Phytoremediation for improving the quality of effluents from a conventional tannery wastewater treatment plants.

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Short title: Phytoremediation for the tertiary treatment of tannery wastewater.

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Abstract

In the present study the quality of effluents from a conventional wastewater treatment plant in Italy has been analyzed. Residual level of contamination by 4-n-nonylphenol, mono- and di-ethoxylated nonylphenols has been recorded in the effluents that resulted to be also phytotoxic and genotoxic. The possibility to exploit phytoremediation as a sustainable tertiary treatment for the depletion of the priority pollutants and for the reduction of the residual toxicity has been verified at mesocosm scale. The phyto-based treatment has been performed by the exploitation of *Phragmites australis* by either a bacterial-assisted and not assisted approach. In relation to the bacterial-assisted approach, two new bacterial strains, capable to use the nonylphenols as sole carbon source, have been isolated. One was identified as a plant growth-promoting rhizobacteria (PGPR) belonging to the *Stenotrophomonas* spieces, the second one was classified as a *Sphingobium* spieces strain. Both strains were independently bioaugmented in the *Phragmites australis* rhizosphere. In relation to the not assisted approach, the phyto-based process determined 87%, 70% and 87% for 4-n-nonylphenol, mono-ethoxylated nonylphenols and di-ethoxylated nonylphenols respectively. The toxicological assessment of the process evidenced the complete depletion of either the phytotoxicity and the genotoxicity of the treated effluents. With reference to the bacterial-assisted approach, the PGPR *Stenotrophomonas* spieces strain resulted to be capable to significantly increasing the efficiency of the phyto-based process in nonlyphenols depletion up to 88% for the 4-n-nonylphenol, 84% for the monoethoxylated nonylphenol and 71% for the diethoxylated nonylphenol.

Keywords

Bacterial-assisted phytoremediation; Genotoxicity; Nonylphenols; Phytoxicity; Plant growth-promoting rhizobacteria; *Stenotrophomonas* sp..

1. **Introduction**

Polyethoxylated nonylphenols (NPnEOs) are non-ionic surfactants that find application, among many others, as wetting agents and emulsifiers in the tannery industry (Langford and Lester 2002). The NPnEOs are only partially degraded in conventional wastewater treatment plants (WWTPs). The main products of their degradation are a mixture of branched nonylphenols (NPs), comprising the linear 4-nNP, and their immediate metabolic precursors, the mono- and di-ethoxylated nonylphenols (NP1EO and NP2EO respectively). Due to their physical-chemical characteristics, these molecules are very recalcitrant to further oxidation (Koh et al. 2005), and accumulate and persist in sewage sludge, river sediments, and several environmental compartments. Recently toxic effects on aquatic organisms such as plants, invertebrate and vertebrate have been reported. Actually, these effects were not restricted to the known estrogenic activity; noteworthy, they were related to the alteration of the cell membrane integrity, to the induction of oxidative stress, to the interference with the cell cycle and cell division, to the induction of apoptosis (Kudo et al. 2004; Yao et al. 2006) and more in general to genotoxic effects (Adam and El-Ashry 2010; Frassinetti et al. 2011).

Chemical analyses of effluents from full-scale WWTPs actually demonstrated that NPs, NP1EO and NP2EO occur quite frequently as stable intermediates at the end of the pipe of the facilities, with a higher incidence in those plants treating industrial wastewaters or civil effluents, deriving from highly populated urban areas (Langford and Lester 2002). NPs were actually designated as priority pollutants in the Water Framework Directive (Directive 2000/60/EC). The use of NPnEOs has been banned in Europe for several industrial uses, including tannery processing. An exception is made for industries owing WWTPs able to perform the removal of these contaminants and of the corresponding metabolites from their effluents (Directive 2003/53/EC). Despite the legislation in force, nonylphenols are still frequently recorded in tannery wastewaters and discharged in receptor aquifers (Pothitou and Voutsa 2008).

Recently the objective of removing recalcitrant priority pollutants from already treated wastewaters has been tentatively approached by physical-chemical technologies. These treatments resulted to be barely sustainable in term of costs and tend to produce not characterized and potentially toxic break-down products. On the other hand, the phyto-based technologies have been recognized as inexpensive, environmentally friendly remediation methods, worthy of serious consideration in the context of the sustainability of the intervention for treatments of either civil and industrial waste flues (Korkusuz 2005). However, the exploitation of phytotechnologies in Europe is limited, as compared with USA (Van der Lelie et al. 2001) and India (Prasad 2007). Recently many pilot and field studies on real case of contamination have been approached with success in Italy (Di Gregorio et al 2013; Marchiol et al 2011) and all over the Europe (Mench et al. 2010; Schröder et al 2007; Vangronsveld et al. 2009). Results obtained encourage the establishment of the technology also in the EU. It is worth mentioning that phytotechnologies can find application in the depletion of heavy metals (Mani et al. 2012 a;b), organics (Di Gregorio et al. 2013), in the case of co-contamination (Arjoon et al 2013) and in the recovery of the resilience of the treated matrices (Mani and Kumar 2013). Moreover, phytoremediation can be approached either as bacterial-assisted and not assisted process. The bacterial-assisted approach is based on the interaction between plants and specific bacteria, massively bioaugmented in plant rhizosphere, to tentatively increase the performance of the phytoremediation process (Glick 2011). The bacteria of interest are mostly classified as Plant Growth Promoting Rhizobacteria (PGPR), capable of facilitating the growth of plants, even in stress conditions, using a wide range of different mechanisms (Glick 2011). On the other hand, microbial strains capable of transforming specific contaminants have been reported for their positive effects on the phyto-based processes of corresponding contaminant's depletion. (Uhlik et al 2009).

In this work the evaluation of the quality of the waste-flues at the end of the pipe of a conventional tannery wastewater treatment plant in Italy showed residual phytotoxicity and genotoxicity and the presence of the priority pollutants 4-n-nonylphenol, mono- and di-ethoxylated nonylphenols.

The possibility to exploit phytoremediation as a sustainable tertiary treatment for the depletion of the priority pollutants and for the reduction of the residual toxicity has been verified at mesocosm scale by using *Phragmites australis*, a plant species well adapted to tannery wastewater in terms of survival and propagation (Calheiros et al 2007). The process has been planned either as a not assisted and as a bacterial-assisted one. For the bacterial-assisted approach 1) a PGPR strain deriving from the rhizosphere of *P. australis* plants irrigated with the effluents and 2) a bacterial strain directly deriving from the contaminated effluents have been isolated. Both strains were selected for their capacity to growth in the presence of NPs as sole carbon sources, suggesting their capacity to transform the contaminants and to promote their depletion in the treated effluents. The two isolates were independently bioaugmented in the *P. australis* rhizosphere to compare the effect of a PGPR-based and a not PGPR-based bioaugmentation strategy on the process efficiency. The metabolic activities of the bioaugmented strains have been monitored by RT-DGGE analysis of the 16S rcDNA derived from the meta-transcriptome of the bacterial communities characterizing the treating modules. The chemical assessment of the process was focused on the recording of the depletion of residual nonylphenols by GC-MS. The toxicological assessment of the phyto-based process has been performed by the phytotoxicity bioassay on *Lepidium sativum* L. and on *Vicia faba* L. The *V. faba* model plant has also been used for the genotoxicity bioassay.

2. Materials and Methods

2.1 Chemicals, plants and wastewaters

Chemicals used throughout the experiments were of analytical grade. The technical nonylphenol (t-NP), and d(deuterium)-4-nNP d(deuterium)NP1EO and d(deuterium)NP2EO were purchased from Sigma-Aldrich (Milan, Italy). The d(deuterium)-4-nNP d(deuterium)NP1EO and d(deuterium)NP2EO were used as analytical standards for the quantification of 4-nNP, NP1EO and NP2EO by GC-MS analysis. The technical nonylphenol (t-NP) has been used as sole carbon source in the enrichment cultures for the isolation of bacterial candidates. Wastewaters have been collected at the end of the pipe of an activated sludge treatment plants collecting the waste-flues from different local tanneries in Tuscany, Italy. The COD (EN ISO 9439:2000) and BOD_5 (EN ISO 9408:1999) of the wastewater after filtration (45 μm) are reported in Table 1. The pH of the wastewater after filtration was 7.4 ± 0,7. The expanded clay (Leca®) was purchased by a local distributor. *Phragmites australis* plants were collected from a local nursery.

2.2 Cultivation media

Brunner mineral medium (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ Medium457.pdf) was used for strain enrichment, isolation and verification of their capacity to utilize t-NP as a sole carbon source.

2.3 Isolation and characterization of bacteria

P. australis plants growing in vessels containing soil were irrigated twice a day for three months with the tannery wastewater collected at the end of the pipe of the WWTP. Enrichment cultures for rhizobacterial strain were prepared as described in (Penrose and Glick 2003), collecting soil aliquots

(1 g) from the soil-fraction tight attached to the root apparatus of *P. australis* plant at the end of the irrigation period. A total of 25 bacterial isolates obtained were analyzed for the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity as described in (Penrose and Glick 2003) and for the capacity to produce Indole-3-Acetic acid (IAA) as described in (Brick et al. 1991). The positive strains for both metabolic activities, 7 in total, were clustered in different Operational Taxonomic Units (OTUs) by amplified ribosomal DNA restriction analysis (ARDRA). The ARDRA was performed digesting the amplification products with Sau3A, AluI, and HaeIII. All the analyses were performed twice for each isolate. The gene encoding for the 16S rRNA of one microorganism for each OTU was amplified, sequenced on both strands, and aligned to the sequence databases using BLASTN.

Strains deriving from the tannery wastewater capable to utilize t-NP as sole carbon source were isolated by preparing enrichment cultures in Brunner medium supplemented with 1000 ppm t-NP as sole carbon source. Enrichment cultures were carried out in 250-ml Erlenmeyer flasks containing 100 ml Brunner medium amended with 1000 ppm t-NP and 10 ml of the wastewater collected at the end of the pipe of the WWTPs. Flasks were incubated at 28 ± 1°C on an orbital shaker (250 rev/min). After 1-week of incubation, 1 ml of the suspension was incubated in flasks with 100 ml fresh Brunner medium for 1 week. The passage was repeated 7 times. Afterwards serial dilutions of the culture medium were plated on agarized Luria Bertani (LB) broth plates. The plates were incubated at 28 ± 1°C for 5 days. Three colonies were collected and clustered in different OTUs by ARDRA as previously described. The isolated strains from the rhizosphere of *P. australis* and from the tannery wastewater were tested for the capacity to use t-NP as sole carbon source in Brunner medium supplemented with 1000 ppm t-NP. Substrate utilization was verified by determining the growth of the bacterial isolates on LB plates plated with serial dilution of the liquid cultures. The evaluation of bacterial growth in presence of t-NP as sole carbon source was performed twice for each isolate. The capacity of the strains isolated from the WWTPs effluent to produce IAA and to express ACC-deaminase was also verified as described in Brick et al. (1991) and Penrose and Glick (2003).

168 **2.4 Preparation of mesocosms**

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170 A total of 36 experimental replicates (pots), each containing 1 kg of Leca® (substrate for
171 vegetation) and 2 L of filtered (0.45 µm) tannery wastewater, were prepared in plastic pots and
172 maintained in a temperature (24 ± 1°C) and lightening controlled growth chamber (14 hrs light/10
173 hrs dark) for 48 and 144 hours (2 and 6 days). The 2 L of wastewater were added to the pots at the
174 beginning of the experimentation. A total of 12 replicates, out of 36, were inoculated with
175 *Sphingobium* sp. bacterial culture (10^6 CFU/g Leca®) and 6 of them were vegetated with 1 plant of
176 *P. australis* per pot. Twelve replicates, out of the remaining 24, were inoculated with
177 *Stenotrophomonas* sp. bacterial culture (10^6 CFU g/Leca®) and six of them were vegetated with 1
178 plant of *P. australis* per pot. A total of six of the remaining 12 not bioaugmented replicates were
179 vegetated with one plant of *P. australis* per pot. The remaining six pots were neither vegetated nor
180 inoculated. Bioaugmentation inocula were prepared by massive cultivations of *Stenotrophomonas*
181 sp. Phr013 and *Sphingobium* sp. NP001 in Luria Bertani (LB) and triptic soy broth (TSB) media
182 respectively. In order to reach the expected bacterial inoculum (10^6 CFU/g Leca®), appropriate
183 volumes (mL) of massive cultures of *Stenotrophomonas* sp. Phr013 and *Sphingobium* sp. NP001
184 have been collected and gently centrifuged. The bacterial pellets obtained have been washed twice
185 with a saline solution (NaCl 0.9% wt/vol) and inoculated in the 2L of wastewaters distributed in the
186 pots.

187

188 **2.5 Phenolics extraction and GC-MS analysis**

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190 Three pots for each set of condition were separately sacrificed and analyzed for phenolic content at
191 48 and 144 hours of incubation. 4-nNP, NP1EO and NP2EO were quantified in the treated
192 wastewater, in plant tissues and as portions adsorbed to Leca®.
193 Phenolic compounds were extracted from wastewaters following the protocol described in Yang et
194 al. (2011). Samples of 10 ml of treated wastewater per pot were acidified with HCl solution (6M)

and extracted three times with an equal volume of dichloromethane (DCM) for 10 min. The DCM extracts were combined and concentrated to lower volumes with a rotary evaporator, further concentrated under a gentle flow of dry nitrogen and transferred in capillary tubes for derivatization with BSTFA [N,O-bis(trimethylsilyl)trifluoroacetamide].

A total of 10 g of Leca® were washed three times with one volume of DCM, combing the resulting washing solution (30 ml) that were concentrated as previously described before derivatization. Phenolics from plant tissues were extracted as described in (Siöström et al. 2008).

Internal standard were added before each extraction procedures. A total of 25 ng of d-NP1EO and d-NP2EO, were added as internal standards to account for purification losses. Quantification was accomplished by GC-MS analysis by a Saturn 2200 quadrupole ion trap mass spectrometer coupled to a CP-3800 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with a MEGA 1 MS capillary column (30 m; 0.25 mm i.d., 0.25 µm film thickness, MEGA s.n.c., Milan, Italia). The carrier gas was helium, which was dried and air free, with a linear speed of 60 cm/s. The oven temperature was maintained at 80°C for 1 min, increased to 210°C at a rate of 15°C /min, further increased to 235°C at a rate of 5°C/min, further increased to 300°C at a rate of 20°C/ min. Full scan mass spectra were obtained in EI^+ mode with an emission current of 10 µA and an axial modulation of 4 V. Data acquisition was from 150 to 600 Da at a speed of 1.4 scan/sec. Final data were the means of three biological replicates.

2.6 Process efficiency and mass balance

The process efficiency (PE) of the different combination of plant and microbial inocula has been calculated as the ratio of the amount of phenolics (ng) depleted per mesocosm to the unit of dry weight (g) of the vegetating plant. A mass balance has been calculated for each of the contaminants and the portion of contaminants that has been metabolized and/or volatilized by plant and/or microorganisms (transformed fraction) has been calculated as the difference between the depleted portions of the phenolics and their portions accumulated in the plant and adsorbed onto the Leca®.

To the scope wastewater evapotranspiration has been quantified as the portion of volume of wastewater not recovered at the end of the experimentation. All the described analysis have been performed after 144 hours of incubation.

2.7 Molecular techniques

Standard procedures were used for nucleic acid manipulation and agarose gel electrophoresis. Bacterial genomic DNA was extracted using the Nucleospin Tissues Kit (BD Bioscences Clontech, Milan, Italy) following the manufacturer instructions. Total RNA was extracted by the biofilm adsorbed on the Leca® of each pots and extracted by washing 10 g of Leca® in 1 volume of sterile water for three times. The combined volumes of washing-water (30 ml) were filtered under gentle vacuum on a sterile 0.45 µm filter. The filter was finely chopped and extracted by using the RNA PowerSoil® Total RNA Isolation kit (Cabru S.A.S., Milan Italy). DNA was manipulated using enzymes purchased from Sigma-Aldrich (Milan, Italy) and sequenced using a PRISM Ready Reaction DNA terminator cycle sequencing Kit (Perkin–Elmer, Milan, Italy) running on an ABI 377 instrument. Nucleotide sequence data were assembled using the ABI Fractura and Assembler computer packages and analyzed using ClustalW and Omiga (version 1.1) (Oxford Molecular Group, UK).

2.8 Reverse transcriptase denaturing gradient gel electrophoresis analyses

The V-3 region (position 341–534, *E. coli* numbering) of bacterial 16S rcDNA was amplified by PCR using primers p3/p2 (Muyzer et al. 1993). The 16S ribosomal copy (rcDNA) was obtained by reverse-transcriptase PCR (RT-PCR) with the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), RNA H Minus, Point Mutant (Promega, Milan, Italy), from 70 ng of total RNA from the Leca® of the different treatment units by using the p2 primer (primer annealing at 42 °C for 10 min, extension at 50 °C for 1 h). An appropriate dilution of the obtained product was

249 used as template for PCR reactions with the p3/p2 primer set. The PCR products were separated on
250 polyacrylamide gels (8% [wt/vol], 37.5:1 acrylamide-bisacrylamide) with a 30–60% linear gradient
251 of urea. Denaturing gels were run using the Dcode Universal Mutation Detection System (Bio-Rad,
252 USA).
253 The gels images were acquired using the ChemDoc (Bio-Rad) gel documentation system. The
254 Denaturing gel electrophoresis (DGGE) profiles, concerning the presence and intensity of the
255 bands, were analyzed using GelCompar_II software (VERSION 4.6; Applied Maths, Sint-Martens-
256 Latem, Belgium). Detected band patterns were transferred to an absence/presence matrix. Band-
257 matching position tolerance was set at 1%, with an optimization of 0.5%. The binary matrix was
258 transformed into a similarity matrix using the Bray-Curtis measure. Dendrograms were generated
259 by unweighted pair group mean average (UPGMA) cluster analysis. DGGE banding data were used
260 to estimate diversity, H (Shannon and Weaver, 1963) and equitability, E (Pielou, 1975) indexes.
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262 **2.9 Genotoxicity and phytotoxicity tests**
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264 Seeds of *Vicia faba*, following the procedure previously described in (Giorgetti et al 2011) were
265 germinated at 24 ± 1°C for 72 hours in different solutions: a) 10 ml of distilled water (control); b)
266 10 ml of filtered (45 µm) wastewater at the end of the pipe of the WWTP; c) 10 ml of filtered
267 wastewater after 144 h incubation with *P. australis* plants; d) and e) 10 ml of filtered wastewater
268 after 144 h incubation with *P. australis* plants bioaugmented with NP001 or Phr013 strains
269 respectively.
270 Five fixed and Feulgen stained root tips *per* experimental group were used for preparing slides, and
271 1,000 nuclei per slide were examined. Micronucleus frequency assay (MNC), mitotic activity
272 (mitotic index, MI = number of mitosis per 100 nuclei) and mitotic aberrations (aberration index, AI
273 = number of aberrations per 100 nuclei) were determined.
274 Phytotoxicity test was carried out with garden cress, *L. sativum*, which is recognized as a sensitive
275 bioassay for phytotoxic compounds (Gehringer et al. 2003) and in parallel with *V. faba*. Four

replicate of ten seeds for each sample were germinated at 24 ± 1°C in dark conditions in the same a), b), c), d) and e) solutions as described above. As parameters of toxicity both root length (cm) and seed germination rate (%) were measured; Index of Germination (IG %) was determined according to the equation:

IG % = (Gs Ls)/(Gc Lc)×100

where Gs and Ls are the seed germination and root elongation (mm) for the sample; Gc and Lc the corresponding values for controls.

2.10 Statistical analysis

Data were elaborated with the aid of the two ways ANOVA and means were separated by the Bonferroni's multiple comparison test ($P \leq 0.001$) using the specific software Statgraphics 5.1 (Statistical Graphics Corp., USA).

3. Results and Discussion

The chemical and biological oxygen demand are broad-spectrum parameters that, in the case of industrial wastewater, can masks a plethora of specific contaminants that can be noxious for the environment when the already treated wastewaters are regularly discharged. In this study the effluents of a tannery wastewater plant, showing residual COD and BOD values compatible with their controlled and authorized discharge, resulted to be contaminated by nonylphenols. The NPs detected were NP1EO, NP2EO and 4 nNP at concentrations (0.066 ± 0.001 ng/mL for NP1EO, 0.152 ± 0.003 ng/mL for NP2EO and 0.332 ± 0.002 ng/mL for 4-nNP) already recorded at similar concentrations in similar effluents (Pothitou and Voutsa 2008). With reference to the toxicity of nonylpheols and the recurrence of their presence in municipal and industrial wastewaters, sustainable technologies dedicated to their complete depletion, even as tertiary treatments, are desirable. The goal of this study was the evaluation of the possibility to exploit a phytobased approach for the above mentioned scope, either as not bacterial-assisted or as bacterial assisted process. To the scope, in order to isolate PGPRs resistant to the tannery wastewater, selective enrichments of the candidates were set up from the rhizosphere of *P. australis* plants irrigated for three months with the contaminated waste flues. Bacterial selection was focused on candidates capable of producing IAA and of expressing ACCdeaminase activity, metabolic traits harbored by PGPRs (Glick 2011). A total of seven isolates capable of producing IAA and of expressing ACC-deaminase activity were recovered and analyzed by ARDRA. They showed the same ARDRA profile, and they were grouped in a single OTU. The partial sequencing of the corresponding 16S rRNA gene indicated that the isolate (Phr013) belonged to the *Stenotrophomonas* sp. [98 % homology to *Stenotrophomonas* sp. SAP52_1, accession number JN872547.1 (Alvarez-Pe´rez et al. 2012)]. In parallel, to isolate bacterial strains capable of using NPs as a sole carbon source eventually transforming these latter and promoting their depletion, enrichment cultures were set up from waste flues collected at the end of the pipe of the WWTPs. A total of three strains were recovered and grouped in a single OTU after ARDRA analysis. The partial sequencing of the

corresponding 16S rRNA gene indicated that the isolate selected as representative of the OTU (NP001) belonged to the *Sphingobium* sp. [97 % homology to *Sphingobium* sp. IT-4, accession number AB491320.2 (Toyama et al. 2011)]. The capacity of the *Sphingobium* sp. NP001 to produce IAA and to express ACC-deaminase activity has been verified, and the strain

failed in both metabolic capacities. The capacity of the *Stenotrophomonas* sp. Phr013 and the *Sphingobium* sp. NP001 to utilize NPs as a sole carbon source was determined by measuring the growth of the strains in minimal Brunner medium in the presence of t-NP as a sole carbon source. The corresponding growth curves are reported in Fig. 1. Both strains are capable of growing on minimal medium added with t-NP as a sole carbon source, reaching significant cell density in only 8 h. The NP001 reached a higher density with respect to Phr013. Thus, a batch experimentation has been performed by the combination of 36 pots simultaneously assayed after 48 and 144 h of incubation. Pots treating the NP-contaminated waste flues were set up as follows: (1) not vegetated and not bioaugmented; (2) vegetated with *P. australis*; (3) bioaugmented with 106 *Stenotrophomonas* sp. Phr013CFU/g Leca_; (4) bioaugmented with 106 *Stenotrophomonas* sp. Phr013CFU/g Leca_ and vegetated with *P. australis*; (5) bioaugmented with 106 *Sphingobium* sp. NP001; and (6) bioaugmented with 106 *Sphingobium* sp. NP001 and vegetated with *P. australis*. Results, reported in Fig. 2, show a progressive depletion of the phenolics in the presence of *P. australis* plants, revealing that phytoremediation can be exploited for the depletion of residual NPs present in the waste flues. Any depletion has been observed in the presence of the sole Phr013 and NP001 inocula. After 144 h of incubation of the effluents with *P. australis* plants, the reduction of 70 % of the residual content for NP1EO, 61 % for NP2EO and 87 % for 4 nNP has been observed. At the same time, the massive inoculation of the PGPR *Stenotrophomonas* Phr013 in the rhizosphere of *P. australis* plants improved the already recorded capacity of the plant to deplete the contaminants.

In fact, the teamwork of Phr013 and *P. australis* determined an increase in the percentages of NP reduction up to 84 % of the residual treated wastewater content for NP1EO, 71 % for NP2EO and 88 % for 4 nNP with reference to 70 % for NP1EO, 61 % for NP2EO and 87 % for 4-nNP. On the

other hand, the same effect has not been observed in the case of massive inoculation with the *Sphingobium* sp. NP001 (Fig. 2).

In order to compare the efficiency of the bacterialassisted and the not bacterial-assisted phyto-based approach in contaminant depletion and to eventually evaluate the contribution of the two bacterial strains, the process efficiencies (PEs) in NP depletion in the different incubation conditions have been calculated after 144 h of experimentation. Results obtained are shown in Fig. 3. The PE was here defined as the ratio between the amount of phenolics (ng) depleted per mesocosms and the unit of dry weight (g) of the vegetating plant. The highest PE for all the three phenolics has been recorded in pots bioaugmented with Phr013. On the other hand, when compared to not bioaugmented pots, the NP001 bioaugmentation significantly decreased the PE for either 4-nNP or NP2EO (Fig. 3). The increase in PE observed with the bioaugmentation of Phr013 can be associated with the capacity of the strain to express the ACC-deaminase responsible for the lowering of plant ethylene synthesis, contrasting plant stress symptoms related to the accumulation of xenobiotics, eventually favoring their accumulation in plants (Di Gregorio et al. 2006; Glick 2011). At the same time, the decrease in PE induced by NP001 might be related to the fact that plant response to rhizosphere inoculation with selected bacterial strains is depending on the general trophic conditions, e.g., massive microbial inoculation can cause competition for macro- and micronutrients up to the net decrease in contaminant uptake by the plant (Lampis et al. 2009). To study more in detail the mechanism of NP depletion here observed, the mass balances of the processes after 144 h of incubation have been evaluated. The present results (Fig. 4) showed the occurrence of a transformed portion for all the three contaminants. These portions consist of the fraction of contaminants metabolized and/or volatilized by plant and/or microorganisms in the different incubation conditions. Essentially, these portions (Tr fractions) were calculated as the difference between the total amounts of NPs recovered in the effluents at the beginning of the experimentation and the portions that, at the end of process, are still present in the effluents plus the portions recovered in the plant biomass and adsorbed on the Leca. The NP1EO and NP2EO transformed fractions were significantly higher with respect to the 4-nNP transformed one, showing different

384 mechanisms of depletion for the different contaminants. The 4-nNP resulted to be depleted
385 principally by plant absorption, whereas the NP1EO and NP2EO mechanism of depletion was not
386 restricted to plant absorption but also to their transformation. Noteworthily, the adsorption of the
387 different NPs onto the Leca was negligible in all the incubation conditions adopted, suggesting a
388 nonsignificant contribution of the process to their depletion (Fig. 4). Bioaugmentation of the phyto-
389 based modules determined an increment in NP1EO and NP2EO reduction. More in detail, NP001
390 bioaugmentation induced the increment of the depletion of the sole NP1EO. Phr013 induced the
391 increment of depletion of both NP1EO and NP2EO. The increment in their depletion was consistent
392 with the increment of the corresponding transformed portions. In this context, it is worth
393 mentioning that either *Stenotrophomonas* or *Sphingobium* genera have been frequently reported as
394 responsible for the transformation of NPnEOs, either in activated sludge plants and in engineered
395 bioremediation process (Di Gioia et al. 2009). Most bacteria previously reported as able to use NPs
396 as a sole carbon source belong to the *Sphingomonas* group (Fujii et al. 2001). These strains were
397 principally isolated from activated sludge and waste flues of WWTPs. However, a *Sphingobium* sp.
398 and a *Stenotrophomonas* sp. strain, both capable of using NPs as a sole carbon source, have been
399 isolated from the rhizosphere of *P. australis* plant growing on NPs spiked sediments (Toyama et al.
400 2011). In this context, it should be mentioned that many PGPRs result to belong to the
401 *Stenotrophomonas* genus (Hayward et al. 2010); however, to our knowledge, this is the first report
402 describing a PGPR, belonging to the *Stenotrophomonas* genus, that is also capable of using NPs as
403 a sole carbon source and might be directly involved in its depletion from environmental matrices.
404 On the other hand, it is reasonable to assume that also the plant capacity to transform the two
405 contaminants can be involved in the process of their depletion. In fact, plants are reported as
406 capable of transforming low ethoxylated NPs (Dettenmaier and Doucette 2007). Consequently, in
407 this context, it is reasonable to assume that the increased PE for NP1EO and NP2EO in the case of
408 Phr013 bioaugmentation can be associated with a reciprocal stimulation in phenolic transformation
409 between the plant and the bioaugmented strain. Due to the spreading of NPs in the environment, a
410 widespread capacity of different bacteria to transform the phenolics can be expected and, in relation

411 to NP1EO and NP2EO transformation, the involvement of the whole microbial communities,
412 characterizing the different experimental sets, cannot be excluded. In fact, the bioaugmentation with
413 the two strains, Phr013 and NP001, determined a different distribution of the metabolically active
414 bacterial taxonomic units in the different experimental sets. The profiles of the bacterial strains that
415 were active in the different experimental conditions have been investigated by RT-DGGE analysis
416 of the 16S rcDNA of the meta-transcriptome of the bacterial communities characterizing the
417 different pots. Results are shown in Fig. 5. The 16S rDNA of Phr013 and NP001 was exploited as
418 molecular markers to monitor the presence of bands corresponding to the microbial inocula in the
419 different profiles. The putative bands indicating the persistence of Phr013 and NP001 as
420 metabolically active strains after 48 and 144 h of incubation in the different profiles were gel
421 excised and sequenced in order to verify their identity, resulting to match with Phr013 and NP001.
422 After 48 and 144 h of incubation, the amplification products of interest were above the detection
423 limits of DGGE analysis, indicating the persistence of the bioaugmented Phr013 and NP001 in the
424 systems as metabolically active strains. Moreover, the cluster analysis of the DGGE profiles of the
425 different bacterial communities in the different experimental conditions indicated that the
426 bioaugmentation of the two strains induced the speciation of different metabolically active bacterial
427 populations [similarity of the different profiles 61.2 % (Fig. 5, panel b vs c)]. On the contrary, the
428 effect of the time span of incubation of the effluents in the phyto-based modules was less
429 significant, showing similarity of the DGGE profiles spanning approximately from 95.9 to 98.2 %
430 for bioaugmentation with NP001 (Fig. 5, panel b) and from 93.2 to 94.6 % for bioaugmentation
431 with Phr013 (Fig. 5c). As a net result, the two bioaugmented strains determined the speciation of
432 different populations of metabolically active bacterial strains in the rhizosphere of *P. australis* that
433 reasonably differently contributed to the transformation of NP1EO and NP2EO. Possibly, Phr013
434 bioaugmentation might have induced the numerical predominance of a bacterial population, more
435 efficient in the transformation of either NP1EO or NP2EO. However, in addition to the bacterial
436 intervention, our experimentation indicated that *P. australis* was pivotal for the depletion of the
437 phenolics. In fact, the intervention of the sole bacterial strains did not determine any depletion of

438 the contaminants from effluents. Similar results have been obtained in t-NP spiked sediments
439 (Toyama et al. 2011) where the authors actually suggested that *P. australis* was exerting a rhizo-
440 effect on the sediment, spanning from transporting oxygen in the anoxic substrate, eliciting the
441 metabolic activity of bacteria that are competent for the aerobic degradation of the contaminants, to
442 the production of plant exudates, which, as carbonaceous sources, generically favors the metabolic
443 activity of the same microbial community. In our experimental system, similar effects can be
444 assumed. However, while in Toyama et al. (2011) the rhizospheric microbial activity was actually
445 considered as mainly responsible for contaminant depletion, our quantification of phenolics in plant
446 tissues indicates *P. australis* as an important element in determining their depletion by absorption.
447 Noteworthily, the phyto-based approach, besides determining the depletion of NPs, determined also
448 the net decrease in the COD and the BOD5 values (Table 1), and the positive effect of the Phr013
449 bioaugmentation was still evident, determining a higher depletion of the two parameters with
450 reference to the sole *P. australis* and to the inoculation with NP001. Positive results on the quality
451 of the treated effluents, beyond the depletion of NPs, were here expected and were actually assessed
452 by the performed toxicological assays performed. In this context, it should be mentioned that in
453 order to evaluate the eventual efficacy of an applied remediation strategy, numerous bioassays have
454 been already standardized in relation to the different environmental matrices and the assessment of
455 the ecological impact of water contaminants on plants is considered a fundamental assay since
456 plants come into direct contact with contaminated water through their root system (Abdel Migid et
457 al. 2007). As a matter of fact, the large use of plants in phytotoxicity and genotoxicity tests has
458 already been reported by several authors (Giorgetti et al., 2011). The phytotoxicity of the effluents
459 before and after the phyto-based approach here described has been evaluated by bioassays carried
460 out on seeds of *L. sativum* and in *V. faba*. Two end points, seed germination and root elongation,
461 were evaluated after 72-h exposure of seeds to the different types of collected effluents. To provide
462 an integrative interpretation, the two end points were combined into an index of germination (IG%)
463 in which IG% values \40 % are considered very toxic, the range 40–80 % moderately toxic, the
464 range 80–120 % without toxic effect and[120 % effect of phytostimulation. Results of phytotoxicity

test are reported in Fig. 6a, c. Concerning root elongation, the phytotoxicity of the effluents before the phyto-based treatment was observed in the two plants: both in *L. sativum* and in *V. faba*, the mean value of root length significantly decreased when compared to their controls. In *L. sativum*, the mean value of root lengths from seeds germinated in the presence of untreated effluents was almost halved if compared to the control. The same results were obtained in *V. faba* with greater inhibitory effects. The not bacterial-assisted treatment of the effluents reduced the phytotoxic effects both in L. sativum and in *V. faba*. However, the difference with the respective controls was still significant (Fig. 6). After the bacterial-assisted phyto-based treatment of effluents, any phytotoxic effect was observed in *L. sativum* or in *V. faba* and root elongation was comparable to that of the controls (Fig. 6). The IG% for the two tested plant species is reported in Fig. 6b, d. Both *L. sativum* and *V. faba* showed the most affected IG% when germinated in the presence of untreated effluent. In particular, the phytotoxicity was evident in *L. sativum* (IG% = 6.8 %), less severe but still evident in *V. faba* (IG% = 36.072 %). After the not bacterial-assisted phytobased treatment, the IG% accounted for 79.87 % in *L. sativum* and for 50.39 % in *V. faba*; therefore, moderately phytotoxic effects were evidenced. In the case of the bacterial-assisted approach, any phytotoxic effect was detectable for *L. sativum* and *V. faba* (IG% values[80 %). In general, bioaugmentation with Phr013 gave best results when compared with NP001, but the differences between the two types of inocula resulted not significant when statistically analyzed.

The results of genotoxicity analysis obtained on primary root tip apices of *V. faba* L. are summarized in the histogram of Fig. 7. The MI, AI and MCN were considered. Mitotic activity was heavily reduced from 12.26 % of the control root tips to 4.26 % of roots grown in untreated effluents. After the not bacterial-assisted phyto-based treatment, a recovery of mitotic activity was observed (8.39 % of mitosis), but the effect on MI decrease was still evident and statistically significant. When effluents were treated by the bacterial-assisted approach, any negative effect on MI was detectable. Actually, MI values increased (NP001, MI = 13.98 %; Phr013, MI = 15.87 %), although the increases were not statistically significant. Moreover, a large number of cytogenetic aberrations and micronuclei were found in *V. faba* root apices exposed to untreated effluents (AI =

13 %, MCN = 5.22 %) (Fig. 7b, c). After treatment with *P. australis*, with or without microbial inocula, both AI and MCN in *V. faba* root meristems were comparable to the control roots grown in distilled water. Figure 8 illustrates some representative cytological appearance of the detected anomalies in root meristematic cells of *V. faba*.

Our results showed that the effluents at the end of the pipe of the tannery WWTP resulted to be phyto- and genotoxic. Noteworthily, the phyto-based treatment determined the complete depletion of either the phyto- or genotoxicity of the treated effluents. In fact, the toxicological assessment performed after 144 h of the phytobased treatments showed a complete removal of the phytotoxicity, with a stronger reduction in the case of bioaugmentation with Phr013. However, our data demonstrated that the treatment of the effluents with the sole *P. australis* was already able to reduce the phytotoxic effects. Interestingly, when the effluent treatment was operated by *P. australis* bioaugmented with Phr013, physiological effects similar to phyto-stimulation were evident in *L. sativum* (IG = 112 %). Concerning cytogenetic investigations, our results showed that the sole *P. australis* was capable of depleting the genotoxicity of the effluents. Indeed, MI, AI and MCN, heavily altered in *Vicia* root tips incubated in untreated effluents, recovered values comparable to the control when treated in the presence of *P. australis*. The same results were obtained when the effluents were treated with *P. australis* bioaugmented with NP001 or Phr013 strains. In those cases, the highest mitotic index values, especially with Phr013 bioaugmentation, have been observed. However, the presence of the two strains was not crucial for the genotoxicity depletion.

Conclusion

This study demonstrates that a sustainable approach in terms of costs such as phytoremediation is feasible for the depletion of residual priority pollutants, such as 4-nNP, NP1EO and NP2EO, in effluents of conventional tannery WWTPs. A nearly complete depletion up to 87 % of the initial wastewater content of 4-nNP and a reduction in NP1EO and NP2EO up to the 70 and 61 %, respectively, have been obtained. The intervention of plant absorption and plant–bacterial

519 transformation as mechanisms responsible for the depletion of the different phenolics from
520 wastewater has been described. A new PGPR strain, the *Stenotrophomas* sp. Phr013, capable of
521 using NPs as a sole carbon source, has been isolated. Its bioaugmentation in the rhizosphere of *P.*
522 *australis* plants induced the enhancement of depletion for all the three phenolics: from 87 to 88 %
523 for 4-nNP, from 70 to 84 % for NP1EO and from 61 to 71 % for NP2EO. The Phr013 strain can
524 improve the effectiveness of the phyto-based approach by increasing the plant absorption of 4-nNP
525 and the NP1EO and NP2EO transformation. As a net result, our data are in complete accordance
526 with the assessment that bioaugmentation is mostly a winning strategy to improve the efficiency of
527 phyto-based approaches when PGPRs are exploited. Moreover, even though the present approach
528 must be tested on a real scale, the feasibility of the phyto-based tertiary treatment for the
529 improvement of the quality of tannery effluents was confirmed by the toxicological assessment of
530 the process that showed a complete depletion of phytotoxicity and genotoxicity of effluents.
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532
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537 Operativo Regionale – obiettivo "Competitività Regionale e Occupazione) Tuscany Region for
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581 the 26th time Council Directive 76/769/EEC relating to restrictions on the marketing and use of
582 certain dangerous substances and preparations (nonylphenol, nonylphenol ethoxylate and cement)
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690 **Figure captions**

691

692 **Figure 1**

693 Growth curves of Phr013 and NP001 cultivated in minimal medium containing t-NP as a sole
694 carbon source.

695

696 **Figure 2**

697 The total concentrations of phenolics in the wastewater at the beginning of the experimentation
698 (WW), after 48 (panel a) and after 144 hours (panel b) of incubation in presence of *P. australis* (*P.*
699 *austr*), in presence of *P. australis* inoculated with Phr013 (Phr013), in presence of *P. australis*
700 inoculated with NP001 (NP001), in absence of plants and microbial inocula (notVnotI), in presence
701 of the sole Phr013 (notVPhr013), and in presence of the sole NP001 (notVNP001) (* significant
702 differences from samples at $p < 0.05$; all the other values refer to differences from sample at $p <$
703 0.001; bars and error bars represent mean and standard error (+SE), respectively, of three parallel
704 samples)

705

706 **Figure 3**

707 Phytoremediation performance (PE) calculated as the ratio of the ng of phenolics removed per pots
708 to the grams of plant dry weight after 144 hours of incubation in presence of the sole *P. australis*
709 (*P. austr*), in presence of *P. australis* inoculated with Phr013 (Phr013), in presence of *P. australis*
710 inoculated with NP001 (NP001) (significant differences from samples at $p < 0.001$; bars and error
711 bars represent mean and standard error (+SE), respectively, of three parallel samples)

712

713 **Figure 4**

714 Mass balances of the different phenolics after 144 hours of incubation in presence of the sole *P.*
715 *australis* (*P. austr*), in presence of *P. australis* inoculated with Phr013 (Phr013), in presence of *P.*
716 *australis* inoculated with NP001 (NP001). Re, amount of phenolics removed from the waste water;

Pl, amount of phenolics accumulated in the plant; Le, amount of phenolics adsorbed to the Leca®; Tr, amount of transformed phenolics; T0, amount of phenolics in the raw wastewater (bars with the same latter, significant differences from samples at $p < 0.05$; all the other values refer to differences from sample at $p < 0.001$; bars and error bars represent mean and standard error (+SE), respectively, of three parallel samples)

Figure 5

PCR amplified V3 regions of: Panel a, Lane 1, rDNA of NP001; Panel b: Lane 2, rcDNA of the bacterial community colonizing the Leca® vegetated with *P. australis* and bioaugmented with NP001 at the beginning of the experimentation; Lane 3, after 48 hours of incubation; Lane 4, after 144 hours of incubation; Panel c, Lane 5, rDNA of Phr013; Panel d, Lane 6, rcDNA of the bacterial community colonizing the Leca® vegetated with *P. australis* and bioaugmented with Phr013 at the beginning of the experimentation; Lane 7, after 48 hours of incubation; Lane 8, after 144 hours of incubation.

Figure 6

Phytotoxicity test on *Lepidium sativum* L. (Panel a, b) and on *Vicia faba* L. (Panel c, d) expressed as a) root length (cm), and b) germination index (IG %). Control, distilled water; WW, raw wastewater without any treatment; *P. aust,* wastewater treated with the sole *P. australis*; NP001 and Phr13 wastewater treated with *P. australis* plants bioaugmented with NP001 or Phr013 strains respectively. Histogram values represent mean +SE (* significant differences from Control at $P < 0.05$; all the other values refer to differences from Control at $P < 0.001$).

Figure 7

Genotoxicity evaluation in *Vicia faba* L. by Mitotic index (a), aberration index (b) and micronuclei frequencies (c) in different germination substrates: Control, distilled water; WW, raw wastewater without any treatment; *P. aust*, wastewater treated with the sole *P. australis;* NP001 and Phr13,

744 wastewater treated with *P. australis* plants bioaugmented with NP001 or Phr013 strains
745 respectively. Histogram values represent mean +SE (* significant differences from Control at $P < 0.05$; all the other values refer to differences from Control at $P < 0.001$).
746
747
748 **Figure 8**
749 Different types of aberration induced by the treatment with row wastewater in root tip meristems of
750 *Vicia faba* L.: (a, b) micronucleus occurrence at metaphase; (c, d) micronucleus occurrence at
751 different stages of anaphase; e) anaphase with chromosomal bridge, f) anaphase with chromosomal
752 lagging; g) pro-metaphasic cell with evident vacuoles in the cytoplasm (h) micronucleus in
753 vacuolated cell; i, j) multiple micronuclei.
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771 **Fig. 1**

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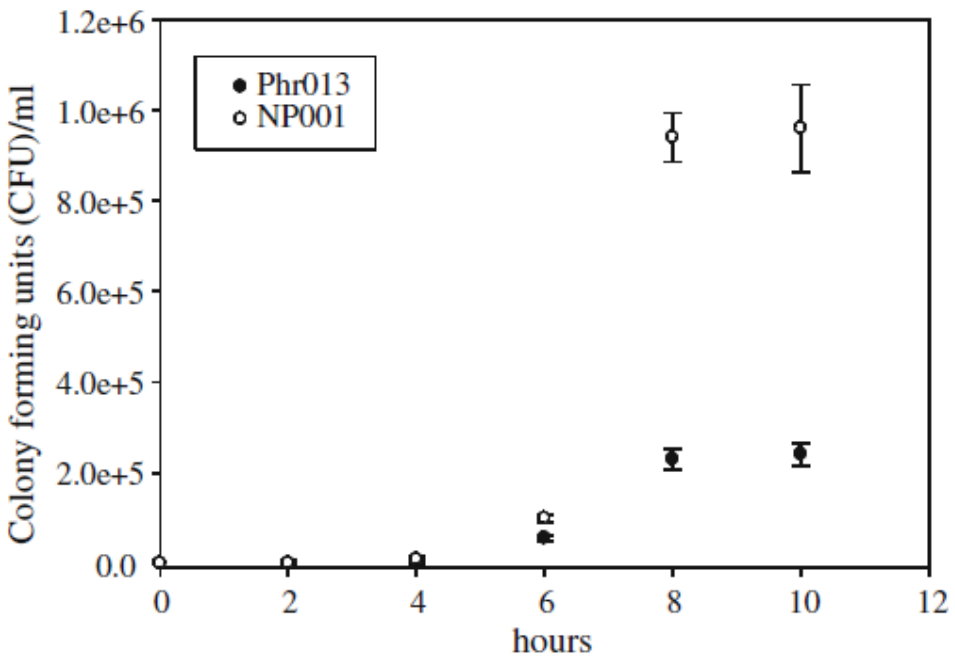

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799 **Fig. 2**

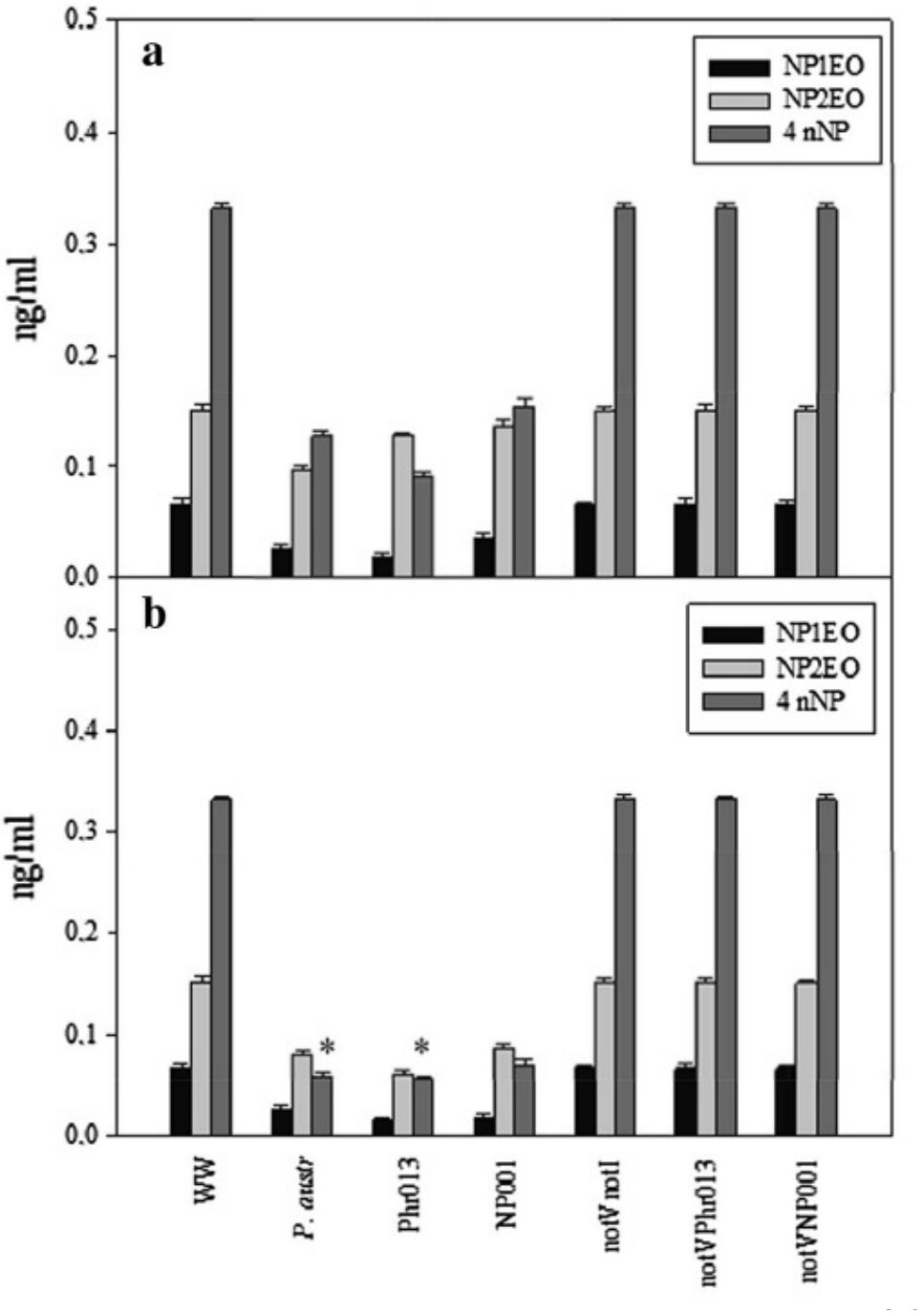

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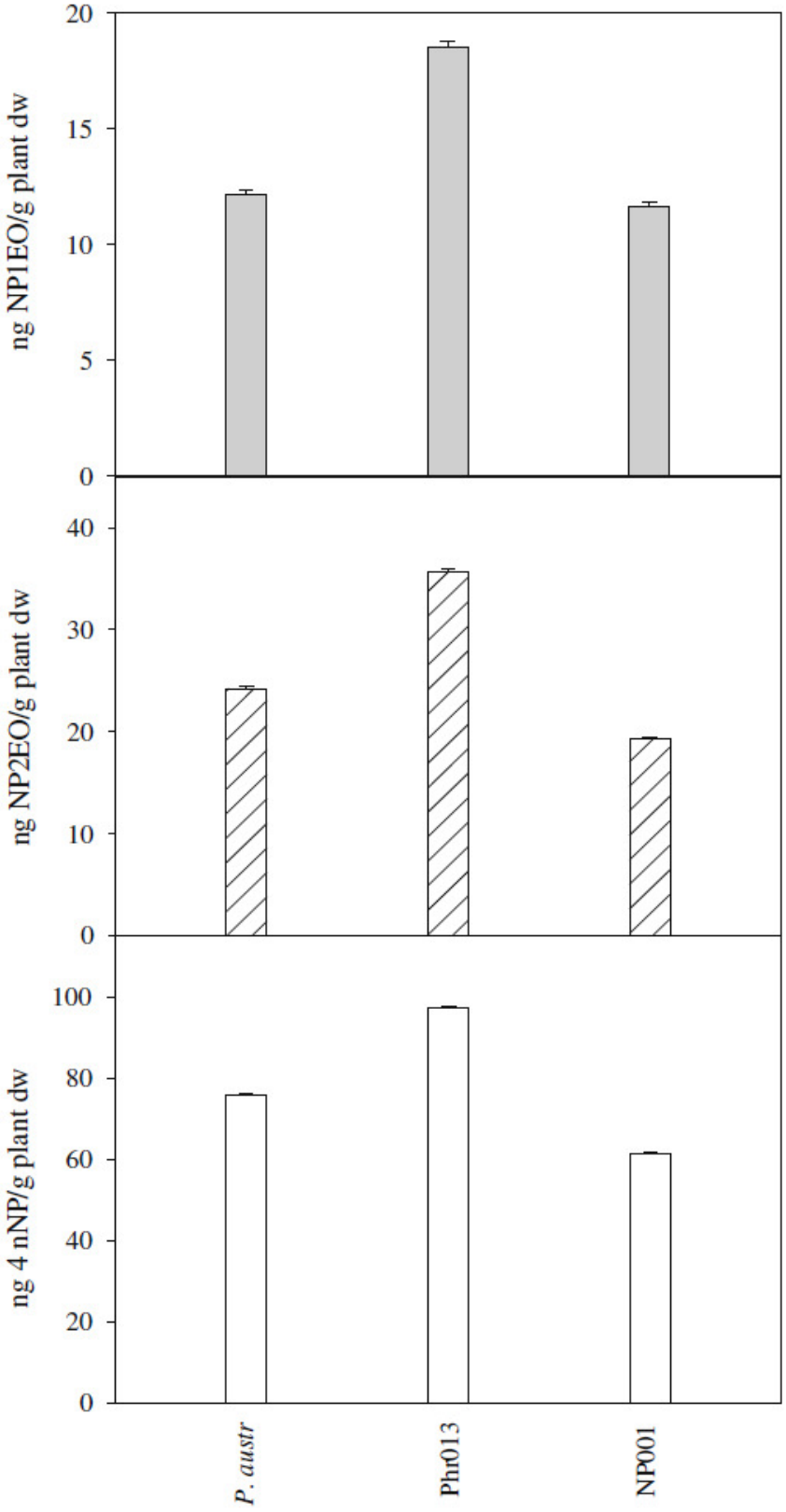
Fig. 3


ng NP1EO/g plant dw
20
15
10
5
0
ng NP2EO/g plant dw
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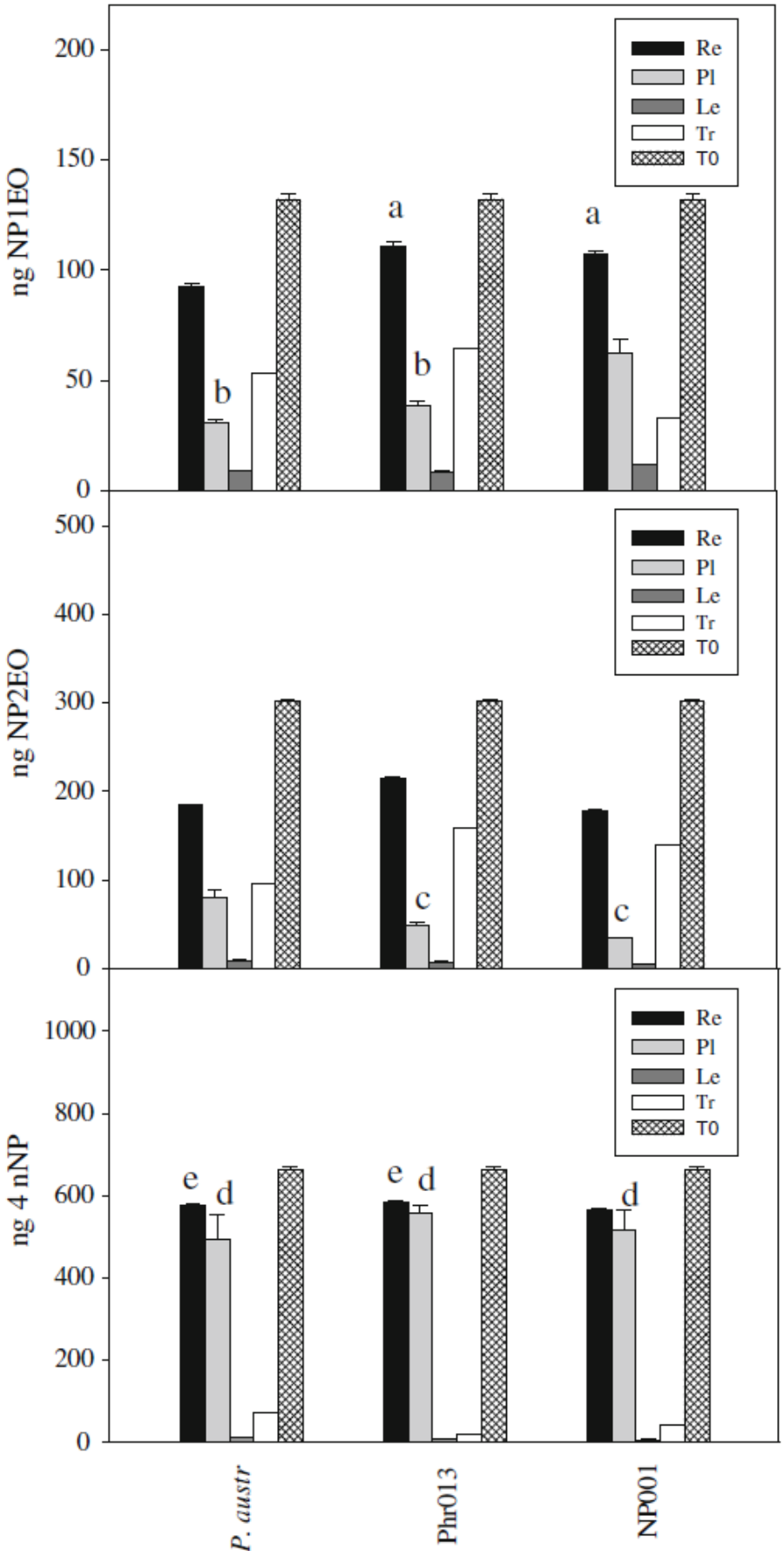
ng 4 nNP/g plant dw
100
80
60
40
20
0
P. austr
Phr013
NP001

Fig. 4


ng NP1EO
200
150
100
50
0
Re
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b
ng NP2EO
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P. austr
Phr013
NP001

879 **Fig. 5**

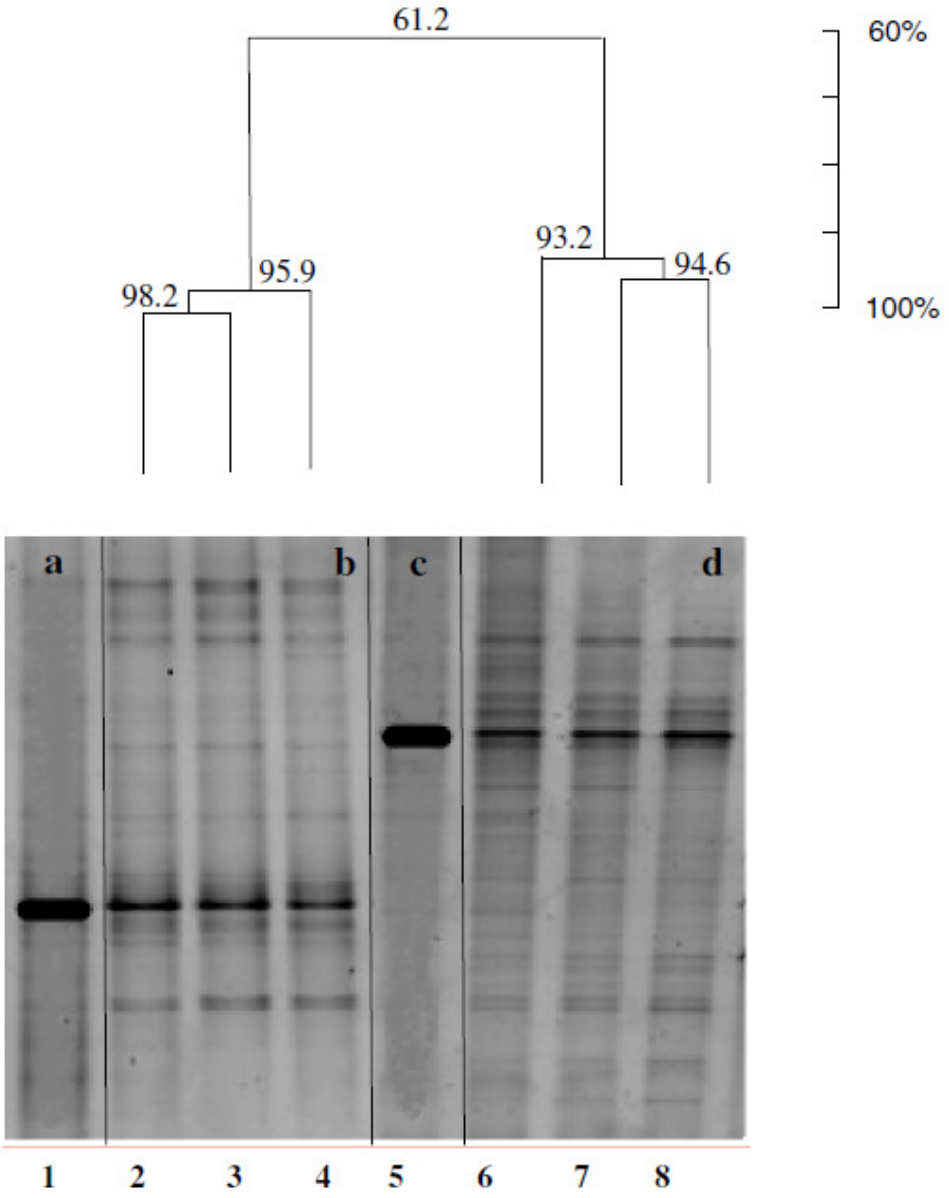

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Fig. 6

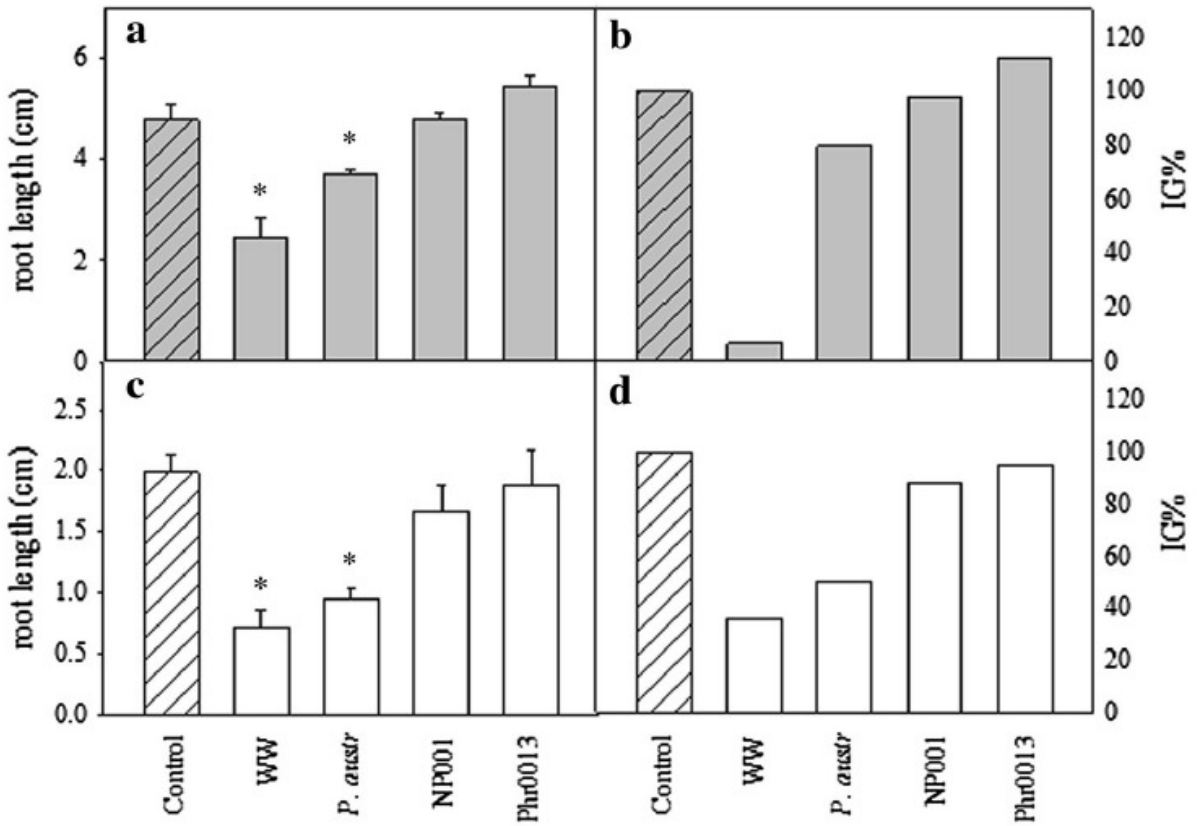
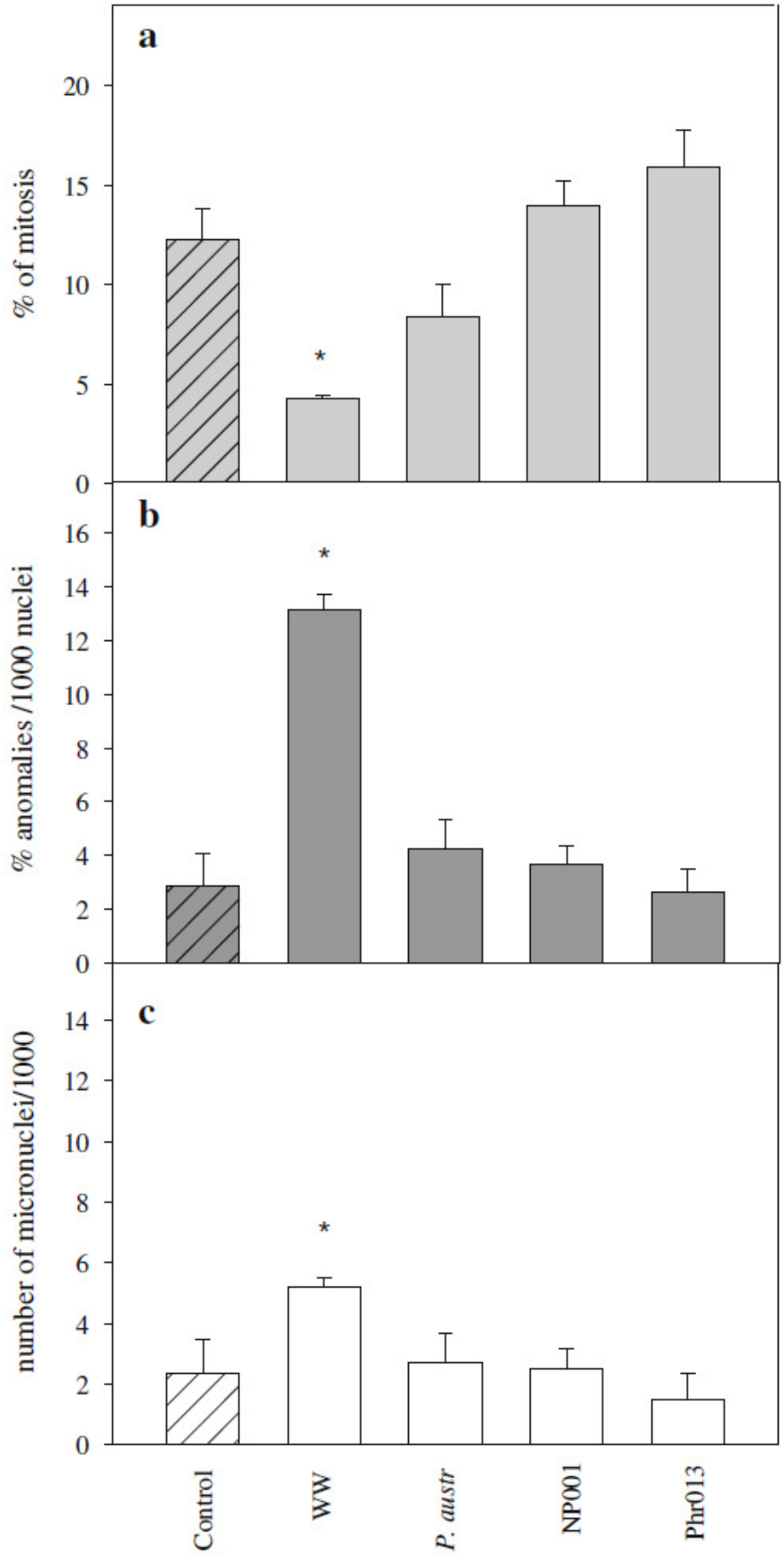


Fig. 7


a
% of mitosis
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15
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b
% anomalies /1000 nuclei
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Control
WW
P. austr
NP001
Phr013

Fig. 8