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Lemna minor tolerance to metal-working fluid residues: implications for rhizoremediation

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Keywords

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ABSTRACT

For the first time in the literature, duckweed *(Lemna minor)* tolerance (alone or in combination with a consortium of bacteria) to spent metal-working fluid (MWF) was assessed, together with its capacity to reduce the chemical oxygen demand (COD) of this residue. In a preliminary study, *L. minor* response to pre-treated MWF residue (ptMWF) and vacuum-distilled MWF water (MWFw) was tested. Plants were able to grow in both residues at different COD levels tested (up to 2300 mg·l^{-1}), showing few toxicity symptoms (mainly growth inhibition). Plant response to MWFw was more regular and dose responsive than when exposed to ptMWF. Moreover, COD reduction was less significant in ptMWF. Thus, based on these preliminary results, a second study was conducted using MWFw to test the effectiveness of inoculation with a bacterial consortium isolated from a membrane bioreactor fed with the same residue. After 5 days of exposure, COD in solutions containing inoculated plants was significantly lower than in non-inoculated ones. Moreover, inoculation reduced β+γ-tocopherol levels in MWFw-exposed plants, suggesting pollutant imposed stress was reduced. We therefore conclude from that *L. minor* is highly tolerant to spent MWF residues and that this species can be very useful, together with the appropriate bacterial consortium, in reducing COD of this residue under local legislation limits and thus minimise its potential environmental impact. Interestingly, the lipophilic antioxidant tocopherol (especially the sum of β+γ isomers) proved to be an effective plant biomarker of pollution.

INTRODUCTION

Industrial development involves emission of a high variety of pollutants (solid, liquid and gas) that results in environmental damage. Metal-working fluids (MWF) have been introduced into industrial cutting practice in order to improve the characteristics of the processes occurring on contact surfaces between the tool and the work piece. They are used for cooling work pieces and tools, lubricating the process and flushing away chips, fines, swarf and residues (Moscoso *et al.* 2012). Once their functional properties are compromised, spent MWF need appropriate management, since they become hazardous waste in terms of international legislation (Directive 2008/98/EC). In fact, disposal costs of the cutting fluids can be two to four times their purchase price in the United States and Europe, respectively (Hong & Zhao 1999).

Treatment and disposal of these operationally exhausted MWF fluids is a serious environmental issue due to their high pollution potential, *i.e.* chemical oxygen demand (COD), biological oxygen demand (BOD) and elevated ecotoxicity of their components. One single MWF or metal-cutting fluid can contain up to 60 different components, including emulsifiers such as fatty alcohols or amino alcohols, corrosion inhibitors (fatty acids, amines and borates), extreme pressure additives, foaming inhibitors and biocides, but the precise composition and percentages of each compound are usually trade secrets of the MWF manufacturers. Thus, considering the potential ecotoxicological risk of some of those components, these residues can pose a serious ecotoxicological threat (Sokovic & Mijanovic 2001; Shokrani *et al.* 2012). Indeed, many scientific studies indicate the need for wider toxicological assessment of these industrial effluents and the aquatic environments affected by their disposal, despite the fact chemical parameters (such as COD) are maintained below regulatory discharge limits. Moreover, aquatic organisms, and more specifically aquatic plants, seem to be the best option for toxicological assessment and phytoremediation of these complex residues, considering that plants are directly exposed to the released spent MWF in all their developmental stages, from germination to reproduction (Wild *et al.* 1992; Grijalbo *et al.* 2015).

Three methods are usually considered for MWF wastewater treatment, which can be classified as (i) physical methods (Hilal *et al.* 2004; Gutiérrez *et al.* 2007); (ii) chemical methods (Sánchez-Oneto *et al.* 2007; Kobya *et al.* 2008); and (iii) biological processes (Van der Gast *et al.* 2003; Perez *et al.* 2006, 2007; Jagadevan *et al.* 2013; Rodriguez-Verde *et al.* 2014). Although the first (physico-chemical) are more effective than biological methods (bioremediation, phytoremediation), they are expensive, have high-energy demand, consumption of many chemical reagents and generate residues. Therefore, disposal of this

waste through an optimised biological route is an attractive option. Biological methods (bioremediation and phytoremediation) are cost-effective and environmentally sustainable technologies. Thus, integrated management of remediation technologies could benefit from both approaches. Therefore, the efficiency of the combination of the two technologies needs to be assessed.

Phytoremediation is a biological technology based on the use of plants to extract, sequester or detoxify pollutants. This emerging technology is being considered for remediation of contaminated sites due to its cost-effectiveness, aesthetic advantages and long-term applicability (Lee *et al.* 2002). Organic pollutants can be effectively transformed or degraded through plant uptake (and metabolic processes) or through the release of compounds in the rhizosphere. Several studies have shown that aquatic and terrestrial plants can be used to remediate organic and inorganic contaminants from soil and water (Rmer & Keller 2001; Mattina *et al.* 2002; Kamel & Aly 2003). However, plants are not always considered as the only mode of rhizosphere remediation (rhizoremediation, rhizodegradation). In fact, plants can create a niche for rhizosphere microorganisms to perform the degradation. Thus, when organic pollutants are degraded in the plant rhizosphere with the intervention of microorganisms (also called phytostimulation), the success of the process would depend not only on the effectiveness of the microbial community to degrade the target organic pollutant (Chaudry *et al.* 2005) but also on the plant tolerance to that pollutant (Barrutia *et al.* 2011).

Regarding selection of microbial populations for bioremediation, previous studies suggest that assemblage of an inoculum, based on comprehensive knowledge of the indigenous microbial community, in the target habitat is a highly effective approach (Van der Gast *et al.* 2004). In fact, there is growing interest in exploiting the biocatalytic potential of microorganisms to biodegrade MWF, and this can best be achieved by improving our understanding of microbial diversity within metal-working fluids (Gerulova *et al.* 2010).

Recently, a phytoremediation study combining maize plants and microorganisms decreased MWF residue COD, organic compound concentration and ecotoxicity (Lucas-García *et al.* 2013). However, immersion of the rhizosphere of terrestrial plants to remove pollutants from the effluent solution will present some constraints that will need to be explored under field conditions for sustainable remediation. Therefore, aquatic plants, such as *Lemna* species, combine unique properties for effective aquatic phytoremediation (Mkandawire & Dudel 2007): (i) fast growth; (ii) high biomass production; (iii) easy to remove from water; (iv) ability to decrease organic contaminants; (v) resilient to pollution; (vi) microbial communities are associated with the plant; and (vii) wide distribution in natural aquatic ecosystems.

Despite the interest in *Lemna* species for aquatic phytoremediation, there are limited studies on microbial associations with these plants with the aim of removing organic pollutants. The aim of the present work is to assess plant tolerance to MWF residue in terms of oxidative stress, photosynthetic efficiency, pigments, tocopherols and xanthophyll cycle pigments. In addition, the capacity of *L. minor* to reduce COD of non-treated or physicochemically treated spent MWF with and without a selected consortium of bacteria was also addressed.

MATERIAL AND METHODS

Obtaining metal-working fluid (MWF) residues

The MWF residue used in this study and provided by John Deere Ibérica (Madrid, Spain), as an operationally exhausted synthetic fluid (Houghton Iberica, Spain) used in large-scale continuous metal-working processes. Main chemical constituents of the residue included a formaldehyde-based biocide, alkyl benzotriazole (metal passivator), C16/C18 fatty alcohol polyglycol ether (corrosion inhibitors), isopropanolamine (lubrication agents) and 3-iodo-2-propynylbutylcarbamate. Prior to use in machining operations, this company typically dilutes the concentrated stock fresh MWF with water to form a 2% v/v working fluid. Once it loses its cooling and lubricating properties, after repeated use, the company stores this spent MWF inside tanks where oils are separated from the remaining residue due to differences in density. The emerging oil layer above the residue is then removed, leaving the remaining liquid residue (called pre-treated MWF, ptMWF). This pre-treated residue still contains emulsified oil that cannot be separated by density. Therefore, ptMWF is further treated by the company to break down this emulsion using a vacuum distillation system (Vacudest 200) at 87 °C and 500 mbar. Under these conditions, the water evaporates from the ptMWF and the distillate produced is condensed, collected and stored as MWF water (MWFw). The above-mentioned company provided us with these two types of residue: ptMWF and vacuum-distilled MWFw to conduct the current study (for more information about composition of the MWF see Lucas-García *et al.* 2013). Although oil and pollutant levels (such as metals) are reduced in MWFw residues after decantation and vacuum distillation, they could not be released to the environment because chemical properties such as COD and pH are usually above the regulatory values (pH > 9 and COD > 1750 $mg \cdot l^{-1}$), according to regional law 10/1993 on industrial waste discharges into urban sanitary sewer systems.

Chemical oxygen demand and pH determinations

The COD and pH were measured following specifications of the Environmental Protection Agency (EPA) method 410.4 (determination of COD with semi-automated colorimetry), and EPA method 150.1 (pH, electrometric method), respectively. COD was determined in pre-filtered samples using 0.2 μm pore size syringe filters (Millipore, Wallford, UK) through colorimetric analysis using a Merck Photometer SQ 118 with COD cuvette test kits (range 500–10,000 $mg \cdot l^{-1}$; Merck, Darmstadt, Germany) as previously reported (Lucas-García *et al.* 2013). Sample pH was directly measured using a CRISON micro pH 2100 pH meter (Crison, Barcelona, Spain).

Plant material and growth conditions

Cultures of duckweed (*Lemna minor* L. strain zabale/3) were grown autotrophically for 1 month in 2-l plastic containers placed in a growth chamber under controlled environmental conditions (temperature 25 °C/18 °C day/night, relative humidity 60/80% day/night, photosynthetic photon flux density 300 $\mu mol \cdot photon \cdot m^{-2} \cdot s^{-1}$ and 14-h photoperiod). Hutner's medium (Hutner 1953) was used as nutrient solution, and pH adjusted to 6.4. This solution was made without EDTA

because of it is an organic compound that could interfere with COD measurement. This solution was renewed every 3 days.

Zabale/3 strain was collected from a small pond in Zabale (Gernika, Bizkaia, Spain; 43°26′ N, 10°0′ W). It had been multiplied in the laboratory for many generations, and is used by our work group to study response to different kinds of abiotic stress (García-Plazaola *et al.* 2002; Artetxe *et al.* 2006).

Experimental design

In order to test *Lemna minor* tolerance to MWF residues, we initially carried out the experiment described below. After the acclimation period, *L. minor* (0.155 g fresh weight) were placed in 6-cm diameter polystyrene Petri dishes containing 15 ml of either ptMWF or MWFw with increasing levels of final COD concentration obtained by dilution of residues using tapwater and nutrient solution (Hutner's medium): 309 (T1), 821 (T2), 1396 (T3), 1772 (T4) and 2324 (T5) COD $mg{\cdot}l^{-1}$ for ptMWF, and 185 (T1), 737 (T2), 1563 (T3), 1819 (T4) and 2220 (T5) COD $mg{\cdot}l^{-1}$ for MWFw. A control set was maintained with plants growing on tapwater and Hutner's medium without MWF residues. All solutions contained half-strength Hutner's growth medium, and final pH was adjusted to 6.4. All treatments were prepared in quadruplicate. After 5 days, plants were harvested and fresh weight recorded. Plants were then frozen in liquid N_2 and stored at −80 °C for later biochemical analyses. Remaining solution in the dishes was also sampled and stored at −80 °C until COD determination.

Based on results of the above-described experiment, a second experiment to assess the benefits of bacterial inoculation was performed using MWFw with 1968 $mg{\cdot}l^{-1}$ COD. Under the same experimental conditions described above, *L. minor* were inoculated with 1 ml of a bacterial consortium formed of MBR-A11 (*Pseudomonas* sp.), MBR-A12 (*Acinetobacter johnsonii*), MBR-A16 (*Acinetobacter johnsonii)* and MBR-A23 (*Sphingobium xenophagum*), at a concentration of each of 10^8 $cfu{\cdot}ml^{-1}$. These four bacteria were selected out of 100 colonies cultured from the sludge of a membrane bioreactor fed with a 6000 $mg{\cdot}l^{-1}$ COD degraded MWF solution (for more information see Grijalbo *et al.* 2015). These bacteria represent the most abundant species determined through the partial sequencing of the gene 16S rRNA, and its later comparison using the BLASTN 2.2.26 (Zhang *et al.* 2000) algorithm in the GenBank database. Sequences of these bacteria were deposited in GenBank with the numbers: JF937328, JF937329, JF937331 and JF937337, respectively.

To produce the inoculum, each bacterial strain was grown in 100 ml nutrient broth (DIFCO, Leeuwarden the Netherlands) in a 250-ml Erlenmeyer flask placed on a shaker (125 rpm) at 28 °C for 24 h. The culture was centrifuged (350 *g* for 10 min), washed with sterile water and the pellets suspended in sterile 10 mM $MgSO_4$ to achieve 10^8 $cfu{\cdot}ml^{-1}$. Enumeration and calculations were carried out following the 'drop method' (Hoben & Somasegran 1982).

Plant parameters

Chlorophyll *a* fluorescence was measured using a modulated chlorophyll fluorometer OS5-FL (Opti-Sciences, Hudson, NH, USA). Initial (F_o) and maximum fluorescence (F_m) were measured in dark-adapted plants with a saturating pulse using modulated light of 660 nm. The maximum photochemical efficiency of PSII (F_v/F_m) was estimated from the ratio $F_v/F_m = (F_m - F_o)/F_m$ (Barrutia *et al.* 2009).

For determination of pigments (Chlorophyll *a* and *b*, and carotenoids: neoxanthin, antheraxanthin (A), violaxanthin (V), lutein, zeaxanthin (Z) and β-carotene) and tocopherols (isomers α and β+γ), frozen plant samples were homogenised with a tissue homogeniser (Tearor model 395, Dremel, Mexico) in pure acetone solution buffered with $CaCO_3$. The extracts were centrifuged at 16,100 *g* for 20 min. Supernatants were filtered with 0.2-μm PTFE filters (Teknokroma, Barcelona, Spain). Pigment separation was performed using HPLC with a reverse phase C18 column (Waters, Massachusetts, USA; Spherisorb ODS1, 4.6 mm × 250 mm) following the method described in García-Plazaola & Becerril (1999) with modifications described in García-Plazaola & Becerril (2001). The relative de-epoxidation state of the xanthophyll cycle pigments was estimated from the ratio (A + Z)/(V + A + Z).

Statistical analysis

One-way ANOVA was performed to compare data. Regarding plant parameters, Duncan *post-hoc* test was performed to discriminate among treatments, after a Cochran test to check for homogeneity of variances. The differences in average COD values were checked with LSD tests. In both cases, significant differences between the analysed variables were considered when $P < 0.05$. All analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA).

RESULTS

As a general rule, plants decreased COD concentration of both residues after 5 days (Table 1). This decrease was not noticeable when solution initial COD was close to its lowest detection

Table 1. Chemical oxygen demand (COD) ($mg{\cdot}l^{-1}$) in the studied solutions at the beginning and end of the study after supporting *L. minor* growth for 5 days.

ptMWF					
initial COD	309 a	821 a	1396 a	1772 a	2324 a
final COD	466 ± 30 b	772 ± 29 a	1416 ± 51 a	1618 ± 35 b	2264 ± 75 a
MWFw					
initial COD	185 a	737 a	1563 a	1819 a	2220 a
final COD	377 ± 7 b	866 ± 50 b	1188 ± 16 b	1638 ± 3 b	1952 ± 18 b

ptMWF = pre-treated MWF; MWFw = vacuum-distilled ptMWF water.

Letters a and b indicate statistical differences. Different letters denote significant differences ($P < 0.05$) in COD concentration at the beginning and end of the experiment.

limit (500 mg·l^{-1}). Nevertheless, COD reduction was more consistent in MWFw treatments, since phytoremediation presence was more effective under all treatments with COD > 1000 mg·l^{-1}.

Plants of *L. minor* survived when exposed to increasing levels of COD of the two MWF residues tested, although growth rate was inhibited in a dose-dependent manner (Fig. 1A). Reduction of growth rate relied more on levels of COD than the origin of the MWF residue. Especially significant was daily growth rate inhibition (60% and 80% inhibition at highest ptMWF and MWFw doses, respectively). Other physiological parameters determined, such as photochemical efficiency (Fig. 1B), was similarly affected under all treatments (ptMWF and MWFw), although this inhibition was low. Fig. 2 shows data on photosynthetic pigments and de-epoxidation index of the V cycle (AZ/VAZ). Chlorophyll and carotenoid concentrations were not significantly affected with ptMWF and MWFw; only T5 treatment using MWFw with the highest COD concentration showed a 20% inhibition of these parameters. Photoprotection-related xanthophylls (VAZ pool) did not vary significantly in plants exposed to the two residues, although some plants growing under T4 (1819 mg·l^{-1} COD) in ptMWF showed an increase in de-epoxidation index.

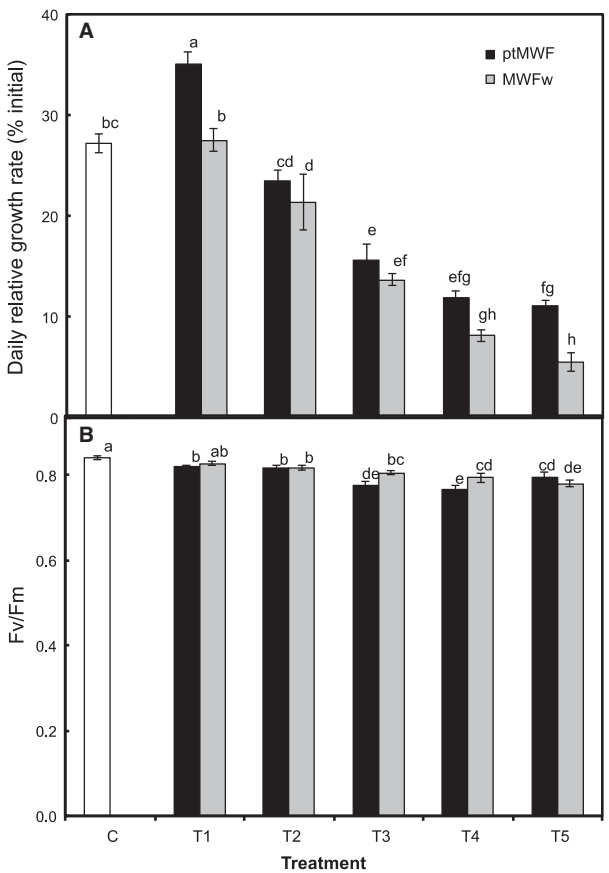

Fig. 1. (A) Daily relative growth rate (% initial) and (B) photochemical efficiency (F_v/F_m) of *L. minor* exposed for 5 days to two kind of MWF residue (ptMWF: pre-treated; MWFw: physicochemically treated). COD concentration (mg·l^{-1}) of the different treatments (T1 to T5) studied were 309, 821, 1396, 1772, 2324 for ptMWF residue and 185, 737, 1563, 1819 and 2220 for MWFw residue. Data represent mean values of four replicates. Bars represent SE. Different letters denote statistically significant differences at $P < 0.05$ among treatments.

Concerning plants antioxidant system, tocopherol levels significantly varied in plants exposed to the residues, and in a different way depending on the type of residue (Fig. 3). Especially noteworthy was the great increase in β+γ-tocopherol levels in MWFw exposed plants (two-fold increment). This induction was independent of the tested MWFw residue dose and was not observed in ptMWF exposed plants. The most abundant tocopherol in *L. minor* plants, α-tocopherol, was not significantly affected except under T4 of ptMWF. This treatment also presented highest de-epoxidation index (Fig. 2) and low F_v/F_m (Fig. 1B).

In order to test the effect of MWF-tolerant bacteria on plant tolerance and the phytoremediation process, a consortium of bacteria was inoculated to *L. minor* exposed to MWFw at 1968 mg·l^{-1} of COD. Inoculation did not protect plants from growth rate inhibition (50%; Fig. 4A). A slight decrease in F_v/F_m was only observed in inoculated plants growing in MWFw (Fig. 4B). Photosynthetic pigment composition of control plants was not affected by bacterial inoculation (Fig. 5). However, a slight increase in chlorophyll, carotenoids and xanthophyll cycle components (VAZ pigments) was observed on modulated plants exposed to MWFw. Inoculated and non-inoculated plant de-epoxidation index decreased when exposed to MWFw (Fig. 5). Inoculation did not affect tocopherol content of control plants. Consistent with previous data (Fig. 3), plants exposed to MWF had increased β+γ-tocopherol content in both inoculated and non-inoculated plants (Fig. 6). This effect was higher in non-inoculated plants.

Plants that were not inoculated significantly reduced 16% COD concentration of MWFw (from 1968 mg·l^{-1} to 1645 mg·l^{-1}), *i.e.* in a similar proportion to the previous assay (Table 1). However, when plants were inoculated with the bacterial consortium, this COD decrease was highly augmented, reaching 41% reduction after 5 days (from 1968 mg·l^{-1} to 1153 mg·l^{-1}). Bacterial consortium without plants did not produce any changes.

DISCUSSION

For the first time in the literature, phytoremediation of MWFs and its physiological effects on *L. minor* are presented. *Lemna* species have gained broad application in ecotoxicological research as model organisms (Zezulka *et al.* 2013), and there are many works using these plants to remediate heavy metals or other inorganic compounds (Mishra *et al.* 2008; Rai 2010; Bhaskaran *et al.* 2013; Goswami *et al.* 2014). However, there are very few studies that have used these plants with organic compounds (Zezulka *et al.* 2013; Török *et al.* 2015).

In our study, *L. minor* were able to significantly reduce COD in MWF residues at values above 1000 mg·l^{-1} COD. Most significant and promising results were observed when plants were growed with MWFw (Table 1), achieving a 24% reduction at initial 1563 mg·l^{-1} COD. Maize plants used with the same objective and in the same conditions were able to reduce COD by around 53% (Grijalbo *et al.* 2013, 2015). Despite the difference between the two species, the use of *Lemna* may be more advantageous, since corn cultivation in this residue is more complex than with *Lemna*.

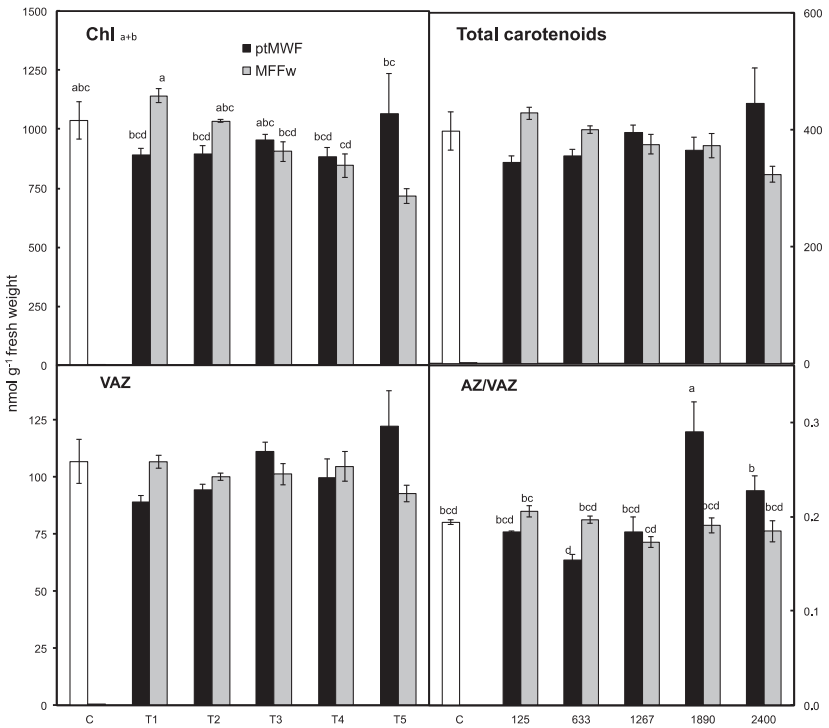

Fig. 2. Pigment composition of *L. minor* exposed for 5 days to two kinds of MWF residue (ptMWF: pre-treated; MWFw: physicochemically treated). Chl *a+b*: Chlorophyll *a+b*; V violaxanthin; A: antheraxanthin; Z: zeaxanthin. COD of the treatments studied as in Fig. 1. Data represent mean values of four replicates. Bars represent SE. Different letters denote statistically significant differences at $P < 0.05$ among treatments.

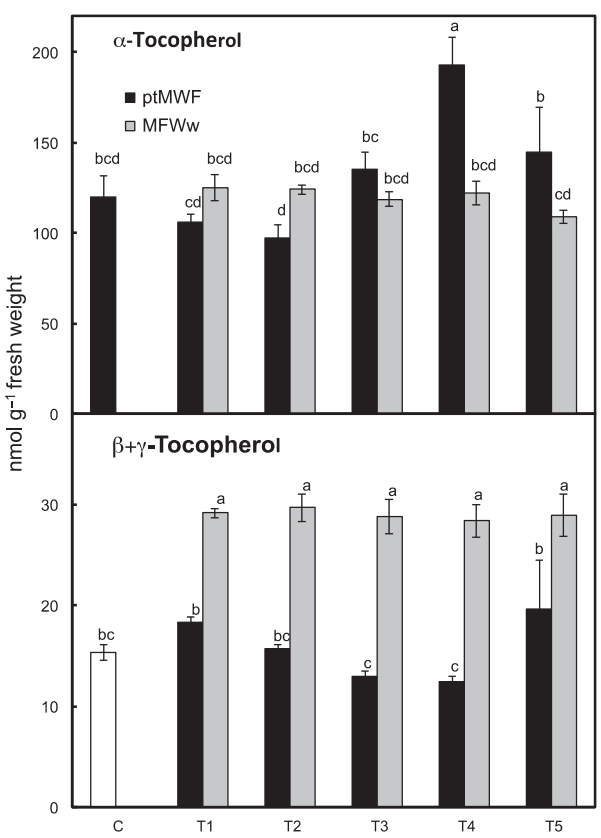

Fig. 3. Tocopherol (isomers α and β+γ) content of *L. minor* exposed for 5 days to two kinds of MWF residue (ptMWF: pre-treated; MWFw: physicochemically treated). COD of the treatments studied as in Fig. 1. Data represent mean values of four replicates. Bars represent SE. Different letters denote statistically significant differences at $P < 0.05$ among treatments.

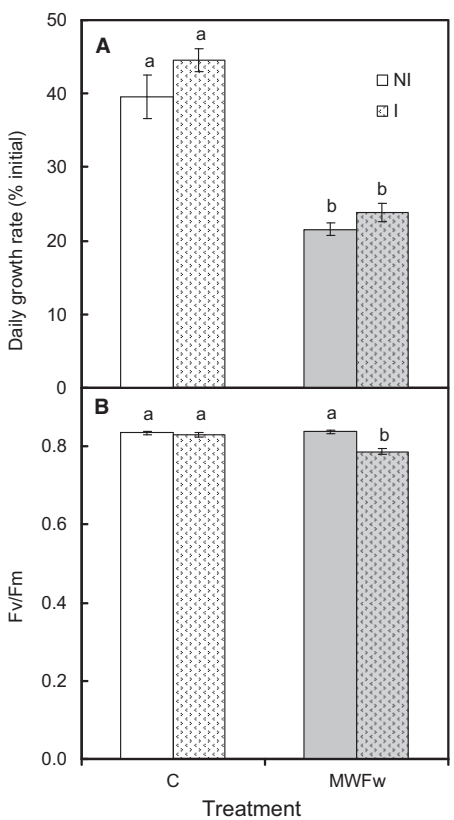

Fig. 4. (A) Daily relative growth rate (% initial) and (B) photochemical efficiency (F_v/F_m) of non-inoculated (NI) and inoculated (I) *L. minor* at the end of the assay (5 days). C: tapwater; MWFw: MWF physico-chemically treated with 1968 mg·l^{-1} COD. Data represent mean values of four replicates. Bars represent SE. Different letters denote statistically significant differences at $P < 0.05$ among treatments.

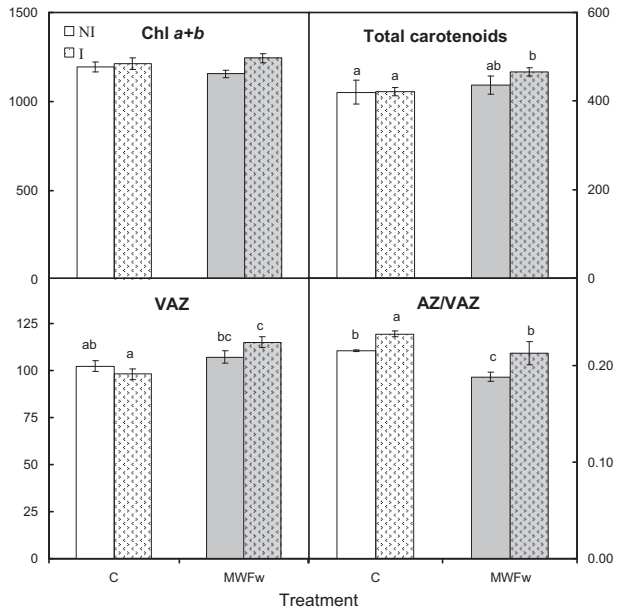

Fig. 5. Pigment composition of non-inoculated (NI) and inoculated (I) *L. minor* at the end of the assay (5 days). C: tapwater; MWFw: MWF physico-chemically treated with 1968 mg·l^{-1} COD. Chl *a+b*: Chlorophyll *a+b*; V violaxanthin; A: antheraxanthin; Z: zeaxanthin. Data represent mean values of four replicates. Bars represent SE. Different letters denote statistically significant differences at $P < 0.05$ among treatments.

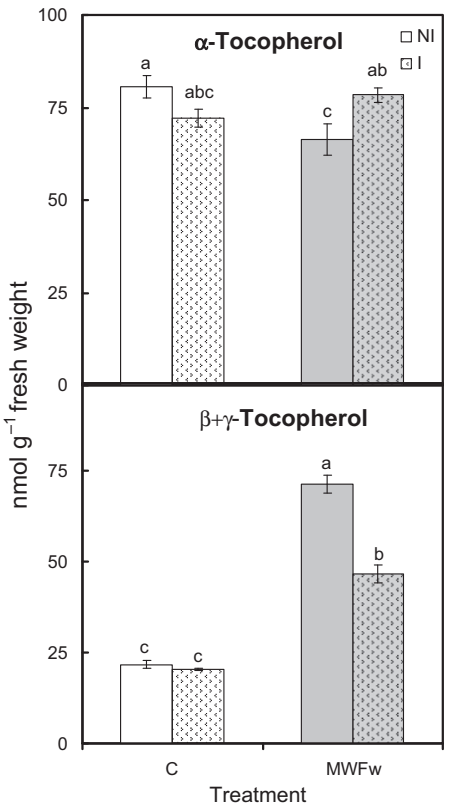

Fig. 6. (A) Tocopherol (isomer α) and (B) tocopherol (isomers β+γ) of non-inoculated (NI) and inoculated (I) *L. minor* at the end of the assay (5 days). C: tapwater; MWFw: MWF physico-chemically treated with 1968 mg·l^{-1} COD. Data represent mean values of four replicates. Bars represent SE. Different letters denote statistically significant differences at $P < 0.05$ among treatments

Preliminary assays using ptMWF or a fraction collected after vacuum distillation (MWFw) at different doses reflected high tolerance of *L. minor* exposed to these residues, since they were able to grow under all the COD doses tested, showing few phytotoxicity symptoms (Figs 1–4). *L. minor* have a high growth rate and, under our experimental conditions, doubled their biomass in 2.5–3.5 days. When exposed to MWF or MWFw, their growth rate was reduced following a dose–response pattern. In fact, plant relative growth rate was the most affected parameter among all plant parameters tested (Fig. 1A). Growth regulation, with no phytotoxicity symptoms in response to the pollutant used, is an inherent characteristic of tolerant plants (Barrutia *et al.* 2009). Indeed, the observed small alterations in photosynthetic apparatus efficiency, photosynthetic pigment contents and the absence of activation of photoprotective mechanisms could confirm the high tolerance of this species to the residues tested. Actually, *L. minor* has proven to be highly tolerant to several abiotic stresses, including heavy metals (Artetxe *et al.* 2002), probably as a consequence of the highly plastic photosynthetic apparatus of this species (Demmig-Adams 1990; Demmig-Adams & Adams 1992), which allows duckweed to grow under different conditions and to quickly adapt to changes in the environment (García-Plazaola *et al.* 2002).

Nevertheless, the observed slight decrease in photochemical efficiency (Fig. 1B) could be a mechanism to protect plants from photooxidative damage (Muller *et al.* 2001). Energy dissipation in the antenna and reduction of over-excitation of the photosynthetic apparatus can also be achieved by increasing the amount of de-epoxidised xanthophylls of the xanthophyll cycle (Demmig-Adams & Adams 1992). However, photoprotection mechanisms seem not to be induced in the current assay, since the de-epoxidation index was not increased (Fig. 2) and did not correlate with photochemical efficiency (Fig. 1B). Another mechanism to prevent oxidative damage is activation of the antioxidant defence system, which comprises several enzymes and low molecular mass scavengers such as tocopherols, ascorbic acid and glutathione (Foyer *et al.* 1994). In this sense, it is pertinent to note induction of β+γ-tocopherol levels in MWFw-exposed plants independent of the COD level of the residue tested. This is an interesting observation since the most abundant tocopherol isomer in plant leaves is α-tocopherol (Szymanska & Kruk 2008a,b) and, indeed, most studies dealing with tocopherols and stress response and adaptation of plants focus on this latter isomer (Munné-Bosch & Alegre 2002; Collin *et al.* 2008). Thus, to date, little is known about the specific content and roles of other tocopherol isomers in response to abiotic stress. The isomers β- and γ-tocopherol differ from α-tocopherol in the number and position of methyl substitutions, which largely determine their antioxidant properties (Packer & Obermuller-Jevic 2002). α-Tocopherol shows maximum *in vivo* vitamin E activity but poor *in vitro* protection, whereas β- and γ-tocopherol are more powerful *in vitro* antioxidants (Pongracz *et al.* 1995). Indeed, some studies have noted a more powerful role of γ-tocoherol in stress responses (Abbasi *et al.* 2007). The results of this experiment indicate that *L. minor* can remediate MWF, especially MWFw, without a substantial effect on physiological status, through adjusting their growth rate to increasing COD values.

In order to assess the effect of bacterial inoculation on plant physiological response and potential improvement of the phytoremediation process of MWF residues, we performed a second assay with 1968 $mg \cdot l^{-1}$ COD MWFw because: overall plant physiological response to vacuum-distilled MWFw was more consistent and dose-responsive (Figs 1B, 2), COD reduction was more significant when growing *L. minor* in this residue (Table 1), and induction of the sum of β- and γ-tocopherol levels observed under this residue was encouraging to find new potential biomarkers (Fig. 3). For the assisted phytoremediation assay, the consortium used for inoculation was isolated from an MWF-fed membrane bioreactor. Selected COD dose was around 1968 $mg \cdot l^{-1}$, while in order to check the ability of the plant–bacteria consortium system to phytoremediate MWFw the COD levels were maintained below regulatory limits for its disposal (<1750 $mg \cdot l^{-1}$ COD). In this second study, after 5 days, plants alone were able to reduce 16% residue COD levels (thus meeting legislative requirements). This reduction was not as effective as that described in Lucas-García *et al.* (2013) for maize grown for 5 days under similar initial COD levels of MWFw (55% reduction). Nevertheless, when *L. minor* were inoculated with the consortium of tolerant bacteria, the remediation process was highly stimulated, and finally a 41% reduction of COD concentration was achieved after 5 days. These results are close to those obtained with maize (Lucas-García *et al.* 2013). Thus, selection of the bacterial consortium from the target waste (*i.e.* MWF) proved to be an effective approach to assist phytoremediation. These populations not only are adapted to extreme conditions of the wastes, but have also been shown to be more resistant to predation (Fewson 1988; Otte *et al.* 1994; Hamer 1997). Moreover, remediation with carefully selected bacterial strains avoids the risk associated with the use of undefined microorganisms from sewage, a common practice in bioreactors, since these heterogeneous and potentially dangerous communities can harbour potential pathogens (Hamer 1997).

Plants that were not inoculated showed a high tolerance to MWFw, and physiological responses were quite similar, *i.e.* main alterations in plant parameters were growth reduction and a significant induction of β- and γ-tocopherol (Figs 4A, 6). Although some authors have suggested that inoculation may negatively affect plant performance because strains can work as strong sinks for photosynthates (Lambers *et al.* 2009; Drogue *et al.* 2012), in this study the bacterial consortium did not affect plant growth and had minor effects on other plant parameters. Most noteworthy was the decrease in β- and γ-tocopherol levels when inoculating MWFw-exposed plants (Fig. 6), although values were still more than twofold those observed in control non-exposed plants. Since results from the previous study indicated that the sum of these tocopherol isomers does not respond in a quantitative way to the COD dose of MWFw, the decrease in levels of this biomarker indicated stress reduction due to exposure to the residue.

CONCLUSIONS

In the current study we observed that despite the fact that *L. minor* responded in a similar way to both ptMWF and vacuum-distilled MWFw at the same CODs, plant physiological response was more consistent and dose responsive when exposed to the latter residue, and a more significant COD reduction was achieved. We also verified that the effectiveness of the rhizoremediation of MWFw using *L. minor* can be enhanced through inoculation of a bacterial consortium selected from a membrane bioreactor fed with MWF. This cost-effective biological system allows the reduction of residual COD levels below local regulatory limits. Regarding risk assessment, *L. minor* has proven to be highly useful for assessing ecotoxicity of aquatic environments and, in particular, β- and γ-tocopherol demonstrate great potential as effective biomarkers to monitor pollutant presence in industrial effluents and the receiving waters.

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