaya de Ouargla (Algeria) in 2007. Our results show that the wastewater treatment of the pilot station accomplished a significant reduction of the organic load in an arid climate over the course of the study and produced an effluent of high-quality water, which meets the requirements and is being used for agricultural/horticultural irrigation. At the same time, from the initially higher biodiversity of plant species, only the plants that are well-adapted to these wetland and environmental conditions were maintained. Hence, in the present investigation we provide a comprehensive set of plant species which might be used for a long treatment program and for large-scale wastewater treatment in arid areas.

2. Materials and methods

2.1. Pilot station and samplings

The trial was conducted in a subsurface, horizontal flow, constructed wetland in Temacine designed (Lat. 31° 36' N, Long. 5° 54' 6" N and 32° 18' E, alt. 49 m) by Wastewater Gardens International (Fig. 1). The climate is Saharan and arid with an average annual rainfall of approximately 60 mm. The mean temperature ranges between 10 °C and 35.8 °C. The experimental design contained a wastewater sedimentation and primary treatment tank in the form of a 2-chamber septic tank with a final filter followed by a 400 m² treatment basin with 3% bottom slope, which had a 60 cm gravel bed (8–12 mm particle size, with an average porosity of 29%) with 5 cm of dry gravel covering the wastewater in the plant-assisted metaremediation treatment basin, thus insuring no bad odour or human contact occurs. The sample-collecting pipes are located as indicated in Fig. 1. These collecting points were used to collect samples for the biochemical and microbiological analyses.

A large plant biodiversity was established at the installation of the pilot station to optimize the absorption and biodegradation of organic and inorganic molecules contained in wastewater. Special attention was given to the inclusion of plants with high added values. The choice of plants was made according three main criteria: (i) plants adapted to arid environments and developing different root systems: deep, medium and shallow, (ii) plants with high utility and productivity and/or pleasing aesthetics, and (iii) plants which were to be tested (some of which were previously used in Wastewater Garden systems but not yet tested in this particular arid climate). The plants were initially planted from April to May 2007. Several supplementary species were added the following year to increase plant biodiversity.

Wastewater was discharged in the pilot station with a gravity-led horizontal flow of 15 m³/day. This corresponds to a retention time of 2.7 days in the sedimentation tank and 5 days in the metaremediation constructed wetland basin. The water flows reached the wet zone by gravity at an average speed of 4 L per second. The treated water was conducted through a drainage network for drip irrigation of an adjacent planted field.

2.2. Biochemical and bacteriological analysis

Samples were collected twice a month in the morning and then analysed in the laboratory of Saharan Bioresources at Ouargla

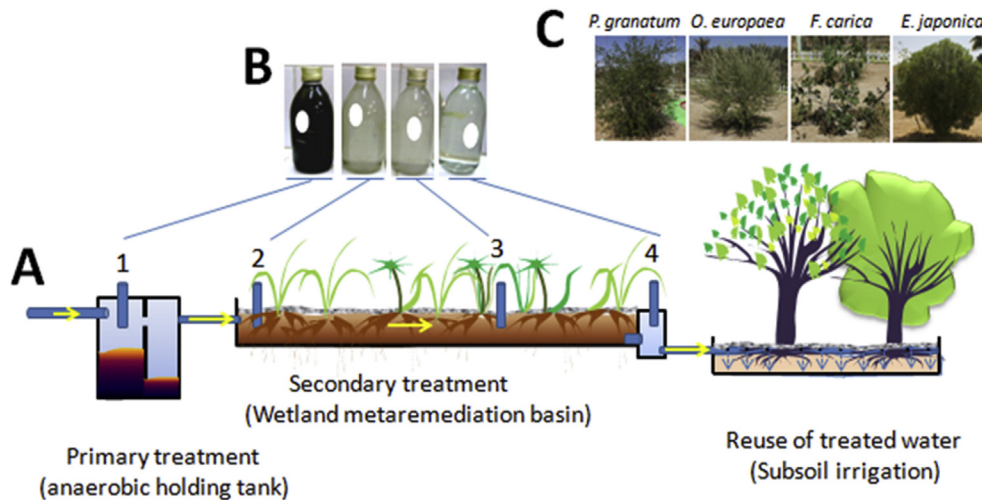


Fig. 1. Schematic representation of the pilot station. A. The primary treatment occurred in an anaerobic septic tank. The yellow arrows show the gravity flow. The resulting treated water was reused by gravitational flow to irrigate a field with crops and trees. B. Visual observation of wastewater samples collected at different point as depicted by the pipes 1, 2, 3 and 4. C. Phenotypical and morphological aspects of agronomical and commercial species developed in the subsurface irrigation field during the eight-year trial. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

University using the AFNOR method for the determination of BOD (Biological Oxygen Demand), COD (Chemical Oxygen demand), TSS (Total Suspended Solid), nitrite ($\text{NO}_2\text{-N}$), nitrate ($\text{NO}_3\text{-N}$), and total phosphorus (TP). Temperature, pH, electrical conductivity (EC) and dissolved oxygen (DO) were measured *in situ*. Total coliform bacteria and faecal Streptococcus were determined by inoculation in a liquid medium (Ausland et al., 2002). Meteorological data (air temperature, solar radiation, humidity, air speed and direction of wind, and precipitation depth) were collected from the weather station at INRAA Touggourt.

2.3. Phenanthrene quantification

The determination of the phenanthrene concentration in the effluent was performed as follows. Samples were collected from the indicated points of the pilot station and then immediately lyophilized at $-20\text{ }^\circ\text{C}$. The resulting materials were weighed. Phenanthrene was extracted by an accelerated solvent extractor (ASE 200, Dionex) with dichloromethane at $100\text{ }^\circ\text{C}$ and under a pressure of 100 bars. The extracts were dried under a gentle flux of nitrogen and finally weighed. One microliter of the extract was injected onto a Shimadzu QP2010+MS gas chromatograph/mass spectrometer (Shimadzu, Tokyo, Japan). The injector used was in splitless mode and maintained at a temperature of $310\text{ }^\circ\text{C}$. The chromatographic separation was performed on a fused silica SLB-5 ms capillary column (from Supelco, length = 60 m, diameter = 0.25 mm, film thickness = $0.25\text{ }\mu\text{m}$) under the following temperature program: $70\text{ }^\circ\text{C}$ (held for 1 min) to $130\text{ }^\circ\text{C}$ at $15\text{ }^\circ\text{C}/\text{min}$ and then $130\text{ }^\circ\text{C}$ to $300\text{ }^\circ\text{C}$ (held for 15 min) at $3\text{ }^\circ\text{C}/\text{min}$. The helium flow was maintained at 1 ml/min. The chromatograph was coupled to the mass spectrometer by a transfer line heated to $250\text{ }^\circ\text{C}$. The analyses were performed in SIM mode (Selective Ion Monitoring). Quantification was based on the internal standard phenanthrene- d_{10} , which was added to the sample post-extraction and prior to the analysis by GC-MS.

2.4. Molecular phylogenetic analysis

We searched for rbcL sequences in GenBank databases (<http://www.ncbi.nlm.nih.gov/>). Among the 25 taxa used herein, 18 had available rbcL sequences. For the remaining 7 taxa, we used rbcL

sequences from a congeneric relative (such as for the genera *Jasminum*, *Lonicera*, *Pelargonium*, *Morus*, *Musa* and *Psidium*) only if there were no other congeners used in the experimental design (such as for *Rosa*). Therefore, 28 rbcL sequences were included in a data matrix and aligned using EMBL-EBI bioinformatics web (Li et al., 2015). After adjusting the data matrix by cutting the incomplete edges, we searched for the best-fit substitution model for the rbcL sequences alignment (based on the lowest Bayesian Information Criterion) prior to performing a phylogenetic analysis using the Maximum Likelihood (ML) method conducted in MEGA7 (Kumar et al., 2016). The bootstrap method was used to evaluate the robustness of the nodes (Felsenstein, 1986).

2.5. Scanning electron microscopy

Plant species were harvested from the wetland; leaf cross-sections were cut out with a razor and placed on a support. The plant materials were sputter-coated with gold for 240 s to avoid charging and then observed and imaged through scanning electron microscopy (JSM 7100F).

2.6. Statistics

Statistical analyses for the comparison of means between the concentrations of water parameters and between seasons were studied by ANOVA. Statistical analyses were performed using Excel.

3. Results and discussion

3.1. Constructed wetlands for wastewater treatment and reuse

Many pilot constructed wetland systems based on the Wastewater Garden (WWG) International design, which is based on the metabolic activities of plants and their associated microbiomes, have been implemented in 14 countries worldwide to test their performance and reliability in a myriad of climate conditions (Nelson and Wolvert, 2011). The WWG wastewater treatment and reuse technology have been shown to produce very high-quality water. However, the efficacy of WWG and other constructed wetlands in retaining or eliminating organic and mineral pollutants, total suspended particles and pathogenic bacteria has

generally been tested over a shorter period than the current study (Chen et al., 2015; Choi et al., 2016; Hadad et al., 2006; Kadlec RH and Wallace SD, n.d.; Nelson and Wolverton, 2011; Ranieri and Gikas, 2014; Ranieri, 2012). Therefore, there are little data about how these constructed wetlands may function over longer periods of years and how the efficacy of remediation may change. In this work, our attention was focused on the investigation of a pilot station located in an arid area in Temacine (Algeria). The station was constructed in 2007, and a high biodiversity of plants and a large selection of rhizosphere-associated microbiomes were used in an effort to optimize the metabolic potential. Twenty-five local plant species (see Table 1) were planted; 1 to 3 plants (depending on size) were planted per square meter, and plants with shallow, moderately deep, and very deep roots were combined to ensure effective penetration of all of the gravel. A large number species was used for the experiments because there is no prior experience in the region with constructed wetlands, and there was an expectation of losses due to the stresses of the site's environmental conditions and uncertainties about the suitability of some species in water-saturated wetland substrates. Fig. 1 shows the gravity flow of wastewater from the first treatment zone (septic tank) to the metaremediation basin. The treatment basin received a total of 15 m³ of wastewater per day. The treated water was conducted through a drainage network for drip irrigation of a planted field. Although the constructed wetland was designed for 15 m³ of wastewater per day, the volume of the effluent fluctuated depending on the season, the weather conditions, and the activity of the homes and buildings connected to the system. Water losses in the metaremediation wetland basin system are higher in spring and summer than in autumn and winter. We hypothesized that these variation are mainly related to evapotranspiration by the plants.

3.2. Eight years of pilot system operation leads to selection of better-adapted plants

Throughout the eight years of the remediation system experiment, some species failed to develop and died, especially some local plants such as *Vetiver zizanioides*, *Lantana camara*, *Lonicera*

caprifolium and *Atriplex halumis*. They were planted several times but failed to grow or persist. Indeed, as sessile organisms, higher plants must cope, in their habitats, with imposed environmental constraints. When these conditions are far from the optimal conditions, plants species adapt their whole genome expression, their physiologic behaviour and, consequently, their phenotypes. This physiological and phenotypical plasticity defined tolerant and sensitive species to the given environmental factors.

At the beginning of the trial, we assumed that wetland wastewater remediation based on high biodiversity is desirable for a large metabolic and physiologic potential to clean up the polluted water. However, over the eight-year trial, several species repeatedly died after planting. Meanwhile, other species continued to grow and showed significant development in these conditions. It is not surprising that this mesocosm polluted by the influent human wastewater and characterized by extreme temperatures, humidity and salinity allowed the development of highly adapted/tolerant plants. Systems aiming to treat wastewater from a tool industry in Argentina resulted in the disappearance of 6 out of 8 transplanted macrophyte species in a pilot-scale wetland (Hadad et al., 2006). The authors noted that interspecific competition and tolerance to residual pollution highly impacted adaptation and plant performance in the constructed wetlands for wastewater treatment. Interspecific competition is an interaction between coexisting species within a community, which compete for a limited environmental resource by direct or indirect mutual inhibition. The differences in competitive ability among species are essentially due to the differences in properties of the species and their adaptability to the environment (Wang et al., 2015). In parallel, many ecological factors may impact interspecific competition. During the preparation of this manuscript, a very interesting work highlighted the role of mycorrhizal hyphal complexes in connecting multiple host plants to form common mycelial networks that affect plant competitive outcomes and community composition through differential resource allocation (Workman and Cruzan, 2016). To better understand how plants species are selected in artificial constructed wetlands on a longer time scale, metagenomic investigations are needed to unravel how the microbiome of this special environment may shape plant community.

Table 1
Plant biodiversity used in assisted metaremediation constructed wetland basin, list used at the beginning of the trial (2007).

Number	Species	Families	Quantity
01	<i>Laurus nobilis</i>	Lauraceae	03
02	<i>Cymbopogon</i>	Poaceae	407
03	<i>Ficus carica</i>	Moraceae	03
04	<i>Jasminum grandiflora</i>	Oleaceae	03
05	<i>Lantana camara</i>	Verbenaceae	10
06	<i>Chrysopobon</i>	Poaceae	03
07	<i>Rosa Banksia</i>	Rosaceae	03
08	<i>Cyperus papyrus</i>	Cyperaceae	44
09	<i>Hibiscus rosa sinensis</i>	Malvaceae	40
10	<i>Nerium oleander</i>	Apocynaceae	61
11	<i>Mentha spicata</i>	Lamiaceae	06
12	<i>Canna indica</i>	Cannaceae	105
13	<i>Typha latifolia</i>	Typhaceae	08
14	<i>Euonimus japonica</i>	Celastraceae	90
15	<i>Lonicera caprifolium</i>	Caprifoliaceae	03
16	<i>Pelargonium rosa</i>	Geraniaceae	06
17	<i>Punica granatum</i>	Punicaceae	04
18	<i>Morus nigra</i> L	Moraceae	03
19	<i>Carica papaya</i>	Caricaceae	04
20	<i>Musa ssp</i>	Musaceae	05
21	<i>Psidium guajava</i>	Myrtaceae	04
22	<i>Atriplex halumis</i>	Chenopodiaceae	03
23	<i>Juncus effusus</i>	Juncaceae	110
24	<i>Washingtonia robusta</i>	Arecaceae	03
25	<i>Lolium perenne</i>	Poaceae	02

3.3. Molecular and phylogenetic analysis of tolerant plants

To evaluate whether a correlation can be established between the evolutionary origin and diversity of the plants and their ability to tolerate exposure to wastewater during several years, we performed a phylogenetic analysis of these species to discover the most tolerant lineages compared with the eliminated ones.

Because the *rbcl* gene has been widely studied in plants and is commonly used for angiosperm phylogenies (Kress et al., 2009), we constructed an *rbcl* sequence data matrix to represent the phylogenetic diversity of the plant set used in this experiment following the procedure presented in the methods section. The analysis involved 28 *rbcl* nucleotide sequences representing the 25 plants used at the onset (2007) trial, which comprised 1 magnolid (in red-brown in the tree), 9 monocots (in green in the tree), and 15 eudicots (in blue in the tree). Three additional taxa (Magnolia, Nuphar and Amborella) were introduced in the analysis to serve as the outgroup and to root the tree. The Maximum Likelihood phylogenetic tree generated from this data matrix illustrates well the wide phylogenetic diversity of the plants used at the onset of the construction of the pilot station, which belongs to three main and early diverging angiosperm lineages: monocots, eudicots and magnolids (see Fig. 2). As summarized in the Venn diagram (Fig. 3A), only 7 species among the 25 initially used tolerated the experimental conditions over the course of the 8 years. Among the

15 genera representative of various rosid and asterid eudicot lineages, only *Nerium oleander* (Apocynaceae; Asterids) showed a tolerant phenotype. On the other hand, 6 of the 9 monocot species, all deriving from various commelinid lineages (Zingiberales, Poales, Arecales) tolerated the eight-year pilot experiment, whereas only the magnolid representative (*Laurus*) failed to grow in these conditions. Thus, these results provide some evidence supporting the selection of monocots in such wastewater-constructed wetland conditions.

It is tempting to speculate that monocots species express a particular set of detoxifying genes in these conditions, which featured high accumulation followed by high degradation of organic pollutants. Among tolerant plants, *J. effusus* and *T. latifolia* have previously been suggested to be able to absorb and degrade organic xenobiotic and to also to stimulate microbial degradation in the gravel bed substrate in wastewater wetland mesocosm pilot stations (Klink et al., 2016; “Use of Poaceae f. species to decontaminate soil from heavy metals,” n.d.). Additionally, *L. perenne* (“Use of Poaceae f. species to decontaminate soil from heavy metals,” n.d.) and *T. latifolia* (Klink et al., 2016) were found to accumulate high concentrations of heavy metals. Interestingly, in our previous investigation (El Amrani et al., 2015; Ramel et al., 2007), and in other research based on wide genome analysis (Weisman et al., 2010), we showed that under xenobiotic induced stress, plants express a specific set of genes involved in xenobiotic

degradation and tolerance. It was proposed that “the whole expressed genome responsible for the signalling, transport, and detoxification of xenobiotics” constitutes the xenome (Edwards et al., 2011). These genes belong to multigenic groups such as many original sets of transcription factors potentially involved in organic pollutant signalling; ascorbate peroxidases, glutathione-S-transferases, glycosyltransferases, and CYPs have been described as participating in the conjugation step and ATP-binding cassette (ABC) transporters in the compartmentalization of pollutants. A model for the detoxification of organic pollutants by plant cells has been proposed and dubbed the “green liver” because of the analogy to the mammalian liver (Sandermann, 1992). Hence, genome expression analyses are necessary to bring new insights about the differences between detoxification mechanisms in monocots and eudicots in plant-assisted wetland metaremediation. On the other hand, the wastewater treatment resulted in high salt accumulation in the treatment basin; this is in accordance with several other already published works (Herpin et al., 2007; Lu et al., 2016). High-salinity conditions are toxic for plant cells. However, halophytic plants are able to escape this toxic effect, as they have evolved many strategies to survive high-salinity environments (Gupta et al., 2014). Interestingly, most of the selected plants are known to survive to salt stress and are classified as halophytes. This finding is in accordance with our mesocosm conditions.

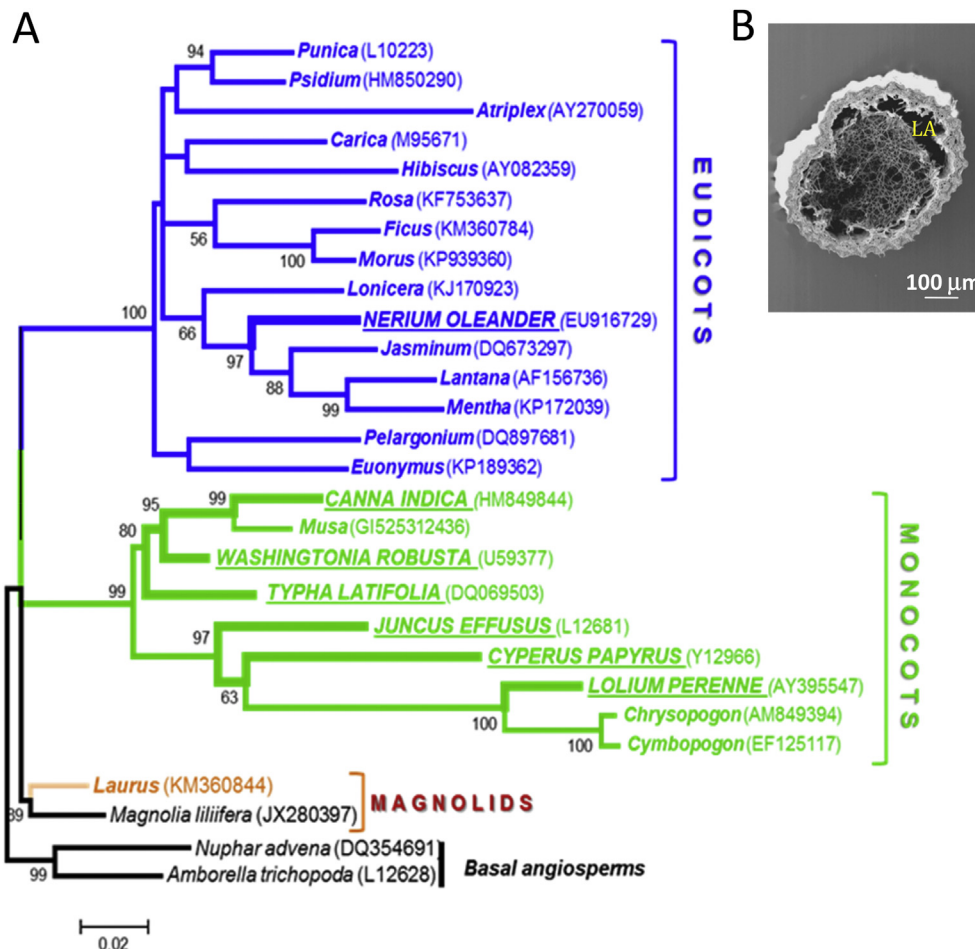


Fig. 2. Molecular phylogenetic analyses (A), and leaf cross-section of a representative monocotyledons species (B). A: The percentages shown next to the branches indicate the clades supported by bootstrap values higher than 50%. After 8 years of phytoremediation trial, only 7 species, 6 monocots and 1 eudicot, they are indicated by thick branches. Their taxon names are in bold capital and underlined. The other taxa that did not survive in this trial are indicated by thin branches in the tree and taxon names in small caps. B: Leaf cross-section of representative monocots, large aerenchyma (LA). Photograph indicated the leaf cross-section of a *Juncus* plant.

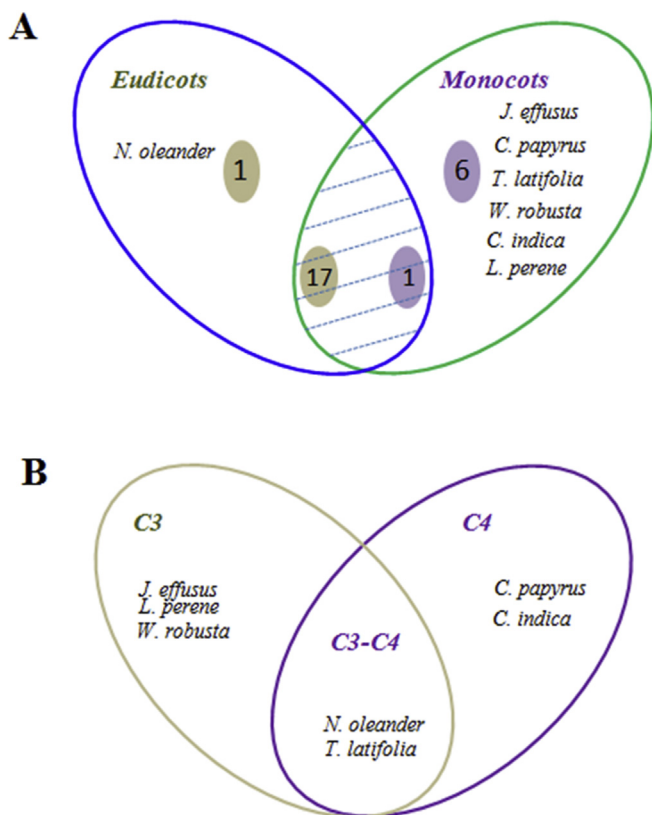


Fig. 3. Venn diagram depicting selected eudicots and monocots and C3/C4 photosynthesis plants at the end of the 8-year trial. (A) After 8 years of plant-assisted wetland metaremediation, 17 species, mainly eudicots died. The shaded area indicates the number of monocots (purple) and eudicots (grey) that died. As shown in the diagram, at the end of the trial only seven species were maintained in the treatment basin, mainly monocot plants. (B) Venn diagram depicting C3, C4 and C3-C4 plants at the eighth year of the trial. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Physiological adaptation to artificially constructed wetland

Photosynthetic C3 or C4 pathways may highly impact plant adaptation in arid wetland conditions (Atkinson et al., 2016). Indeed, C4 plants exceed 50% photosynthesis efficiency compared with C3 plants in arid conditions (Wang et al., 2012). This is due to the mechanism of carbon fixation. Ribulose-1,5-bisphosphate carboxylase (Rubisco) is the only used enzyme to fix CO₂ throughout the Calvin cycle in C3 plants. However, in C4 plants, the carbon fixation is initially catalysed by phosphoenolpyruvate carboxylase (PEPC), and this additional metabolic and histological compartmentation allows CO₂ concentrations to increase around Rubisco. Hence, this CO₂ concentration mechanism suppresses the oxygenation reaction by Rubisco and the subsequent energy-wasting photorespiratory pathway. This evolutionary transition from C3 to C4 photosynthesis occurred independently several times in a number of plant lineages and has resulted in a more efficient use of water and nitrogen. It is reasonable to speculate that such a feature may influence plant tolerance in constrained environments such as polluted wetland ecosystems. As shown in the Venn diagram (see Fig. 3B), two strict C4 plants were identified, and an additional two species are considered as C3-C4 intermediate plants. Even though *Nerium oleander* is considered as a C3 plant, the PEPC activity in this plant increased under stress conditions (Mandal, n.d.). Similarly, it has been reported that *Typha latifolia* exhibits a high carbon assimilation rate similar to C4 plants (Holm

et al., 1997). These data may argue that, in our conditions, C4 photosynthesis may bring a significant ecological advantage to survive polluted constructed wetland conditions.

3.5. Histological adaptation

Wetland conditions limit gas diffusion, and they are characterized by hypoxic and anoxic conditions. There is a strong relationship between soil saturation and the development of communities dominated by plants specifically adapted to such conditions. Hence, tolerance to waterlogged soil in a number of plant species has been shown to be associated with the production of specialized air cavities called aerenchyma (Steffens et al., 2013). These histological changes allow internal oxygen movement/ventilation from the leaves to the submerged roots of the plants. It is reasonable to speculate that selected plants in the pilot station are able to implement an affective aerenchyma network to facilitate oxygen ventilation in the submerged part of the plants, which are mainly in contact with wastewater. This aerenchyma formation has been described for many wetland and aquatic plants. Interestingly, this process has been investigated recently in *Typha latifolia* (found in the selected plant set). The authors showed that in wetland conditions 50% of leaf cross-sections were occupied by aerenchyma as a result of the degradation of some specialized cells designated as programmed cell death-susceptible cells (Ni et al., 2014). To verify these morphological changes, we harvested *Juncus effusus* leaves, and their cross-sections were examined and imaged through scanning electron microscopy. As expected, a large number of parenchyma was observed (Fig. 2B). We conclude that the ability to form aerenchyma by the selected plants is one of the important features of stress tolerance induced by wetland conditions and oxygen deprivation in the plant-assisted metaremediation basin.

3.6. Assessment of the pilot station efficiency over eight years

After several years of plant assisted metaremediation, we assumed that only highly adapted plants were maintained in the treatment basin. It is of high interest to evaluate the treatment reliability of this adapted mesocosm. Hence, to achieve this task, samplings of the influent and the effluent were collected from the sampling points 1 and 4 as shown in Fig. 1A. Typically, three assays were performed monthly; these values were used to generate data corresponding to each season. The data collected over the eight-year trial are depicted as a heat map (Fig. 4).

Typically, the TSS concentration of the sewage passing through the septic tank fluctuated during the four seasons from a minimum of 400 mg/l to a maximum of 560 mg/l. TSS at the output underwent a reduction rate from 91% to 96% in spring and in summer. The ANOVA showed that the difference between the entry and exit system is highly significant ($p < 0.01$).

The analyses showed no significant changes in the efficacy of the pilot station during the eight years of the trial. The residence time of almost 6 days in the constructed wetland allowed the system to provide clear and clean water with a greatly reduced TSS compared with that of the original sewage organic load (see Fig. 1B; samples were harvested in February 2016). We conclude that the removal of the organic load is due to physical filtration by the gravel substrate in addition to biochemical processes associated with the bacteriome and fungi associated with the macrophyte rhizosphere; similar studies outline the multiplicity of physical, chemical and biological processes in constructed wetlands (Nelson and Wolverson, 2011). The reduction of TSS is similar to those in many other wetland treatment systems, e.g., that found by Gersberg et al. (1986) for a residence time of 6 days.

The total phosphorus and BOD presented the same trends

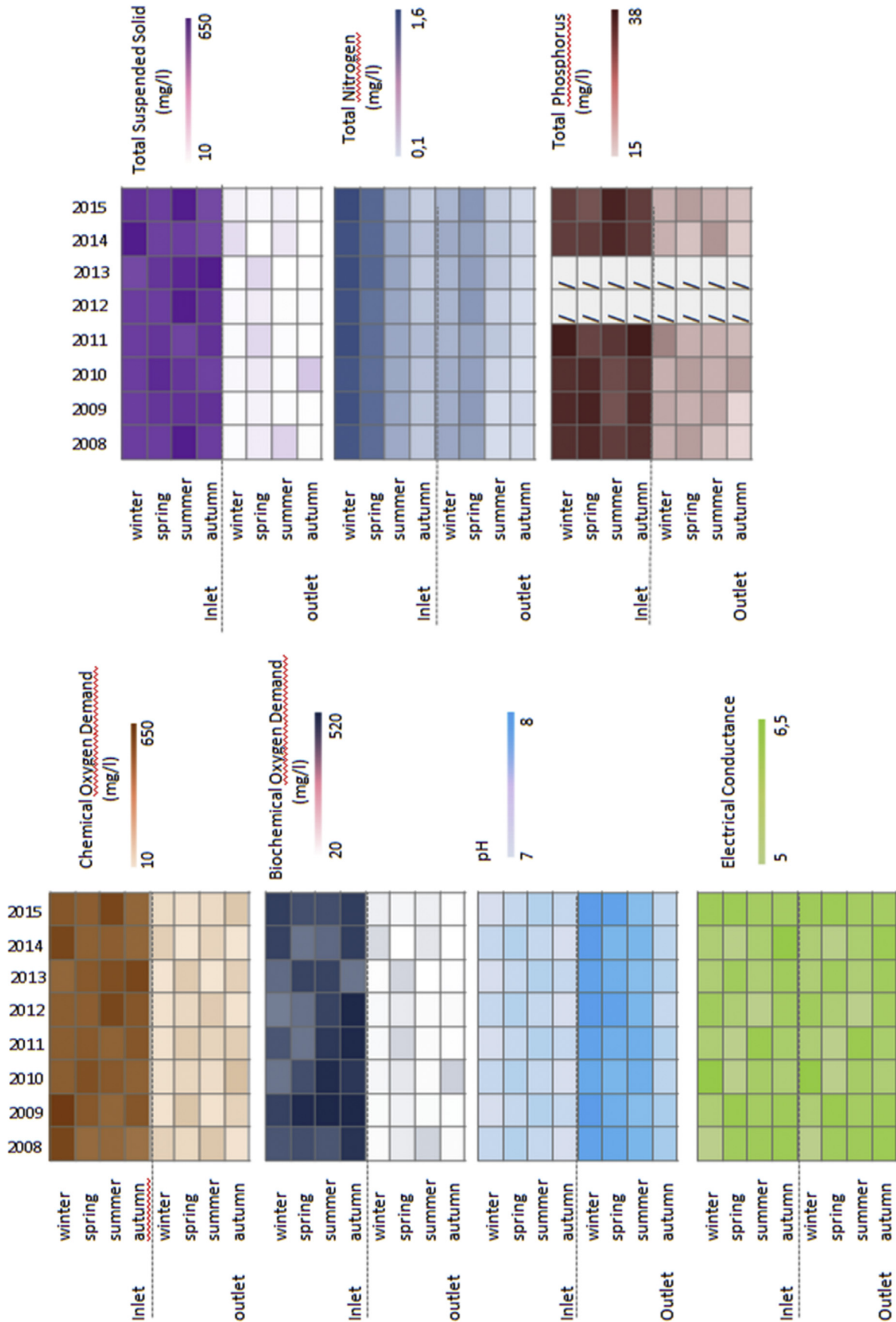


Fig. 4. Heat map of chemical and biochemical factors over eight years of the wetland pilot trial. Colours in the heat map represent the value of a given chemical indicator. The horizontal bar-plot at the right hand side of each map represents the biochemical indicators and the level of these later at the inlet or outlet of the pilot station. "/" indicates missing values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

because high concentrations are found in the wastewater entering the pilot station. Total phosphorus and BOD were reduced by approximately 60% and 80 to 95% at the outlet, respectively. On the contrary, the total nitrogen concentration was high at the inlet in winter and spring, but it decreased in summer and autumn at the outlet. This biochemical indicator presents similar trends but with lower decreases compared with the other contaminants measured. The pilot station showed significant seasonal variations of BOD at the wetland entrance during the eight-year trial. However, the differences between the entry and the outlet of the system evaluated by ANOVA are very highly significant ($p > 0.05$).

Load of influent sewage COD fluctuated widely between 430 mg/l and 540 mg/l in winter with an average of 470 mg/l. The COD was reduced dramatically in the effluent of the system, and it followed the same fluctuations in the treated water over the eight years. The COD removal was approximately 80%. The ANOVA showed that the effectiveness of the treatment for COD removal was highly significant ($p > 0.05$).

The pH of the station underwent a slight variation. The average pH at the entrance was 7. At the outlet of the system the pH was 8. The same tendency was found by other authors (Kadlec RH and Wallace SD, n.d.), who obtained an increase of half a pH unit for a wetland planted with *Typha latifolia*.

Electrical conductivity, in general, was virtually the same in the output (4.5–5.9 mmhos/cm) compared with the input average (4.9–6 mmhos/cm). These data were statistically similar; hence, we conclude that the treatment wetland did not affect EC.

To estimate the reduction efficiency of organic pollutants, we assessed the presence of phenanthrene. This later was considered as a model molecule in several laboratories, and it represents the polycyclic aromatic hydrocarbons (PAHs), which constitute a large family of highly hazardous pollutants (Vandermeersch et al., 2015). In this study we present a typical assay (data not shown) which shows that phenanthrene underwent a dramatic decrease at the outlet of the treatment basin. Indeed, there is much data indicating that macrophyte-assisted metaremediation may act as a meta-organism to degrade and dissipate organic pollutants as they possess high metabolic potential (El Amrani et al., 2015).

Total faecal coliform bacteria and streptococci were also determined to assess the efficacy of the pilot station. A typical analysis (data not shown) where plant-assisted metaremediation achieved an approximately 99% decrease in sewage pathogens. Similar results have been published by other authors (Morató et al., 2014; Stottmeister et al., 2003). Indeed, root exudates have been shown to exert a bactericidal effect on pathogenic germs (Stottmeister et al., 2003). This effect varies depending on the species of plant used. Interestingly, *J. effusus* and *Typha* have been proven to be especially efficient. Experiments conducted in mesocosm-scale tests have revealed that the number of bacteria (colony-forming units) was reduced by 90% after a very short contact time of 7–11 h. The bactericidal effect of highly adapted wetland plants (helophytes) was investigated *in vitro*. The authors concluded that the bactericidal effect is an active process that requires the direct presence of plants (Nelson and Wolverton, 2011). However, one can notice that plant root exudates also stimulate the rhizosphere-associated micro-organisms. Recent investigations have suggested that plants can modulate their microbiota to dynamically adjust to their environment (Vandenkoornhuysen et al., 2015). Additional characterization of the molecular dialogue between the plant and microorganisms is needed to better understand how plant species select microorganisms in the rhizosphere.

4. Conclusion

Overall, in this study, we investigated metaremediation by

plant-assisted constructed wetlands of high biodiversity based on the principle of using plants and their associated microbial rhizosphere as the main ecological engineer to accomplish wastewater treatment. The system studied underwent a selection of species with a greater ability than others to survive and tolerate local environmental conditions while handling wastewater in the local climate of Temacine.

Despite the very significant changes observed in the plant community, 72% of the initial planted species were not found to be well adapted for long period of wetland and polluted environment, and in the rhizosphere-associated microbiome during the eight-year trial, the quality of the treated water demonstrated that the pilot station is highly reliable and efficient.

The pilot station of Temacine in an arid climate, through a system of horizontal subsurface flow wetland treatment, provided good treatment of domestic wastewater and effectively reduced the organic load which underwent a reduction rate from 91% to 96% in spring and in summer and pathogenic bacteria about 99% over an extended period of eight years. This sewage treatment system used no chemicals or electricity and operated by gravity flow rather than with pumps/aerators and other equipment that more high-tech, typical sewage treatment plants require. The treated wastewater exiting the wetland treatment meets high standards and was safely reused for crop and tree irrigation. This method conserves potable water and chemical fertilizers which might otherwise be needed for the irrigation. The constructed wetland also provides a pleasant, green landscape in an arid desert environment.

Author contributions

The experimental work was conducted by Mohamed Mounir Saggai, Abdelhak El Amrani, Mark Nelson and Florence Cattin. Abdelkader Ainouche conducted phylogenetic analysis. The manuscript was written by Abdelhak El Amrani and Mohamed Mounir Saggai. Data analysis was performed by Mohamed Mounir Saggai and Abdelhak El Amrani. All authors approved the final version of the article.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jenvman.2017.06.040>.

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