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Novel nonylphenol-degrading bacterial strains isolated from sewage sludge: Application in bioremediation of sludge

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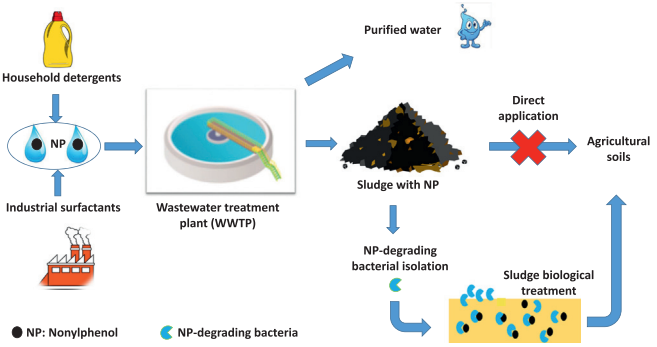
HIGHLIGHTS

- 6 bacterial strains from sludge not previously reported as nonylphenol (NP)-degrader.
- *B. safensis* a powerful novel NP-degrader bacterial species in solution
- Toxic metabolites from NP degradation by *B. safensis.* Toxicity studies are necessary.
- Bioaugmentation with bacteria used for the first time to degrade NP in sewage sludge.
- Effectivity of cyclodextrin to extract NP from sludge to be degraded by *B. safensis.*

GRAPHICAL ABSTRACT



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ABSTRACT

Nonylphenol (NP) is an anthropogenic pollutant frequently found in sewage sludge due to the insufficient degrading effectiveness of conventional WWTPs and has attracted attention as an endocrine disruptor. The aim of this study was to isolate specific NP-degrading bacteria from sewage sludge to be used in the degradation of this contaminant through bioaugmentation processes in aqueous solution and sewage sludge. Up to eight different bacterial strains were isolated, six of them not previously described as NP degraders. *Bacillus safensis* CN12 presented the best NP degradation in solution, and glucose used as an external carbon source increased its effect, reaching DT_{50} degradation values (time to decline to half the initial concentration of the pollutant) of only 0.9 days and a complete degradation in <7 days. Four NP metabolites were identified throughout the biodegradation process, showing higher toxicity than the parent contaminant. In sewage sludge suspensions, the endogenous microbiota was capable of partially degrading NP, but a part remained adsorbed as bound residue. Bioaugmentation was used for the first time to remove NP from sewage sludge to obtain more environmentally friendly biosolids. However, *B. safensis* CN12 was not able to degrade NP due to its high adsorption on sludge, but the use of a cyclodextrin (HPBCD) as availability enhancer allowed us to extract NP and degrade it in solution. The addition of glucose as an external carbon source gave the best results since the metabolism of the sludge microbiota was activated, and HPBCD was able to remove NP from sewage sludge to the solution to be degraded by *B. safensis* CN12. These results indicate that *B. safensis* CN12 can be used to degrade NP in water and sewage sludge, but the method must be improved using consortia of *B. safensis* CN12 with other bacterial strains able to degrade the toxic metabolites produced.

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1. Introduction

Management of the sludge produced in wastewater treatment plants (WWTPs) constitutes a major environmental problem. In Spain, about 1.2×10^6 tons of sewage sludge are produced annually (Ministerio para la Transición Ecológica, 2019), and in Europe, about 11×10^6 tons per year are generated (Patureau et al., 2021). The main methods of sewage sludge management in the EU are agricultural use and incineration (Kacprzak et al., 2017). About 80 % of sewage sludge produced in Spain is valorised as an amendment in agriculture, adding an economical value to something usually considered as a residue. However, this approach has potential risks because of the presence of hazardous pollutants, such as potentially toxic elements (PTEs), pathogens and organic contaminants (Thomaidi et al., 2016; Ghirardini and Verlicchi, 2019). In biosolids intended for agricultural application, the pathogenicity or the PTE content are regulated, but not the contents of organic contaminants (pesticides, industrial compounds, pharmaceuticals and personal care products, hormones, etc.), which can accumulate in agricultural soils and contaminate surface water and groundwater, with potential enrichment in plants and animals (Semblante et al., 2015).

Since the second half of the 20th century, synthesis of new organic chemicals has been increasing exponentially, with some negative effects on human health and the environment. Some of them are toxic and/or persistent in the environment, such as those proposed by the Stockholm Convention (2001), as well as the so-called "contaminants of emerging concern" (CECs), which are not yet included in current environmental legislations (Tijani et al., 2016). Among these molecules, alkylphenols (APs) and, in particular, their polyethoxylated derivatives, nonylphenol mono- (NP1EO) and diethoxylates (NP2EO), referred to as NPEs, deserve special attention. They are used as nonionic surfactants in a large variety of industrial and domestic applications (detergents, emulsifiers, wetting and dispersing agents, antistatic agents, demulsifiers, solubilisers, cosmetic products and textiles) (Hecker and Hollert, 2011). Their use results in their disposal and treatment in WWTPs, where they are biodegraded to nonylphenols (NPs) (Zhang et al., 2008; Bai et al., 2016), relatively stable chemical that remain in sewage sludge (Priac et al., 2017). NPs are a mixture of >100 isomers and congeners with variations in substitution positions in the benzene ring and different branching in the side chains (Lu and Gan, 2014). However, about 90 % of NPs from the degradation of NPEs are 4-NP isomers (NPs). Due to their formation in sewer systems, NPs are commonly detected in influent wastewater and sewage sludge, as well as in rivers and seas as a result of wastewater discharge. Once NPs enter into the environment they will get into the food chain and can disrupt endocrine, immune, nervous and reproductive systems of aquatic organisms and human beings (Soares et al., 2008; Cajthaml et al., 2009). NPs are included among the 13 priority hazardous substances defined as in the field of water policy by the European Community and listed in the Water Framework Directive 2000/60/EC. The European Union has launched a working document on sludge (European Comission, 2010), and its third draft limits the values for the sum of NP and NPEs for the land application of sludge in 450 mg kg^{-1} dry matter. As NP is much more hydrophobic than NPEs, it has a high affinity for sludge flocs and is recalcitrant to wastewater and sludge treatments, which makes NP the main alkylphenol associated with sewage sludge (90 %; Soares et al., 2008).

Based on the above, it is crucial to find processes to remove 4-NP from the environment and water sources. Different physicochemical methods have been proposed such as membrane filtration or photocatalytic, chemical or electrochemical advanced oxidation (Bhandari et al., 2021), but they are all linked to high costs. On the contrary, bioaugmentation is considered as a relatively cheap and environmentally friendly treatment to further improve the biodegradation of other organic contaminants in sewage sludge (Rodriguez-Rodriguez et al., 2014; Aydin, 2016; Taha et al., 2018).

Microbial degradation of NP has been reported mainly in solution using bacteria from different environments. Most reported 4-NP-degrading bacteria belong to *Sphingomonas* (Fujii et al., 2000, 2001; Ikunaga et al., 2004; Corvini et al., 2004, 2006; Gabriel et al., 2005a, 2012; Kolvenbach and Corvini, 2012; Bai et al., 2017). But NP-degrading bacteria belonging to other genera have also been isolated, such as *Stenotrophomonas, Pseudomonas,* or *Bacillus* (Soares et al., 2003; Yuan et al., 2004; Di Gioia et al., 2008a, 2008b). On the contrary, as far as we know, no studies have been conducted on bioaugmentation using bacteria for 4-NP degradation in sludge. Most studies in this field focused on the effect of sewage sludge endogenous microbiota in WWTPs environments on the degradation of NP and NPEs (Chang et al., 2005; Zhang et al., 2008; Ömeroğlu and Sanin, 2014), and in others, bioaugmentation was applied directly to the activated sludge (Michalska et al., 2020). However, for other types of contaminants (PAHs, PBDEs, pharmaceutical and personal care products), studies have shown the feasibility of using bioaugmentation to remove them from sewage sludge using bacteria (Larsen et al., 2009; Liu et al., 2017) or fungi (Rodríguez-Rodríguez et al., 2014; Taha et al., 2018).

Since WWTPs are the primary source of 4-NP release into the environment, the objective of this study is the reduction of the 4-NP concentration in sewage sludge produced by WWTPs through the implementation of a novel technology using bacterial strains to obtain more environmentally friendly biosolids to be used as amendment in agriculture, preventing the contamination of soils and the environment. The specific aim is therefore to obtain NP-degrading bacteria from NP enrichment cultures of sewage sludge, selecting the most suitable ones for the application in sewage sludge suspensions. One of the most significant novelties of this study is that bioaugmentation has been used for the first time to remove 4-NP from sewage sludge; in addition, most of the bacterial strains isolated in this study have not been previously described in the literature as 4-NP-degrading bacteria. Due to the high hydrophobicity and recalcitrant adsorption of NP in sewage sludge, cyclodextrins were used as availability enhancers (Villaverde et al., 2018; Morillo et al., 2020) to improve NP extraction from sewage sludge, facilitating its degradation by the strains selected.

2. Materials and methods

2.1. Chemical, sewage sludge, biostimulants and availability enhancers

The analytical standard 4-n-nonylphenol (4-n-NP, C15H24O, 99.9 % purity, CAS: 104–40-5) was purchased from Sigma-Aldrich. Its chemical properties are as follows: molecular weight: 220.35 g/mol, solubility in water at 25 °C: 7 mg L^{-1}, soluble in most organic solvents, partition coefficient (log Kow): 5.76; vapour pressure at 25 °C: $8.18*10^{-4}$ mmHg (PubChem, 2021). It behaves as a weak acid in aqueous solution (pKa = 10,7) (de Araujo et al., 2018). The availability enhancers β-cyclodextrin (BCD) and hydroxypropyl-β-cyclodextrin (HPBCD) (purity 97 %) were provided by Cyclolab Ltd. (Budapest, Hungary). Three fresh sewage sludge samples (Sludge 1, Sludge 2, Sludge 3) were collected from different wastewater treatment plants (WWTPs) in Seville (Southwest Spain), and one composted biosolid was collected from the Emasesa sludge composting plant in Seville and designated as Compost (B). A detailed description of their characteristics was provided by Madrid et al. (2020) (Table 1S in Supporting Information). Their contents in organic matter were 58.1 %, 58.7 %, 56.6 % and 45.0 % for Sludge 1, Sludge 2, Sludge 3 and Compost, respectively. In all biodegradation assays described below, a nutrients solution (NS) composed by mineral salt medium (MSM) and a solution of micronutrients (mg L^{-1}, 75.0 $MnCl_2$ $4H_2O$; 37.5 $FeSO_4*7H_2O$; 25.0 $SnCl_2$ $2H_2O$; 12.5 $ZnSO_4*7H_2O$; 12.5 $Al_2(SO_4)_3*18H_2O$; 12.5 $NiCl_2*6H_2O$; 12.5 $CoCl_2*2H_2O$; 10.0 $CaSO_4*2H_2O$; 3.75 KBr; 3.75 KCl; 2.50 LiCl), was used.

2.2. 4-n-NP enrichment cultures

The 4-n-NP-degrader microbial consortia were isolated from Sludge 1, 2 and 3 and from Compost, adding 4-n-NP (100 mg L^{-1}) as the only source of carbon and energy to 1 g of fresh sample, together with 20 mL of sterilised MSM (autoclave Auster-G, P-Selecta with one cycle at 120 °C, inlet pressure of 103 kPa, for 20 min) containing also NS, which was used as a biostimulant (Fenlon et al., 2011). Cultures were incubated with orbital

shaking at 30 °C, and every 7 days, an aliquot of the culture was transferred to another flask containing the same sterile mineral medium plus 100 mg L^{-1} 4-n-NP and incubated again. After four enrichment transfers (28 days), an aliquot of the culture was plated on LB-agar medium (25 g L^{-1} Difco™ LB Broth, Miller, and 20 g L^{-1} agar) and incubated for 72 h at 30 °C. Four consortia were obtained (one from each of the three sludge and one from the compost), stored in Microbank™ cryovials (2-mL microtubes containing R2A medium and 20 porous spheres of 3 mm diameter) and kept at -80 °C.

2.3. Isolation and identification of bacterial strains

From each consortium, different 4-n-NP-degrading bacterial strains were isolated on LB agar medium petri dishes in the presence of 4-n-NP. Briefly, 100 μL of each consortia suspension was incubated at 28 °C for 48 h. Successive isolations were performed, recognising the morphologically different bacterial colonies (according to their colour, size, edge and elevation), which were subsequently stored in Microbank™ cryovials and kept at -80 °C (Villaverde et al., 2012). Identification of the different isolated strains was accomplished by extracting DNA from the liquid culture and amplifying the 16S rRNA genes by PCR, using universal oligonucleotide primers (Villaverde et al., 2019). In total, 17 bacterial strains were isolated. Taxonomic identification was carried out by comparison with the NCBI database (National Centre for Biotechnology Information, Maryland, USA) using the BLASTN algorithm, and their sequences were deposited in the NCBI GenBank with their corresponding accession numbers.

2.4. Inoculum preparation

The bacterial strains were cultivated in LB medium in the presence of 4-n-NP (10 mg l^{-1}) (150 rpm, 30 °C) and harvested at the beginning of the stationary phase. The resultant bacterial pellets obtained were washed twice in MSM solution before the experiment. Bacterial growth was monitored by optical density (OD_{600nm}) on a VWR UV-3100 spectrophotometer and by colony-forming units (CFU) of serial dilutions on LB medium plates. The initial cell density of each strain added to the degradation experiments was 10^8 CFU mL^{-1} (Villaverde et al., 2017).

2.5. 4-n-NP biodegradation experiments in solution

Biodegradation experiments of 4-n-NP in solution in a monosubstrate system were carried out by inoculating the NP-degrader microbial consortia or isolated bacterial strains. Biodegradation experiments were performed in triplicate, and all microcosm components were sterilised before the assays. The 4-n-NP biodegradation in solution was carried out in Corex glass centrifuge tubes, where 300 μL of 4-n-NP 100 mg L^{-1} solution prepared in methanol was added and evaporated for 24 h in a fume hood before mixing with the rest of the solutions. After that, each container received 3 mL of MSM solution to obtain a final concentration of 4-n-NP 10 mg L^{-1} and 3 μL of the micronutrient solution. The containers were inoculated to obtain 10^8 CFU mL^{-1} of the corresponding selected consortium or bacterial strain.

To study the possibility of the cometabolic degradation of *B. safensis*, several external carbon sources were used. Biodegradation assays were carried out in the same way, but the mineral salt medium was supplemented with glucose (1 g L^{-1}), yeast extract (0.5 g L^{-1}), BCD (0.511 g L^{-1}) and HPBCD (0.63 g L^{-1}) (the concentrations of BCD and HPBCD used corresponded to 10 times the concentration of 4-n-NP in the microcosms).

Microcosms were incubated on a temperature-controlled rotary shaker at 150 rpm and 30 ± 1 °C. The glass tubes were periodically opened to ensure sufficient oxygen supply. Non-inoculated sterile controls were also prepared (abiotic degradation controls). At the beginning of the experiment and at periodic intervals, the corresponding glass tubes for each time were removed from the incubator and the remaining 4-n-NP residues determined. To recover the concentration of 4-n-NP both in the supernatant and accumulated in the microbial biomass, the glass tubes went through a process of freezing and thawing three times for breaking the cell walls (López-Pacheco et al., 2019), and subsequently, liquid-liquid extraction was carried out. For this, 3 mL of dichloromethane (1:1, v/v) was added to each microcosm, and the total remaining 4-n-NP was analysed in the dichloromethane extract. The calibration curve was prepared in dichloromethane. The concentration of 4-n-NP was determined by a HPLC analyser coupled to a fluorescence detector (Shimadzu RF-10APXL), with a C-18 column (4 mm diameter, 150 mm length) and acetonitrile: water (90:10) as mobile phase, excitation and emission wavelengths were 222 and 305 nm, respectively, and the limit of quantification was 10 μg L^{-1}. The analysis of 4-n-NP metabolites produced during the degradation process was performed by the injection of 5 μL of sample extract on an ultra-high performance liquid chromatography instrument (UHPLC, Agilent 1290 II Infinity LC system equipment) with a vacuum degasser, a binary pump, an autosampler and a thermostated column compartment (Agilent, USA). Separation of nonylphenol and its metabolites was carried out in an InfinityLab Poroshell 120 EC-C18 (100 × 4.6 mm i.d.; 4 μm) analytical column (Agilent, California, USA). Gradient elution was carried out using as mobile phase a 10 mM ammonium acetate aqueous solution (solvent A) and methanol (solvent B) at a flow rate of 0.6 mL/min, with the column thermostated at 35 °C. The elution program was as follow: 0–15 min: linear gradient from 10 % to 100 % of solvent B, 15–18 min: isocratic 100 % solvent B. Mass spectrometry analysis was carried out on an Agilent 6495c triple-quadrupole-mass spectrometer equipped with an electrospray ionisation source. For the ionisation of the compounds, the following setting was applied: MS capillary voltage: 3000 V, drying-gas flow rate: 14 L/min, drying-gas temperature: 200 °C and nebuliser pressure: 20 psi. Each extract was measured using scan, product ion and Multiple Reaction Monitoring (MRM) modes. First, scan mode was applied to the determination of the retention time of each of the studied compounds; scanning was carried out using negative polarity. Mass spectra were registered from m/z 50 to 500. Precursor ions of m/z 219, 233, 235 (two compounds) and 237, corresponding to the ions [M-1]- of nonylphenol, 4-(6-oxo-1,1-dimethylheptyl)phenol, 4-(6-hydroxy-1,1-dimethylheptyl)phenol, 4-nonyl-4-hydroxycyclohexa-2,5-dienone and 4-Nonyl-4-hydroxycyclohex-2-enone, respectively, were selected. Product ion mode was applied, using negative polarity, for the monitorisation of the product ions of the selected precursor ions. The applied fragmentor was 166 V, and the voltage applied to the ions in the collision cell (collision energy) was 36 V. The MS/MS spectra from m/z 50 to 500 were obtained using a scan time of 300 ms. The presence of the compounds was confirmed using MRM mode. At least two transitions were selected for all compounds, except for 4-Nonyl-4-hydroxycyclohex-2-enone (only one transition was monitored). The conditions applied in MRM mode are shown in Table 1.

2.6. 4-n-NP biodegradation experiments in sewage sludge slurry

Sludge 3 and *B. safensis* CN12 were selected to carry out 4-n-NP biodegradation experiments in sludge suspensions. All microcosm components were sterilised before the assays, which were performed in Corex glass centrifuge tubes containing 100 mg of sludge, spiked with 100 μL of 50 mg L^{-1} 4-n-NP solution prepared in methanol, which was evaporated for 24 h in a fume hood to obtain a contamination of 50 mg kg^{-1} in the sludge. After that, a 5-mL solution containing 4.9 mL MSM and 100 μL SNs was added in all assays, although different treatments were carried out, as shown in Table 2. In those assays where *B. safensis* CN12 was present, the containers were inoculated to obtain 10^8 CFU mL^{-1} of this strain in the suspension. Sterilised sludge was used in some experiments to observe the abiotic dissipation of NP. Sludge was sterilised adding sodium azide to obtain 200 mg L^{-1} in the supernatant to prevent biological activity during the experiment. To undo the biodegradative effect of the sludge endogenous microbial population and observe the effect of only *B. safensis* on NP degradation, sludge was autoclaved before being contaminated with NP (autoclave Auster-G, P-Selecta with one cycle at 120 °C, inlet pressure of 103 kPa, for 40 min). In the assays where glucose was used, it was added to the solution to obtain a concentration of 1 g L^{-1} glucose. Parallel experiments were also carried out adding HPBCD as availability enhancer. In such cases, the appropriate

Table 1
Optimized MS/MS parameters for MRM analysis for 4-n-NP and metabolites.

Compound	Ionisation mode	MRM 1 (*m/z*)	MRM 2 (*m/z*)	MRM 3 (*m/z*)	Fragmentor (V)	Collision energy (eV)
4-n-Nonylphenol	Negative	219 > 133	219 > 149	219 > 107	166	20/20/40
4-(6-hydroxy-1,1-dimethylheptyl)phenol	Negative	235 > 117	235 > 133		166	80/36
4-(6-oxo-1,1-dimethylheptyl)phenol	Negative	233 > 117	233 > 133		166	80/34
4-nonyl-4-hydroxycyclohexa-2,5-dienone	Negative	235 > 122	235 > 121	235 > 235	166	20/20/0
4-Nonyl-4-hydroxycyclohex-2-enone	Negative	237 > 219			166	20

amount of HPBCD was added to MSM solution to obtain 50 mM, a HPBCD concentration used previously by Madrid et al. (2020) with extraction enhancements of NP from sludge of >500-fold higher with HPBCD than with water. Microcosms were incubated on a temperature-controlled rotary shaker at 150 rpm and 30 ± 1 °C for 40 days. Non-inoculated controls were also prepared. The 4-n-NP in the microcosms was measured at the beginning of the experiment and at periodic intervals. The glass tubes went through a process of freezing and thawing three times for breaking the cell walls, the slurry was centrifuged (7000 rpm, 20 min), and the 4-n-NP measured both in the liquid phase, through a liquid-liquid extraction as previously explained (Section 2.5), and in the solid phase, using exhaustive extractions of NP carried out following the method proposed by Fernández-Sanjuán et al. (2009). Briefly, the extractions were carried out by sonication in the same centrifuge tubes as follows: 2.5 g of Na_2SO_4 was added, and after 24 h, 4-n-NP was extracted twice with 5 mL hexane: DCM (1:1) (15 min, sonication). After centrifugation, both supernatants were separated, and the sludge was extracted a third time with DCM: acetone (1:1) (15 min, sonication). The 4-n-NP in the supernatants were measured by HPLC coupled to a fluorescence detector as previously mentioned.

2.7. Model of biodegradation kinetics

All 4-n-NP biodegradation curves were fitted to the best kinetic model employing an excel file provided by the FOCUS (2006) workgroup on degradation kinetics and the Solver tool (Microsoft statistical package). Parameters were optimized adapting the recommendations by FOCUS to our biodegradation processes, using the least-squares method. The biodegradation curves were fitted to two kinetic models: a simple first-order model (SFO) and a biphasic first-order sequential model (Hockey-Stick, HS) using the following equations:

$$M_t = M_o{}^{*} e^{-kt} \quad \text{(SFO)}$$

$$DT_{50} = \ln 2/k \quad \text{(SFO)}$$

$$M_t = M_0 e^{-k1} tb e^{-k2(t-tb)} \quad \text{(HS)}$$

$$DT_{50} = (\ln 100/100-50)/k_1 \text{ if } DT_{50} \le t_b \quad \text{(HS)}$$

$$DT_{50} = t_b + (\ln (100/100-50) - k_1 t_b)/k_2 \text{ if } DT_{50} > t_b \quad \text{(HS)}$$

where M_t and M_0 are the concentrations of the remaining 4-n-NP (mg kg^{-1}) at time t and immediately after spiking the soil, respectively, and k is the rate constant of degradation (day^{-1}). The DT_{50} is the time required for the pollutant concentration to decline to half of its initial value. In the HS model, k_1 and k_2 are the rate constants of degradation for the fast and the slow NP degradation fractions, respectively, and t_b is the time at which the rate constant changes. The chi-square (χ^2) and scaled error values with a least square 0.05 were used to estimate the appropriateness of the model and to assess the accuracy of each resulting fit.

Table 2
Treatments applied in 4-n-NP biodegradation in sewage sludge suspensions.

Treatments without HPBCD	Treatments with HPBCD
Sludge	Sludge + HPBCD
Sterilised sludge	Sterilised Sludge + HPBCD
Sludge + *B. safensis*	Sludge + *B. safensis* + HPBCD
Autoclaved sludge + *B. safensis*	Autoclaved sludge + *B. safensis* + HPBCD
Sludge + Glucose	Sludge + Glucose + HPBCD
Sludge + Glucose + *B. safensis*	Sludge + Glucose + *B. safensis* + HPBCD

2.8. Ecotoxicity bioassays

The inhibitory effects of aqueous 4-n-NP solutions at different times of the biodegradation process on the light emission of freeze-dried luminescent bacteria *Aliivibrio fischeri* were determined using the Acute toxicity test according to ISO 11348-3 (2007). Solutions were filtered through 0.45-μm PVDF filters (Scharlau, Spain) to remove the remaining particulate matter and serially diluted with NaCl (2 %) at 50 %, 25 %, 12.5 % and 6.25 % (v/v)', which were tested and compared with the control. The decrease in luminescence was measured in triplicate after 15-min contact using an Microtox model 500, (Modernwater, USA). For each aqueous sample, the EC_{50} value corresponding to the 4-n-NP concentration (% v/v) having a toxic effect on 50 % of the bacterial population was calculated. Toxicity values were then expressed in toxic units, using the formula $TU = 100/EC_{50}$.

3. Results and discussion

3.1. Isolation and characterization of potential NP degraders present in the fresh sewage sludge samples and the composted biosolid

This study reports an approach for isolating potential bacterial degraders of NP from various sewage sludges and a composted biosolid for their possible use in bioremediation. Conventional enrichment of bacteria with 4-n-NP as the only carbon and energy source yielded mixed cultures able to grow using the organic pollutant. Four microbial consortia were isolated, one from each of the three sewage sludges and one from the compost, and individual bacterial strains were isolated from each of the microbial consortia. Overall, 17 bacterial strains were retrieved through 4-n-NP enrichment cultures and isolation. Their phylogenetic information obtained from 16S rRNA gene sequences showed a match over 99 % to those from bacteria in the NCBI GenBank database (Table 3); 16 isolates belonged to the phylum Proteobacteria. Of these strains, 10 belonged to the class Betaproteobacteria, and all of them were *Alcaligenes faecalis*; 4 belonged to the class Gammaproteobacteria (*Aeromonas* sp., *Enterobacter* sp., *Pseudomonas* sp., Shewanella sp.), and 2 belonged to the class Alphaproteobacteria (*Pseudochrobactrum* sp., *Ochrobactrum* sp.). Only one strain belonged to the phylum Firmicutes and class Bacilli (*Bacillus safensis*). The isolation of these bacterial strains indicates that an indigenous microbial community adapted and acclimatised to NP are likely able to metabolise this pollutant using it as a carbon source (Stasinakis et al., 2010; Tunçal et al., 2011).

Most of the bacterial strains isolated in the present work (except *Ochrobactrum anthropic* and *Pseudomonas putida*) have not been previously described in the literature as NP-degrading bacteria. This is the case for *Shewanella putrefaciens* and *Aeromonas salmonicida*, although Barberio et al. (2001) associated bacteria of the genera *Aeromonas* and *Shewanella* with NPE degradation. Also, *Alcaligenes faecalis* was not previously described as NP, although Tanghe et al. (1999) reported one *Alcaligenes* strain able to degrade branched NP. However, this bacterial strain was the most frequently isolated in the present study from the different consortia after

Table 3
Phylogenetic affiliations of bacterial strains isolated from 4-n-nonylphenol enrichment cultures of consortia from three sewage sludge samples (S1, S2, S3) and the composted biosolid (B).

Strain (accession number)/Consortium	NCBI affiliation (accession number)	Similarity	Phylum/Class; order; family; genus
CN1 (MT159828)/S3	*Aeromonas salmonicida* (MG711833.1)	99.92 %	Gammaproteobacteria; Aeromonadales; Aeromonadaceae; Aeromonas
CN2 (MT159914)/S1	*Alcaligenes faecalis* (CP032521.1)	99 %	Betaproteobacteria; Burkholderiales; Alcaligenaceae; Alcaligenes
CN3 (MT159959)/S1	*Alcaligenes faecalis* (GQ856253.1)	100 %	Betaproteobacteria; Burkholderiales; Alcaligenaceae; Alcaligenes
CN4 (MT159958)/S1	*Alcaligenes faecalis* (GQ856253.1)	100 %	Betaproteobacteria; Burkholderiales; Alcaligenaceae; Alcaligenes
CN5 (MT160086)/S1	*Alcaligenes faecalis* (GQ856253.1)	100 %	Betaproteobacteria; Burkholderiales; Alcaligenaceae; Alcaligenes
CN6 (MT160080)/S3	*Alcaligenes faecalis* (CP036294.1)	99.92 %	Betaproteobacteria; Burkholderiales;Alcaligenaceae; Alcaligenes
CN7 (MT160079)/B	*Alcaligenes faecalis* (GQ856253.1)	100 %	Betaproteobacteria; Burkholderiales; Alcaligenaceae; Alcaligenes
CN8 (MT160081)/B	*Alcaligenes faecalis* (GQ856253.1)	100 %	Betaproteobacteria; Burkholderiales; Alcaligenaceae; Alcaligenes
CN9 (MT160348)/B	*Alcaligenes faecalis* (GQ856253.1)	100 %	Betaproteobacteria; Burkholderiales; Alcaligenaceae; Alcaligenes
CN10 (MT160329)/B	*Alcaligenes faecalis* (GQ856253.1)	100 %	Betaproteobacteria; Burkholderiales; Alcaligenaceae; Alcaligenes
CN11 (MT160350)/B	*Alcaligenes faecalis* (GQ856253.1)	100 %	Betaproteobacteria; Burkholderiales; Alcaligenaceae; Alcaligenes
CN12 (MT160352)/S3	*Bacillus safensis* (MN461559.1)	100 %	Firmicutes/Bacilli; Bacillales; Bacillaceae; Bacillus
CN 13 (MT160351)/B	*Enterobacter bugandensis* (MK389443.1)	99.58 %	Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; Enterobacter
CN 14 (MT163437)/S2	*Pseudomonas putida* (MK318658.1)	100 %	Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas
CN15 (MT163434)/B	*Pseudochrobactrum saccharolyticum* (MK377096.1)	100 %	Alphaproteobacteria; Rhizobiales; Brucellaceae; Pseudochrobactrum
CN 16 (MT1634)/S3	*Ochrobactrum anthropi* (AB841127.1)	100 %	Alphaproteobacteria; Rhizobiales; Brucellaceae; Ochrobactrum
CN17 (MT163436)/S3	*Shewanella putrefaciens* (MF401513.1)	99.68 %	Gammaproteobacteria; Alteromonadales;Shewanellaceae; Shewanella.

enrichment with NP (Table 3). Similarly, *Bacillus safensis* has not been described as NP degrader, although some strains from the genus *Bacillus* have been reported in this context (Di Gioia et al., 2004; Chang et al., 2005; Yang et al., 2011; Hsu et al., 2013; Reddy et al., 2015; Zheng et al., 2018). There is also no information about *Enterobacter bugandensis* as NP or NPE degrader, and, as far as we know, even the genus *Enterobacter* has not been previously described as NP degrader. *Pseudochrobactrum saccharolyticum* was also not described as NP degrader, although a bacterial strain from the genus *Pseudochrobactrum* demonstrated phenol-degrading activities (Mao et al., 2015).

On the contrary, NP biodegradation by *Ochrobactrum anthropic* and *Pseudomonas putida* has been described previously. De Weert et al. (2011) observed that *Ochrobactrum anthropic* was involved in the degradation of linear NPs present in polluted river sediment. Also, Villemur et al. (2013) observed NP degradation in the enrichment cultures of the activated sludge from a WWTP, and *Ochrobactrum anthropic* was present. In addition,

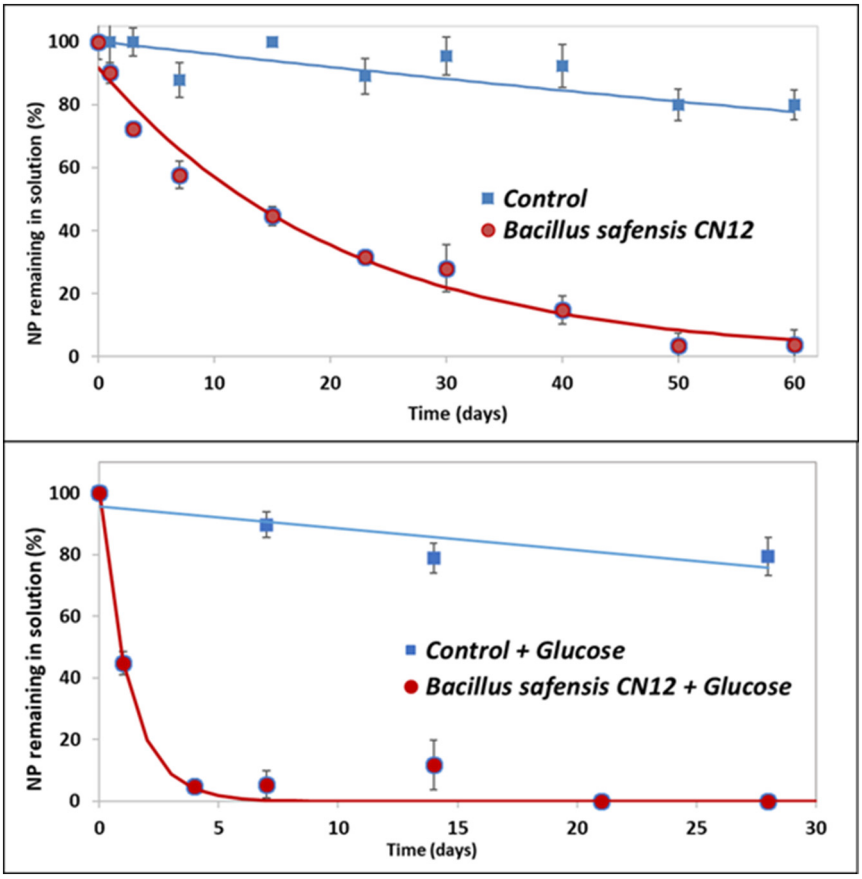

Fig. 1. 4-n-NP biodegradation in solution by *Bacillus safensis* CN12 in the absence (A) and presence of glucose (B).

Ochrobactrum sp. was dominant during the aerobic degradation of nonylphenol in soil (Chang et al., 2007). *Pseudomonas putida* has also been previously described as being able to degrade branched nonylphenol by Tanghe et al. (1999), and Takeo et al. (2006) observed alkylphenol degradation.

3.2. Nonylphenol biodegradation in aqueous solution

The four consortia previously isolated using NP enrichments cultures were used as inoculum for the NP biodegradation assays (Fig. 1S), and the corresponding kinetic parameters are given in Table 4. The dissipation of about 18 % NP from the control samples may be due to abiotic processes such as photolysis and hydrolysis (Medvedeva et al., 2017). Results in NP biodegradation indicated that all consortia contained a microbial biomass capable of degrading NP, and all the bacterial strains isolated from the four consortia were NP-degraders (from 14.1 % to 72.0 %, Fig. 2S), and six of them have been revealed for the first time as specific degraders of NP. Since *Bacillus safensis* CN12 presented the best NP degradation (Fig. 2S), a complete biodegradation curve was carried out over 60 days. Up to 94.9 % of the NP was degraded (Fig. 1A, Table 4), with a DT_{50} value of only 14.4 days, in comparison to the 212 days in the control without inoculation. Four metabolites of the biodegradation were identified (Fig. 2), and their presence throughout the process was measured (Fig. 3A). Metabolite 1 (4-(6-hydroxy-1,1-dimethylheptyl) phenol) appeared after 3 days of incubation and increased up to day 30, disappearing after longer times, whereas NP also disappeared. Metabolite 2 (4-(6-oxo-1,1-dimethylheptyl) phenol) slowly increased until 60 days. Metabolite 3 (4-nonyl-4-hydroxycyclohexa-2,5-dienone) appeared after 7 days, and its concentration increased drastically from day 40 and remained high up to the end of the biodegradation period. Metabolite 4 (4-nonyl-4-hydroxycyclohex-2-enone) appeared after 7 days and disappeared after 40 days of incubation. These results indicate that *B. safensis* CN12 is not capable of provoking the cleavage of the ring and that it was also unable to undergo the reaction to detach the alkyl group from the aromatic part of the molecule.

Metabolites 3 and 4 have previously been observed by Gabriel et al. (2005b) in the 4-n-NP biodegradation by *Sphingomonas xenophaga* Bayram. Such degradation was carried out through cometabolism, using as growth substrate a 4-NP isomer ($4\text{-}NP_{112}$) because Gabriel et al. (2005a) observed that those highly branched isomers presented higher biodegradation rates by *S. xenophaga* Bayram and served as carbon sources (when they used 4-n-NP as the sole carbon and energy source, no degradation was observed). Metabolites 3 and 4 presented a hydroxyl substituent at the 4-position of the ring, indicating that the initial reaction was a hydroxylation at the *ipso*-position, forming Metabolite 3, which subsequently suffered dehydrogenation of one of the two C—C double bonds of the ring to form Metabolite 4. On the contrary, in our case, *B. safensis* CN12 used 4-n-NP as the sole carbon and energy source, without the presence of any other growth substrate, and degraded it to Metabolites 3 and 4, indicating that the degradation occurred through a hydroxylation at the *ipso*-position. For a wide range of phenols containing a quaternary α-carbon structure in the para position, *ipso*-substitution is considered a universal degradation mechanism (Gabriel et al., 2012). According to Gabriel et al. (2005a, 2005b), Metabolite 3 served as substrate and was transformed to Metabolite 4, but in our case, this transformation did not occur from day 40 onward, and Metabolite 3 was accumulated in the system. López-Pacheco et al. (2019) also observed, the presence of Metabolites 3 and 4 in the biodegradation of 4-n-NP.

Although the target contaminant 4-NP was completely eliminated from the microcosms, taking into account the high accumulation of metabolites in the biodegradation system, toxicity measurements using Microtox bioassays were carried out before and after the bioremediation process. As the 4-NP levels decreased, the toxicity increased from a system which presented acute toxicity (TU 5.7) in the non-inoculated microcosms at the beginning of the process to high acute toxicity after 30 days, with a TU value of 10.7, which increased even more after 60 days of incubation (TU 16.5), indicating that the metabolites produced through this process are even more toxic than the parent contaminant.

Experiments were carried out to observe if some external organic sources could increase the percentage of 4-n-NP biodegradation (glucose, yeast extract, BCD and HPBCD). Both CDs are biodegradable (Fenyvesi et al., 2005), and can also be useful as extractants for NP adsorbed on sludge to increase its bioavailability (Madrid et al., 2020; Morillo et al., 2020). Fig. 3S shows that the maximum degradation was achieved with yeast extract and glucose (about 93 % and 95 %, respectively). Glucose was selected as external carbon source and Fig. 1B shows the corresponding degradation curve over 30 days, which fitted well to a single first-order model (Table 4). It is worth noting that *B. safensis* CN12 was able to remove almost 10 mg L^{-1} of 4-n-NP from an aqueous solution in only 4–7 days, with a DT_{50} value of only 0.9 days. Our results are similar to those obtained by López-Pacheco et al. (2019), who indicated that a consortium of two microorganisms could remove a similar concentration of 4-n-NP (9.15 mg L^{-1}) in 6 days. The same four metabolites previously mentioned were also observed (Fig. 3b), indicating that the degradation pathway was the same in the absence and presence of glucose. In this case, the accumulation of Metabolite 3 over the rest was not observed, and all four metabolites were present at the end of the assay (28 days). Toxicity measurements were also carried out, and a similar behaviour as in the absence of glucose was observed,

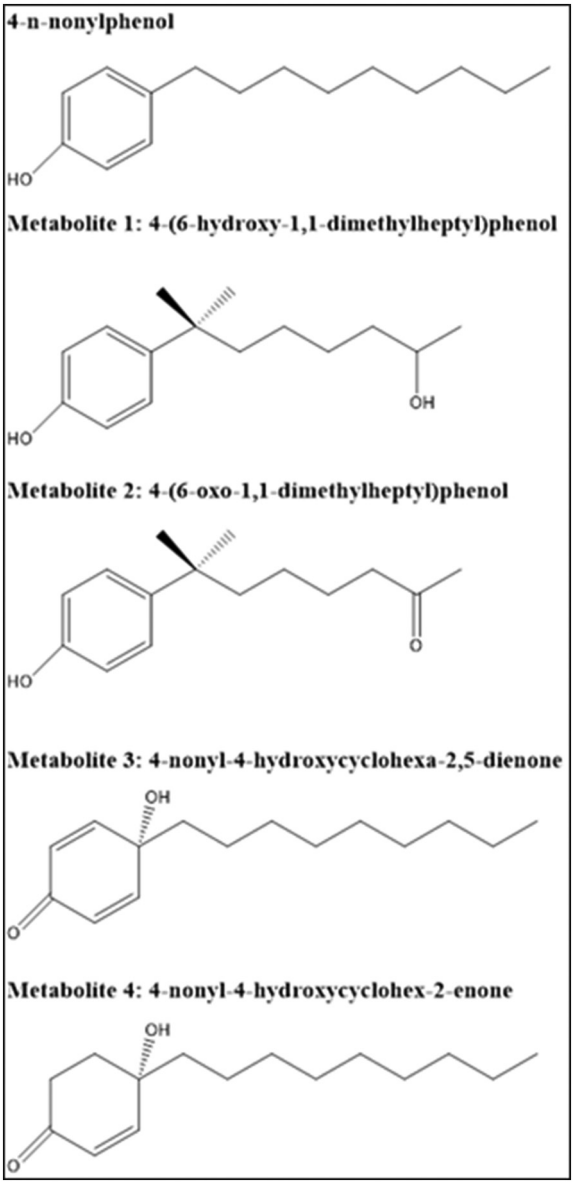

Fig. 2. Chemical structures of 4-n-NP and the metabolites detected during its degradation by *Bacillus safensis* CN12 in solution.

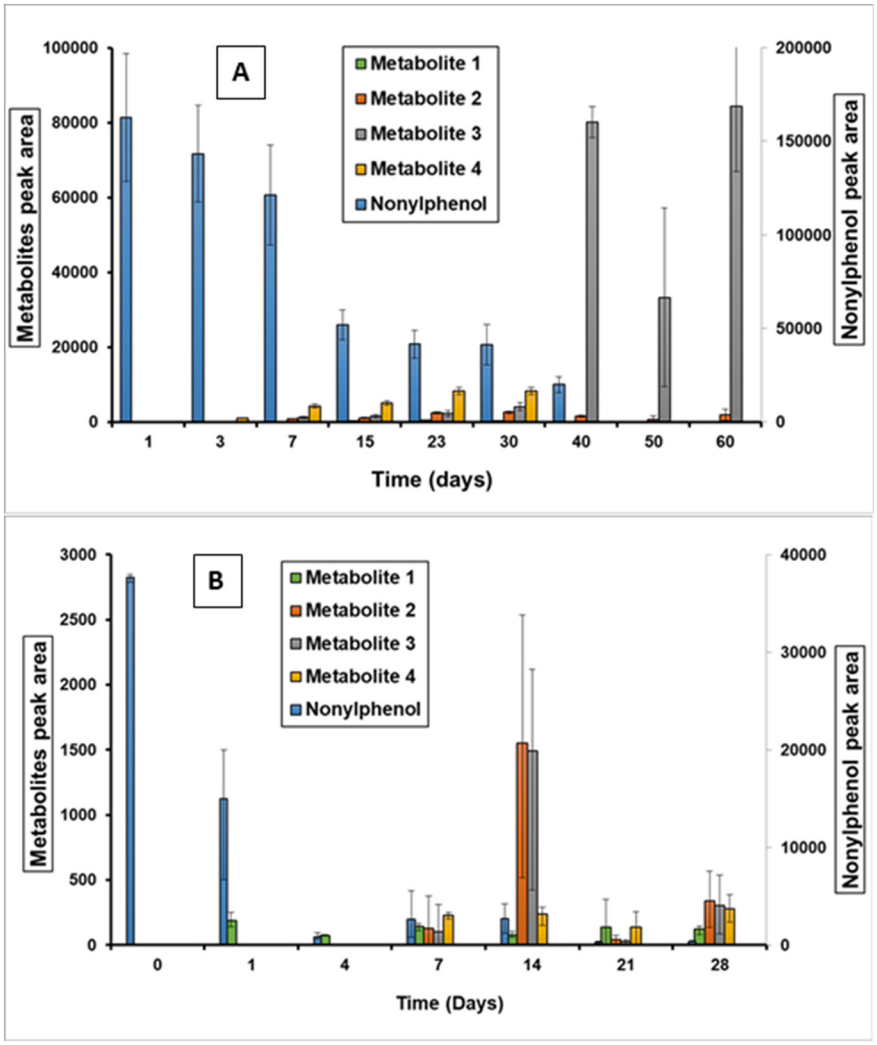

Fig. 3. Evolution of 4-n-NP and the metabolites detected during its degradation by *Bacillus safensis* CN12 in solution in the absence (A) and presence of glucose (B).

with increased toxicity as 4-n-NP was degraded and its metabolites appeared in the system, from TU 9.25 (Acute toxicity) at the beginning of the process (day 0) to TU 12.7, 16.5, 50.2 and 49.4 (high acute toxicity) after 7, 14, 21 and 28 days, respectively, indicating again that the metabolites produced are more toxic than 4-n-NP. Glucose have also increased the metabolism of the other isolated bacteria (Fig. 4), although *B. safensis* CN12 showed the best result. Other two strains of *B. safensis* previously isolated in our laboratories were also tested, reaching good degradation percentages (Fig. 4), but lower ones than those of *Bacillus safensis* CN12 (*B. safensis* CCLP2, 70,1 %; *B. safensis* CTFL5, 45,6 %), indicating that it is a powerful bacterial strain to be applied as an NP degrader.

3.3. Nonylphenol biodegradation in sewage sludge

One of the objectives of the present study was to reduce the concentration of 4-n-NP not only in solution but also in sewage sludge samples to obtain more environmentally friendly biosolids to be used in agricultural practices. Therefore, the next step was to test the bacterial strain which gave the best degradation results in solution, *B. safensis* CN12, to decrease the amount of 4-n-NP in suspensions of sewage sludge artificially contaminated with this pollutant. Fig. 5 shows the percentages of residual 4-n-NP, both in the solid and aqueous phases, under different conditions.

Table 4
Kinetic parameters (*) obtained from 4-n-NP biodegradation in solution after inoculation with 4 bacterial consortia and *Bacillus safensis* CN12.

	Degradation time (days)	Model kinetic	K_1 (day^{-1})	K_2 (day^{-1})	tb (days)	DT_{50} (days)	Extent of degradation (%)	R^2
Control	60	SFO	0.003	–	–	212	18.3	0.9820
Consortium S1	60	SFO	0.43	–	–	1.6	100	0.9710
Consortium S2	60	SFO	0.18	–	–	3.8	100	0.9763
Consortium S3	60	HS	0.22	0.01	3.20	3.1	76.8	0.9351
Consortium B	60	SFO	0.35	–	–	2.0	100	0.9403
Bacillus safensis CN12	60	SFO	0.048	–	–	14.4	94.9	0.9632
Control + glucose	30	SFO	0.009	–	–	79.1	25.8	0.9543
Bacillus safensis CN12 + glucose	30	SFO	0.79	–	–	0.90	100	0.9865

(*) K: mineralization rate constants; tb: Time at which rate constant changes; DT_{50}: Time required for the concentration to decline to half of the initial value.

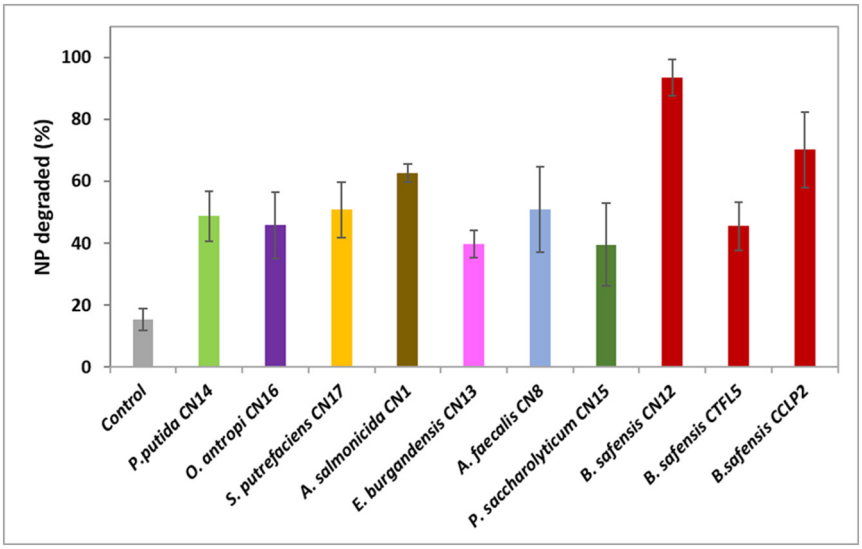

Fig. 4. 4-n-NP biodegradation in solution by different bacterial strains in the presence of glucose after 7 days of incubation.

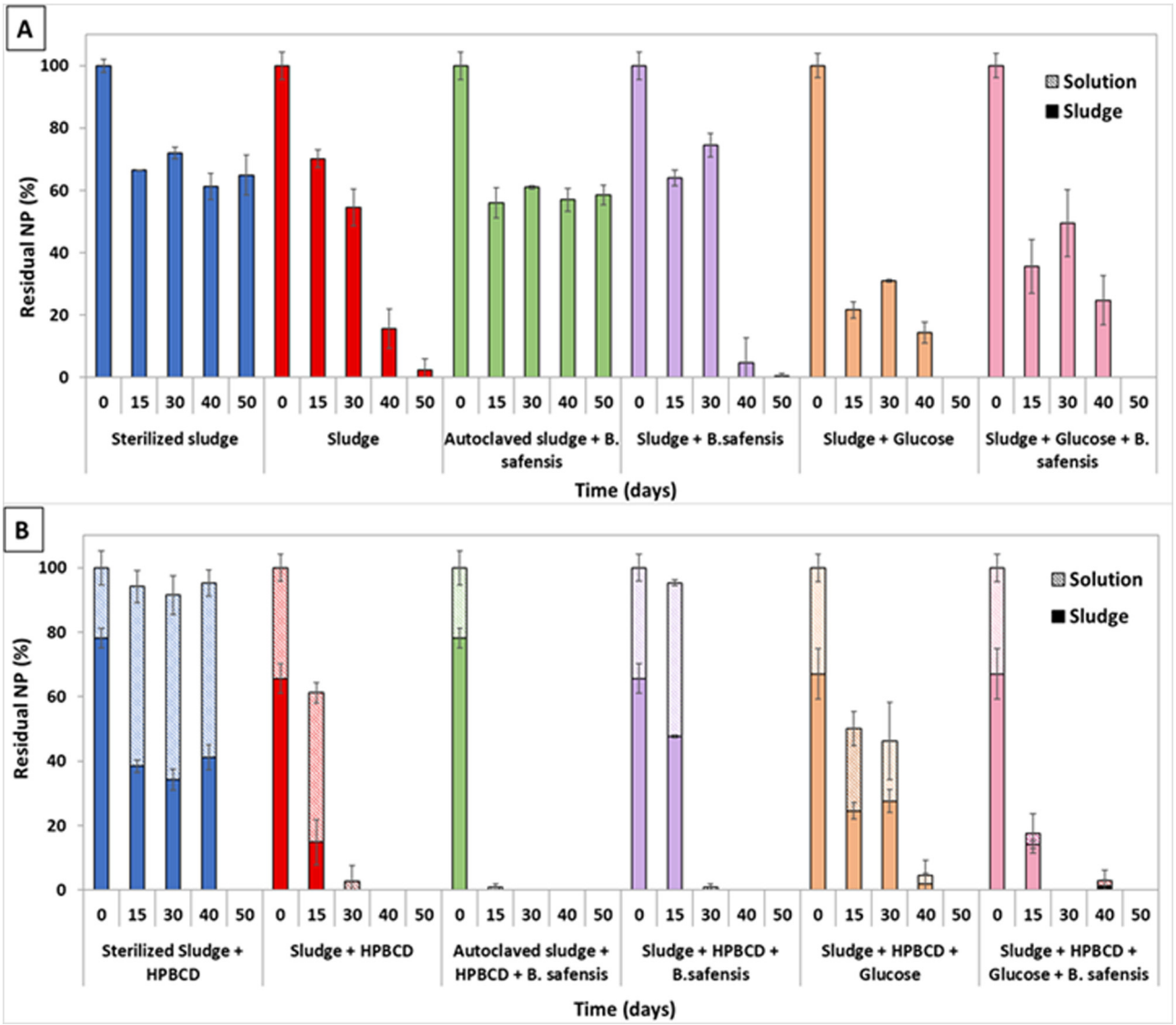

Fig. 5. Percentages of residual 4-n-NP in the sludge and in the aqueous phase after different days of incubation with *Bacillus safensis* CN12 and different conditions in the absence (A) and presence of HPBCD (B).

The assay called "Sterilised sludge" (Fig. 5A), shows the behaviour of the sludge treated with sodium azide to eliminate any biotic effect. Immediately after spiking 4-n-NP on the sludge, 100 % of the NP could be extracted applying the exhaustive extraction method previously described. After longer periods, only 60–70 % could be extracted, indicating that part of the contaminant was strongly adsorbed on the sludge in the first days after treatment and remained as bound residue, but the percentage of this residue did not change with the contact time. The sorption equilibrium of NP in activated sludge can be considered as instantaneous (Dionisi et al., 2006) and NP presents a great tendency to remain adsorbed on the organic matter of sludge as a consequence of the nonpolar and hydrophobic characteristics of both the matrix and NP, with log Kow 5.76 (Sánchez-Trujillo et al., 2014; Madrid et al., 2020).

When the sludge was not sterilised (assay called "Sludge", Fig. 5A), a continuous decrease in the NP present in the solid phase was observed, indicating that the endogenous microbiota of the sludge was capable of degrading >80 % of 4-n-NP after 40 days of incubation despite being strongly adsorbed on sludge. Zhang et al. (2015) observed that the sludge acts as a sorbing matrix, where bacterial strains are capable of developing natural microbial biofilms, which increase bacterial growth rate and cell density, and influence the enhancement of hydrophobic pollutant biodegradation because they promote substrate diffusion, increasing their bioavailability (Smith et al., 2009; Han et al., 2021).

In the assay called "*Autoclaved sludge + B. safensis*", the effects of endogenous microbiota were cancelled but without affecting the possible degradative effect of *B. safensis*, which was added later. The results of this experiment (Fig. 5A) were similar to those of the "Sterilised sludge", where part of the contaminant remained strongly adsorbed on the sludge and only the abiotic dissipation of 4-n-NP could be detected, indicating that the presence of only *B. safensis* does not provoke NP biodegradation. When non-autoclaved sludge was inoculated with *B. safensis* CN12 ("Sludge + *B. safensis*"), the results of NP removal from the sludge were similar to those of the assay called "Sludge", indicating that the endogenous microbiota of the sludge was the one responsible for such degradation. The effect of glucose addition on the degradation of 4-n-NP in sludge ("Sludge + glucose") favoured NP degradation by the sludge endogenous microbiota, but the addition of *B. safensis* ("Sludge + glucose + *B. safensis*") did not increase NP degradation.

Experiments where HPBCD was used as availability enhancer are shown in Fig. 5B. Residues of 4-n-NP were detected in the aqueous phase in all experiments where HPBCD was present.

The "*Sludge + HPBCD*" assay showed a faster NP degradation by the endogenous microbiota, and after 30 days NP was almost completely removed from the system. Up to 34 % NP was extracted from sludge by HPBCD and was present in the solution at the beginning of the experiment, facilitating NP degradation. After 15 days, NP remaining in the system was 61.2 % (>46 % in solution, and only 14.8 % adsorbed on the sludge), indicating that HPBCD is a good availability enhancer for 4-n-NP. Even in the "*Sterilised sludge + HPBCD*" assay, in which almost 100 % of NP added remained in the system, most of it was present in the liquid phase. Neither abiotic degradation was observed nor NP dissipation by bound residue in the presence of HPBCD.

In the assay "*Autoclaved sludge + HPBCD + B. safensis*" (Fig. 5B), 100 % of NP was absent after 15 days. In comparison to the same assay but in the absence of HPBCD (Fig. 5A), it indicated that *B. safensis* had a high capacity to degrade only NP in solution and not the adsorbed NP. However, when *B. safensis* was inoculated in the original sludge ("*Sludge + HPBCD + B. safensis*"), >95 % of 4-n-NP remained in the system after 15 days, divided equally between the solid and the solution. This indicates that the presence of *B. safensis* delayed the NP degradation by the endogenous microbiota of the sludge at the beginning of the incubation, probably due to competition between exogenous and endogenous bacteria (Nwankwegu et al., 2022). However, after an acclimation period, the sludge microbiota was again active, and after 30 days, the NP had been completely eliminated from both the solid and the liquid phases, demonstrating the suitability of HPBCD as availability enhancer. The addition of glucose to this system ("Sludge + HPBCD + glucose + *B. safensis*") resulted in the highest NP degradation. The presence of glucose activated the metabolism of sludge microbiota to degrade NP, whereas HPBCD increased the degradation of NP in solution by *B. safensis*, resulting in residual NP < 20 % after 15 days and its complete degradation after 30 days. The simultaneous addition of glucose and HPBCD cancelled out the competitive effect between endogenous microbiota and *B. safensis*.

4. Conclusions

Of the different species of bacterial strains isolated from sewage sludge and biosolid compost, six could be shown to be, for the first time, as specific nonylphenol-degraders and *B. safensis* CN12 gave the best results of 4-n-NP degradation in solution, with a DT_{50} value of 14.4 days which decreased to only 0.9 days when glucose was used as external carbon source. Four metabolites were detected throughout the biodegradation process and they had a higher toxicity than 4-n-NP. Consortia of *B. safensis* CN12 with other bacteria able to degrade these metabolites are currently being studied. Other two strains of *B. safensis* isolated from agricultural soils also reached high NP degradation percentages, confirming that it is a powerful bacterial species to be applied for NP bioremediation.

The presence of only *B. safensis* CN12 as degrader did not cause the biodegradation of 4-n-NP highly adsorbed in sludge, but the use of HPBCD as availability enhancer extracted a high percentage of NP which could be degraded by *B. safensis* CN12 in solution. The best degradation results were obtained when glucose and HPBCD were added simultaneously since the metabolism of the sludge endogenous microbiota was activated by glucose, favouring the degradation of 4-n-NP strongly adsorbed on sludge, whereas HPBCD increased the NP desorption from sludge, facilitating its degradation by *B. safensis* CN12 in solution.

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CRediT authorship contribution statement

A. Lara-Moreno: Investigation, Methodology, Design of experiment, Writing, Review

I. Aguilar-Romero: Design of experiment, Methodology, Investigation.

M. Rubio-Bellido: Design of experiment, Methodology, Investigation.

F. Madrid: Investigation, Methodology, Data curation, Review.

J. Villaverde: Conceptualization, Design of experiment, Data curation and kinetic modelling, Funding acquisition, Review.

J.L. Santos: Investigation, Methodology, Data curation.

E. Alonso: Investigation, Methodology, Data curation.

E. Morillo: Conceptualization, Design of experiment, Writing original draft, review & editing, Funding acquisition, Supervision.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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