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Novel Bacterial Strains for Nonylphenol Removal in Water and Sewage Sludge: Insights from Gene Expression and Toxicity

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Abstract: 4-Nonylphenols (4-NPs) are persistent endocrine disruptors frequently found in wastewater treatment plant (WWTP) effluents and sewage sludge. This study evaluated the ability of eight bacterial strains that were isolated from sewage sludge to degrade 4-n-NP in an aqueous solution. *Bacillus safensis* CN12, *Shewanella putrefaciens* CN17, and *Alcaligenes faecalis* CN8 showed the highest degradation rates, removing 100%, 75%, and 74% of 4-n-NP (10 mg L^{-1}), with DT_{50} values of 0.90, 8.9, and 10.4 days, respectively. Despite the reduction in 4-n-NP concentrations, ecotoxicity assays revealed that the resulting transformation products (TPs) were more toxic than the parent compound. To investigate the potential degradation mechanisms, in silico and gene expression analyses were conducted on *B. safensis* CN12, revealing a significant upregulation of the multicopper oxidase gene, *cotA* (7.25-fold), and the ring-cleaving dioxygenase gene, *mhqO* (13.9-fold). Although the CN12 strain showed potential for mineralization based on gene expression studies, this was not observed in the aqueous solution. However, when 4-n-NP was adsorbed on sludge and treated with CN12 in the presence of hydroxypropyl-β-cyclodextrin (HPBCD) as a bioavailability enhancer, mineralization reached up to 33%, indicating a synergistic effect with the native sludge microbiota.

Keywords: nonylphenol; biodegradation; mineralization; sewage sludge; *Bacillus safensis*; cyclodextrins

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1. Introduction

Nonylphenols (NPs) are toxic xenobiotic compounds classified as endocrine-disrupting chemicals (EDCs) capable of interfering with the hormonal system of various organisms and are, therefore, considered emerging contaminants. NPs are biodegradation products derived from nonylphenol polyethoxylates (NPEOs), nonionic surfactants widely used by industries involved in the manufacture of pesticides, detergents, emulsifiers, cosmetic products, and textiles, among others [1]. Effluents and sewage sludge from wastewater treatment plants (WWTPs) are the primary sources of NPs in the environment [2] since both industrial discharges and municipal waste streams containing NPEOs arrive at WWTPs, where they are biodegraded to NPs, contaminants that are more persistent and estrogenic [3].

NPs are highly lipophilic and persistent metabolites and are more resistant to biodegradation than NPEOs [4], remaining in wastewater effluents and predominantly adsorbed onto sewage sludge due to their strong hydrophobicity (log Kow range: 5.4–5.92, [5]). Consequently, NPs are found in environmental matrices such as wastewater [6], surface water and sediments [7], soils (due to water irrigation with NP-contaminated sources or due to the use of sewage sludge as an organic amendment) [8], and even in the air [9]. This widespread environmental presence leads to animal and human exposure, primarily through the consumption of contaminated food [10]. In humans, NPs persist in gastrointestinal tissues for extended periods, causing damage to the central nervous system (cognitive functions, memory deficiencies, and depressed behavior) [11] and gut damage (colonic barrier and gut microbiota) [12]. These findings have raised growing concern over NPs, which are now recognized as urgent environmental contaminants requiring action [13]. In this sense, the use of NPEOs and NPs is regulated, and their use has been limited or banned in many applications in the European Union [14–16] or the United States [17,18], among others.

Physical and chemical methods have been tested to remove NPs mainly from water [6], but bioremediation methods based on bioaugmentation are preferred to be applied in WWTPs due to their low cost, high selectivity, and minor secondary pollution, being considered environmentally friendly treatments [1]. Biological processes in secondary treatments in WWTPs (involving aerating the primary effluent to encourage the growth of microorganisms in the activated sludge) and sludge treatment (involving the dewatering of sludge on drying beds and aerobic digestion through composting) present opportunities for the deployment of bioremediation to remove NPs from WWTPs [19,20].

NP biodegradation focused on the use of pure bacterial strains, mainly *Sphingomonas* [21–26], *Stenotrophomonas*, and *Pseudomonas* [27–31], as well as *Rhizobium* and *Sphingobium* [32]. The genus *Bacillus* was also used for NP degradation, as observed by Chang et al. who saw [33,34] that the *Bacillus sphaericus* CT7 strain isolated from sludge samples manifested the best NP degrading ability in sludge, and three strains identified as Bacillus sp. expressed the best biodegradation of NPs adsorbed on mangrove sediment. An NP-degrading enzyme from *Bacillus cereus* could be purified and characterized [35]. Also, Zheng et al. [20] observed that *Bacillus* was the dominant bacterial species in NP biodegradation during aerobic composting of sewage sludge. In this way, we have previously reported that the *Bacillus safensis* strain isolated in our lab was able to degrade 4-n-NP (as a representative of 4-NPs), both in an aqueous solution and when adsorbed on sewage sludge [36]. NPs are anthropogenic contaminants formed by a complex mixture of isomers (about 211) with differently branched and linear carbon chains, but about 90% are 4-NP isomers. Since the primary source of most 4-NPs released into the environment is effluent water and sewage sludge from WWTPs, it is important to find new effective 4-NP degraders as well as bioremediation technologies that can be used shortly to control and reduce the concentration of these anthropogenic contaminants at WWTPs.

In our previous study on 4-n-NP degradation [36], several bacterial strains with degradation capabilities were isolated from sewage sludge, and the main objectives of this study were (i) to investigate the potential of these novel bacterial strains to reduce the 4-n-NP concentration in water through bioaugmentation, compared to *B. safensis*, and to analyze the kinetics of 4-n-NP biodegradation; (ii) to study the potential toxicity of the aqueous solution after the bioremediation process; and (iii) to identify the possible transformation products or metabolites formed. As *B. safensis* CN12 was the bacteria that presented the best results of 4-n-NP degradation in the solution among the eight bacteria tested, the following objectives were planned: (iv) to study its genome to determine the presence of genes that could be responsible for the transformation or mineralization of 4-n-NP and (v) to evaluate 4-n-NP mineralization both in the aqueous solution and in

sewage sludge. As the recalcitrant adsorption of 4-n-NP on sewage sludge is expected due to its high hydrophobicity, cyclodextrins (CDs) will be used as extractants to increase its bioavailability [37], thereby facilitating its degradation by the selected strain.

2. Materials and Methods

2.1. Materials

The 4-n-nonylphenol analytical standard (4-n-NP, CAS: 104-40-5, purity > 98%) was provided by Sigma-Aldrich (Madrid, Spain). Approximately 90% of NPs from the degradation of NPEs are 4-NP isomers, and 4-n-NP was selected for the present research. The radiolabeled compound ring, 14C-4-n-NP (36 mCi·mmol^{-1}, purity 99.9%, and radiochemical purity 100%) was obtained from the Institute of Isotopes (Budapest, Hungary). Hydroxypropyl-β-cyclodextrin (HPBCD, purity 97%) was supplied by Cyclolab (Budapest, Hungary). All other chemicals used were of analytical grade, obtained from Sigma-Aldrich, and used as supplied without further purification. A fresh sewage sludge sample from a WWTP in Seville (southwest Spain) was supplied by Emasesa. It was dried by freezing and lyophilization, crushed, and homogenized before use, and its characteristics are shown in Table S1 (Supporting Information). The nutrients solution (NS) used in biodegradation assays was composed of the mineral salt medium (MSM) and micronutrients (mg/L, 75.0 $MnCl_2{\cdot}4H_2O$; 37.5 $FeSO_4{\cdot}7H_2O$; 25.0 $SnCl_2{\cdot}2H_2O$; 12.5 $ZnSO_4{\cdot}7H_2O$; 12.5 $Al_2(SO_4)_3{\cdot}18H_2O$; 12.5 $NiCl_2{\cdot}6H_2O$; 12.5 $CoCl_2{\cdot}2H_2O$; 10.0 $CaSO_4{\cdot}2H_2O$; 3.75 KBr; 3.75 KCl; 2.5 LiCl). The MSM contained principal and essential trace elements (per liter of deionized water): 0.5 g of KH_2PO_4, 0.5 g of K_2HPO_4, 0.01 g of NaCl, 0.2 g of $MgCl_2{\cdot}6H_2O$, 0.02 g of $CaCl_2$, 1 g of $(NH_4)SO_4$, 0.339 mg of $MnSO_4$, 0.428 mg of $ZnSO_4$, 0.347 mg of $(NH_4)6Mo_7O_{24}{\cdot}4H_2O$, 0.4 mg of $CoCl_2{\cdot}6H_2O$, 5 mg of $FeSO_4{\cdot}7H_2O$, 0.2 mg of $CuSO_4{\cdot}5H_2O$, and 10 mg of EDTA. The pH was adjusted to 7.3 ± 1 with NaOH. Luria–Bertani (LB) broth contained (g L^{-1}) 10.0 tryptone, 5.0 yeast extract, and 10.0 NaCl (BD Difco™, Fisher Scientific, Madrid, Spain).

Eight different specific NP-degrading bacterial strains, which were previously isolated from sewage sludge through the enrichment culture technique with an NP and deposited in the NCBI GenBank with their corresponding accession numbers [36], were used in this study: *Aeromonas salmonicida* CN1, *Alcaligenes faecalis* CN8, *Bacillus safensis* CN12, *Enterobacter bugandensis* CN13, *Pseudomonas putida* CN14, *Pseudochrobactrum saccharolyticum* CN15, *Ochrobactrum anthropi* CN16, and *Shewanella putrefaciens* CN17. These bacterial strains remained stored in MicrobankTM cryovials in the LB medium mixed with a 40% glycerol solution (50/50 v/v) and were kept at −80 °C.

2.2. Inoculum Preparation

The eight bacterial isolates were cultured in the LB medium with 4-n-nonylphenol (4-n-NP) at a concentration of 10 mg L^{-1} under controlled conditions (30 °C at 150 rpm). Cells were harvested by centrifugation (7000 rpm at 10 min) at the beginning of the stationary phase. Bacterial growth was assessed by measuring the optical density at 600 nm (OD600) using a VWR UV-3100 (Avantor, Allentown, PA 18101, USA) spectrophotometer and by counting colony-forming units (CFUs) from serial dilutions plated on LB agar. The pellets of bacteria were washed twice in the MSM solution before each assay to completely remove the residual LB medium and 4-n-NP previously added and then resuspended in the MSM solution. For the degradation experiments, each strain was added at an initial cell density of 10^8 CFUs mL^{-1} [38].

2.3. 4-n-NP Biodegradation in Solution

By inoculating the NP-degrading bacterial strains, 4-n-NP biodegradation studies were carried out in a solution. All the microcosm components were sterilized (autoclaved

for one cycle at 120 °C and a pressure of 101 kPa for 20 min, steam sterilizer model S100, Matachana, Casteldefells, Spain). 4-n-NP biodegradation in the solution was carried out in triplicate. Briefly, 4-n-NP was added to Corex glass centrifuge tubes containing 3 mL of the MSM + micronutrient solution to obtain a final concentration of 4-n-NP 10 mg L^{-1} (about 7 mg L^{-1} should be dissolved and the rest in solid state, according to the aqueous solubility of 4-n-NP). In the previous study where these selected strains were isolated [36], it was observed that the degradation of 4-n-NP by *B. safensis* CN12 increased in the presence of glucose, and the same was observed in the present study for the rest of the isolated strains. The results of these studies are included in Figure S1 (Supplementary Material). For this reason, MSM was supplemented with glucose (1 g L^{-1}) as an external carbon source. The containers were inoculated with 10^8 CFUs mL^{-1} of each bacterial strain, and non-inoculated sterile controls were also prepared. Microcosms were incubated on a rotary shaker (150 rpm, 30 ± 1 °C) and were periodically opened to ensure sufficient oxygen supply. To determine the remaining 4-n-NP in the system, at periodic intervals, some containers were removed from the incubator, frozen, and thawed three times (to also determine 4-n-NP accumulated in the microbial biomass [39]), and a liquid–liquid extraction assay was carried out by adding dichloromethane (1:1, *v*/*v*). The concentration of 4-n-NP in the dichloromethane extract was determined by HPLC with a fluorescence detector (RF-10AXL Shimadzu, Kyoto, Japan), a C-18 column (4 mm diameter, 150 mm length), and the acetonitrile mobile phase: water (90:10), as well as 222 and 305 nm as the excitation and emission wavelengths. 4-n-NP transformation products produced (Figure S2, Supplementary Material) were also analyzed by ultra-high-performance liquid chromatography (UHPLC, Agilent 1290 II Infinity LC system equipment (Agilent, CA, USA)) using an InfinityLab Poroshell 120 EC-C18 (100 × 4.6 mm i.d.; 4 µm) analytical column (Agilent, CA, USA) thermostated at 35 °C. The mobile phase consisted of a mixture of a 10 mM ammonium acetate aqueous solution (solvent A) and methanol (solvent B) at a flow rate of 0.6 mL min^{-1}. The elution program was for 0–15 min, with a linear increment from 10% to 100% of solvent B, followed by 15–18 min in isocratic 100% solvent B. Mass spectrometry detection was performed on an Agilent 6495c triple-quadrupole-mass spectrometer equipped with an electrospray ionization source (more details in Lara-Moreno et al.) [36]. Precursor ions of *m*/*z* 219 corresponding to 4-n-NP, and values of 233, 235, and 237 corresponding to the ions [M^{-1}] of nonylphenol, 4-(6-oxo-1,1-dimethylheptyl)phenol (TP1), 4-nonyl-4-hydroxycyclohexa-2,5-dienone (TP2), and 4-Nonyl-4-hydroxycyclohex-2-enone (TP3), respectively, were selected. 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 Multiple Reaction Monitoring (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 experimental conditions used in MRM mode are shown in Table S2.

2.4. Biodegradation Kinetics Modeling

4-n-NP biodegradation plots were fitted to the optimal kinetic model using an Excel file supplied by the FOCUS working group [40] on degradation kinetics and the Solver tool (Microsoft statistical package). The optimization of the parameters was performed by adapting the recommendations of FOCUS to our biodegradation processes and applying the least-squares method. The biodegradation data were fitted to three kinetic models, namely a first-order simple (SFO) model, a first-order biphasic sequential model (hockey stick, HS), and a first-order multi-compartment (FOMC) model, using the equations below:

$$M_t = M_0\, e^{-kt} \quad \text{(SFO)}$$

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

$$M_t = M_0\, e^{-k1tb}\, e^{-k2(t\,-\,tb)} \quad \text{(HS)}$$

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

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

$$M_t = M_0/((t/\beta) + 1)^{\alpha} \quad \text{(FOMC)}$$

$$DT_{50} = \beta\,(2^{(1/\alpha)} - 1) \quad \text{(FOMC)}$$

SFO is a function describing exponential decline from a defined starting value, where M_t and M_0 are the remaining concentrations of 4-n-NP (mg kg^{-1}) at time t and just after spiking the sample, respectively, whereas k is the degradation rate constant (day^{-1}). The HS model describes two exponential decline functions with a breakpoint between them, where k1 and k2 are the rate constants of degradation for the fast and slow degradation fractions, respectively, and t_b is the time at which the rate constant changes. In the FOMC model, the function describes an exponential decline from a defined starting value, with a decreasing rate constant; α is a shape parameter determined by the coefficient of variation in k values, and β is a location parameter. The DT_{50} is the time required for the pollutant concentration to decline to half of its initial value. The chi-square (χ^2) variable was used to estimate the appropriateness of the model and to assess the accuracy of each resulting fit.

2.5. Acute Toxicity Assessments

The Microtox® bacterial toxicity test was used as an indicator of the Acute Toxicity of 4-n-NP solutions throughout the biodegradation processes. The luminescence of the bacterial strain, *Vibrio fischeri*, is directly related to cell activity and cellular respiration, and the method (Acute toxicity test ISO 11348-3 [41]) is based on the measurement of V. fischeri luminescence inhibition. Data extraction was achieved as reported by Lara-Moreno et al. [36]. Briefly, the solutions were filtered (0.45 µm PVDF filters, Scharlau, Spain) to remove particulate matter and serially diluted with NaCl (2%) at 50%, 25%, 12.5%, and 6.25% (v/v). The decrease in luminescence in comparison to the control was measured (Microtox model 500, Modernwater, York, UK) in triplicate after 15 min of contact. The EC50 value was calculated as the concentration of 4-n-NP (% v/v) that was toxic to 50% of the bacterial population. Toxicity values were expressed as toxic units (TUs = $100/EC_{50}$).

2.6. 4-n-NP Mineralization in Solution

Mineralization studies in a solution using the ^{14}C-ring-labeled 4-n-NP were performed (in triplicate) by the production of $^{14}CO_2$ using respirometers consisting of 30 mL Corex glass centrifuge tubes closed with Teflon-lined stoppers, with a soda trap containing 1 mL of 0.5 M NaOH. All the microcosm components were sterilized as previously mentioned. Tubes contained 3 mL of MSM, the ^{14}C-ring-labeled 4-n-NP to obtain a radioactivity level of approximately 420 Bq per tube, and the unlabeled 4-n-NP to obtain a final concentration of 10 mg L^{-1} in the solution. The containers were inoculated with 10^8 CFU mL^{-1} of selected bacterial strains. In addition, non-inoculated sterile controls were prepared. Microcosms were incubated on a rotary shaker (150 rpm at 30 ± 1 °C). Radioactive production of $^{14}CO_2$ was measured in the soda trap of the respirometer flasks. Regularly, 1 mL of the NaOH solution was sampled from the trap and replaced with fresh alkali. The removed NaOH solution was combined with 3 mL of a liquid scintillation cocktail (Ready Safe, Perkin Elmer, Inc., Waltham, MA, USA) and kept in the dark for approximately 24 h for chemiluminescence quenching, and radioactivity was quantified using a liquid scintillation counter (Perkin Elmer, Tri-Carb 4810TR, Waltham, MA, USA).

2.7. 4-n-NP Mineralization in Sewage Sludge Slurry

4-n-NP mineralization experiments in sludge suspensions were carried out using the same respirometers previously described for mineralization experiments in the solution, and all microcosm components were sterilized before the assays. *B. safensis* CN12 was selected to study the possibility of 4-n-NP mineralization in sewage sludge. Experiments were performed in Corex glass centrifuge tubes containing 100 mg of sludge spiked with a mixture of 4-n-NP in methanol (to obtain a final content of 50 mg kg^{-1}) and the corresponding volume of labeled ^{14}C-4-n-NP to obtain a radioactivity level of approximately 420 Bq ^{14}C-4-n-NP per Corex tube. After methanol evaporation in a fume hood (24 h), 5 mL of a solution containing 5 mL of MSM was added to the Corex tubes. Various assays were carried out in parallel: (I) Non-inoculated sludge: To observe the effect of the sludge endogenous microbial population on 4-n-NP mineralization (assay named "Sludge"), no inoculation was added; (II) Non-inoculated sludge + Glucose: To observe the effect of adding glucose to the endogenous microbial population, glucose was added to the solution to obtain a concentration of 1 g L^{-1} (assay named "Sludge + glucose"); (III) Non inoculated sludge + HPBCD: To observe the effect of adding HPBCD as an availability enhancer on NP mineralization, the appropriate amount of HPBCD was added to the MSM solution to obtain 50 mM (assay named "Sludge + HPBCD"), a HPBCD concentration used previously by Lara-Moreno et al. [36] in NP biodegradation studies; (IV) Inoculated sludge: In those assays where bacterial strains were present, the containers were inoculated to obtain 10^8 CFU mL^{-1} of each strain in the suspension. Corex glass centrifuge tubes were closed with Teflon-lined stoppers, with a soda tramp containing 1 mL of 0.5 M NaOH.

Less than 1/5 of the total volume of the microcosms was occupied by the slurry, and they were incubated on a temperature-controlled rotary shaker at 150 rpm and 30 ± 1 °C to ensure aerobic conditions. The production of $^{14}CO_2$ was measured as radioactivity in the alkali trap (as previously explained) at the beginning of the experiment and at periodic intervals.

2.8. DNA Extraction and Genome Sequencing, Assembly, and Annotation

To characterize *B. safensis* CN12, a pure culture was grown in LB broth at 30 °C for 24 h and then collected for extracting genomic DNA (gDNA). gDNA was extracted using the Canvax Higher Purity bacterial genomic DNA isolation kit (Córdoba, Spain) with RNase treatment. The quality and quantity of the DNA were measured by fluorometric quantification using the Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). To obtain the draft genome, DNA was sequenced by the Macrogen Inc. company (Seoul, Republic of Korea) using the Illumina NovaSeq 600 platform at a coverage of 200×. The generated sequencing reads were assembled using SPAdes-3.14.1 [42]. The sequencing data generated were deposited in the National Center for Biotechnology Information (NCBI) under the BioProject ID PRJNA882784. The whole genome shotgun result of *B. safensis* CN12 was deposited in DDBJ/ENA/GenBank under the accession number JAOEGM000000000.1 Annotation was performed with the Rapid Annotations Subsystems Technology (RAST) [43] and the NCBI Prokaryotic Genome Annotation Pipeline [44].

2.9. Analysis of cotA and mhqO Gene Expression in B. safensis CN12 in the Presence of NP

2.9.1. RNA Extraction, DNase Treatment, and cDNA Synthesis

To evaluate the expression of two putative 4-n-NP-degrading genes (*cotA* and *mhqO*), CN12 cells were collected by centrifugation (12,000 rpm for 10 min) at different times (1, 3, 5, and 7 days), after their cultivation in MSM + 1 g L^{-1} of glucose supplemented with 10 mg L^{-1} 4-n-NP (treatment) and without 4-n-NP (control). The supernatant was removed, and total RNA was extracted from the bacterial pellet using the NZY Total RNA Isolation Kit (NZYTech, Lisbon, Portugal) according to the manufacturer's instructions. RNA samples from

different times were treated first with RNase-free DNAse (ThermoFisher Scientific, Waltham, MA, USA) using 3U of DNase for 3 µg of RNA. The quality of the extracted nucleic acids was assessed through electrophoresis on a 1% (*w*/*v*) agarose gel prepared with 1× TAE buffer (AMRESCO, Solon, OH, USA), incorporating the GreenSafe Premium dye (50 µL L^{-1}, NZYTech, Portugal) for visualization. Subsequently, complementary DNA (cDNA) synthesis was conducted using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) following the protocol recommended by the manufacturer. The resulting cDNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and all samples were diluted in DEPC-treated water to a final concentration of 5 ng·µL^{-1}.

2.9.2. qPCR Primer Design and qPCR Test

The primers for real-time quantitative PCR (qPCR) reactions to analyze the expression of the *cotA*, *mhqO*, and *16s rRNA* genes of *B. safensis* CN12 were designed in silico using the program Primer3 (primer3 https://primer3.ut.ee/ (accessed on 27 May 2025)). During the design of qPCR primers, several strategies were implemented to ensure optimal amplification efficiency: (1) amplicon lengths were restricted to under 200 bp to enhance reaction performance; (2) the potential for primer self-dimers, cross-dimers, and hairpin structures was assessed using the UNAFold Web Server (http://www.idtdna.com/UNAFold (accessed on 27 May 2025), and (3) to prevent non-specific amplification, the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/ (accessed on 27 May 2025) was employed using the *B. safensis* CN12 genome as a reference to verify primer specificity. The primers selected for qPCR analysis included *cotA*-f: CAATCCAACCAGAGGTACAC, *cotA*-r: CGATCCGAATGACTTCACC, *mhqO*-f: AGCAAACGATGGGACTTG, and *mhqO*-r: CGTGGAACAGGCGTAATG. The primers for qPCR amplification of the *16s rRNA* gene were *16s*-f: CTTGACATCCTCTGACAACC and *16s*-r: CTCGTTGCGGGACTTAAC.

cotA and *mhqO* expression were quantified by qPCR with cDNA synthesized immediately after total RNA extraction and using a Relative Quantitation of Gene Expression method. qPCR reactions were carried out using NZY qPCR Green Master Mix (2×) (NZYTech, Portugal). Each 10 µL reaction mixture contained 5 µL of the master mix, 0.4 µL each of the forward and reverse primers (10 µM), 4.0 µL of cDNA (previously normalized to the desired concentration), and 0.2 µL of Milli-Q water. Amplification was performed on a CFX Connect real-time PCR thermocycler (Bio-Rad, Hercules, CA, USA) using the following cycling conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The data were normalized with the 16S rRNA gene as an internal control gene because it is one of the most tested and validated genes in qPCR studies [45]. This study was conducted with the samples obtained from the biodegradation test in MSM + 1 g L^{-1} of glucose spiked with 10 mg L^{-1} of 4-n-NP and control samples in the absence of the contaminant (as a calibrator and a reference sample). Two biological replicates (2 different cultures for each time: 1, 3, 5, and 7 days) were used, and three technical replicates were performed (three independent qPCR reactions for each biological replicate).

2.10. Statistical Analysis

Statistical analysis was carried out using IBM SPSS Statistics v.25. ANOVA to identify significant differences among mean values.

3. Results and Discussion

3.1. 4-n-NP Degradation in Solution by Various Bacterial Strains

4-n-Nonylphenol (10 mg L^{-1}) biodegradation curves were obtained over 28 days using bacterial strains previously isolated from sewage sludge through 4-n-NP enrichment cultures. The bacterial strains selected were *Aeromonas salmonicida* CN1, *Alcaligenes faecalis* CN8, *Enter-*

obacter bugandensis CN13, *Pseudomonas putida* CN14, *Pseudochrobactrum saccharolyticum CN15,* Ochrobactrum anthropi *CN16, and* Shewanella putrefaciens *CN17.* Bacillus safensis CN12 was included as a comparison. Except for *O. anthropi* and *P. putida*, which had been described as NP-degrading bacteria [46,47], the rest of the bacterial strains were observed as possible NP degraders for the first time in our laboratories. The biodegradation curves and kinetic parameters for all these bacterial strains are shown in Figure 1 and Table 1, respectively. Except for the control and *B. safensis* CN12 that fitted to an SFO model, the HS or FOMC model accurately fitted the experimental data of 4-n-NP biodegradation curves by isolated strains, showing a good fit regarding the tabulated chi-square (χ^2) values. The R^2 values were over 0.93 except for *P. saccharolyticum* CN15 (Table 1). Up to 74–75% of the 4-n-NP was degraded by *S. putrefaciens* CN17 and *A. faecalis* CN8, with a DT_{50} of about 9–10 days, in comparison to the control, which showed a DT_{50} of 129 days and only degraded 14.7% of the 4-n-NP. Also, *P. putida* CN14 and *E. bugandensis* CN13 showed a good extent of degradation (66.5 and 63.0%, respectively) and a DT_{50} of only 10.5 and 11.7 days. The other three bacterial strains studied achieved an extent of biodegradation of <51% after 28 days. Special mention should be made to *A. salmonicida*, which showed the highest initial degradation rate after 2 days, but it subsequently reached a plateau at around only 40% degradation. All the bacterial strains tested showed lower degradation capacity than the previously studied *B. safensis* CN12 strain [36], since it was able to remove 100% of the 4-n-NP (10 mg L^{-1}) from the solution in about 7 days, with a DT_{50} value of only 0.9 days (Table 1).

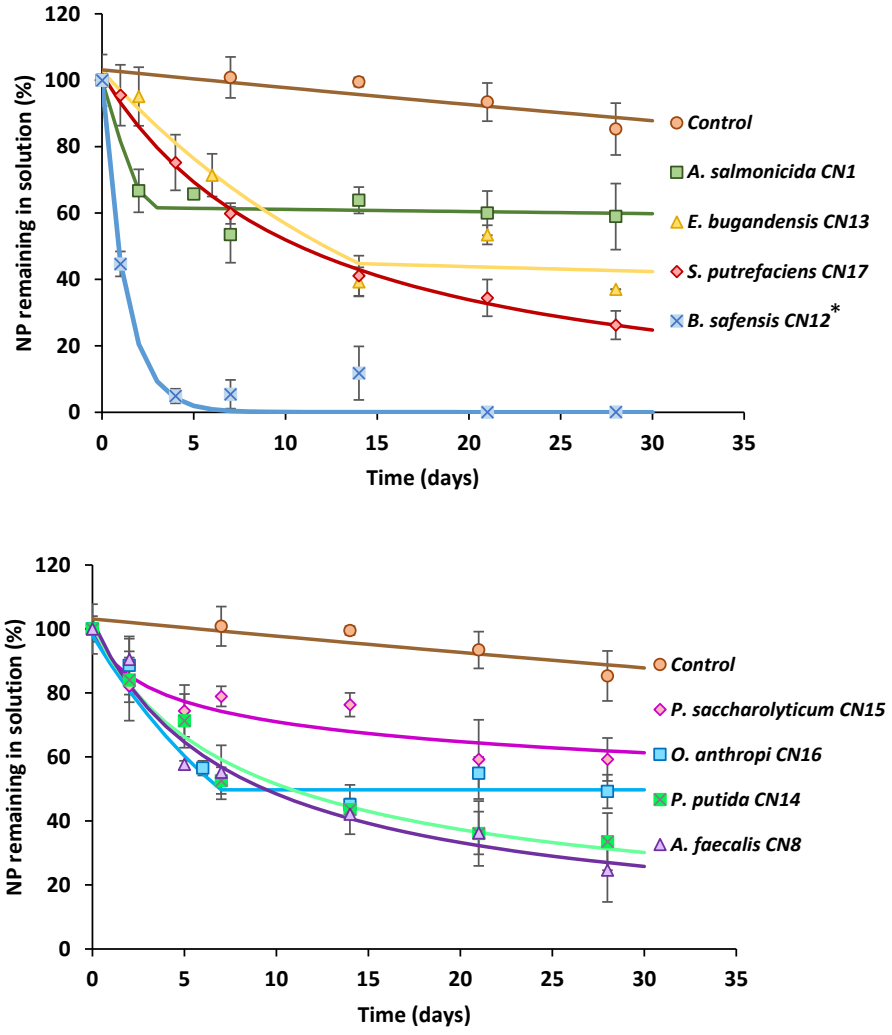

Figure 1. 4-n-NP biodegradation in solution by various bacterial strains isolated from sewage sludge. * *B. safensis* CN12 reported by Lara-Moreno et al. [36].

Table 1. Kinetic parameters from 4-n-NP biodegradation in solution after inoculation with several bacterial strains.

	Kinetic Model	K_1 (d^{-1})	K_2 (d^{-1})	tb (d)	α (d^{-1})	β (d^{-1})	DT_{50} (days)	Extent of Degradation (%)	R^2	χ^2 Err_{scaled}
Control	SFO	0.005	-	-	-	-	129	14.7	0.767	2.27
B. safensis CN12 *	SFO	0.790	-	-	-	-	0.90	100	0.986	5.39
A. faecalis CN8	FOMC	-	-	-	0.785	6.265	8.9	75.4	0.969	3.91
S. putrefaciens CN17	FOMC	-		-	1.182	13.02	10.4	73.8	0.997	1.36
P. putida CN14	FOMC	-	-	-	0.643	5.404	10.5	66.5	0.980	2.86
E. bugandensis CN13	HS	0.059	0.003	14.0	-	-	11.7	63.0	0.952	0.17
O. anthropi CN16	HS	0.093	0.000	7.7	-	-	7.4	50.8	0.973	0.02
A. salmonicida CN1	HS	0.202	0.001	2.4	-	-	203	41.1	0.936	0.78
P. saccharolyticum CN15	FOMC	-	-	-	0.142	1.031	136	40.8	0.879	3.89

* From Lara-Moreno et al. [36].

3.2. Ecotoxicity Studies

Toxicity measurements using Microtox® bioassays were carried out in the solution of 4-n-NP (10 mg L^{-1}) before inoculation with the selected bacterial strains under study and after finishing the bioremediation process (28 days). The results obtained are shown in Table 2. It can be observed that the non-inoculated 4-n-NP solution presented a TU (toxic unit) value of 5.5, corresponding to a level of *Acute Toxicity* according to Persoone et al. [48]. However, when the 4-n-NP concentration in the solution decreased due to biodegradation (Figure 1), the toxicity increased, reaching levels of High Acute Toxicity ($10 < TU < 100$) for all the isolates except for *P. saccharolyticum* CN15, which remained as Acute Toxicity ($1 < TU < 10$). It seems to indicate that the transformation products (TPs) generated by the biodegradation of 4-n-NP must be more toxic than the parent compound, increasing the toxicity of the solution [49]. In Table 2, the isolated bacterial strains are shown in increasing order of 4-n-NP concentration remaining in the solution, but no direct correlation was observed between toxicity increase and the reduction of 4-n-NP in the solution. This suggest that toxicity is more closely related to the presence and concentration of the different TPs generated. It should also be considered that the different bacterial strains could show different degrees of biodegradation towards each specific metabolite produced. In addition, those strains with a lower extent of degradation, as previously mentioned for *A. salmonicida*, which showed the highest TU (Table 2), are those that showed kinetics of the HS type (Table 1). This kinetic model consists of two sequential first-order curves and a faster stage, followed by another with a slower degradation rate. This second slower stage could be provoked by the generation and accumulation of TPs whose toxicity inhibits, to different degrees for each strain, their degradation activity, making it difficult to continue with 4-n-NP degradation.

The toxicity of NP is discussed in various studies. It is widely known that NP (the last end product from NPEO biodegradation) is highly toxic in its recalcitrant form and can have a long half-life in the environment [50], including harmful effects on the nervous, reproductive, immune, endocrine, and metabolic systems [51]. However, as far as we know, the toxicity of the different TPs detected after NP biodegradation has not been discussed in previous studies [39], except in the study of Lara-Moreno et al. [36] where four metabolites were detected throughout the biodegradation process by *B. safensis* CN12 and were shown to have higher toxicity than 4-n-NP.

Table 2. The acute toxicity test towards *A. fischeri* (Microtox assay) in the 4-n-NP solution (10 mg L^{-1}) before inoculation and 28 days after inoculation with different isolated bacterial strains. TU values followed by the same letter are not significantly different ($p < 0.05$).

Bacterial Strain	4-n-NP Remaining (mg L^{-1})	TU	Toxicity
Control	10.0	5.5 a	Acute toxicity
B. safensis CN12 *	0.00	49.4 b	High acute toxicity
A. faecalis CN8	2.46	17.6 ab	High acute toxicity
S. putrefaciens CN17	2.62	19.6 ab	High acute toxicity
P. putida CN14	3.35	28.6 ab	High acute toxicity
E. bugandensis CN13	3.70	31.6 ab	High acute toxicity
O. anthropi CN16	4.92	10.9 ab	High acute toxicity
A. salmonicida CN1	5.89	32.3 ab	High acute toxicity
P. saccharolyticum CN15	5.92	9.20 a	Acute toxicity

* From Lara-Moreno et al. [36].

To observe if the increase in toxicity was related to the presence of certain transformation products, those generated after 28 days of 4-n-NP degradation in the solution in the case of the three bacterial strains that reached higher NP degradation (*A. faecalis* CN8, *S. putrefaciens* CN17, and *P. putida* CN14) were analyzed. Three transformation products (TPs) were identified, which are shown in Figure 2, and their chemical structures are included in Figure S2. TP1 (4-(6-oxo-1,1-dimethylheptyl) phenol) and TP2 (4-nonyl-4-hydroxycyclohexa-2,5-dienone) were detected as degradation products using the three strains studied, and TP3 (4-nonyl-4-hydroxycyclohex-2-enone) appeared only in 4-n-NP degradation using *A. faecalis* CN8. The three TPs found involved the basic structure of 4-n-NP, indicating that none of the three bacterial strains under study could provoke the cleavage of the aromatic ring. As far as we know, TP1 (4-(6-oxo-1,1-dimethylheptyl) phenol) had not been previously detected from NP degradation until the study by Lara-Moreno et al. [36] by *B. safensis* CN12, and now it is detected in this study for *A. faecalis*, *S. putrefaciens*, and *P. putida*.

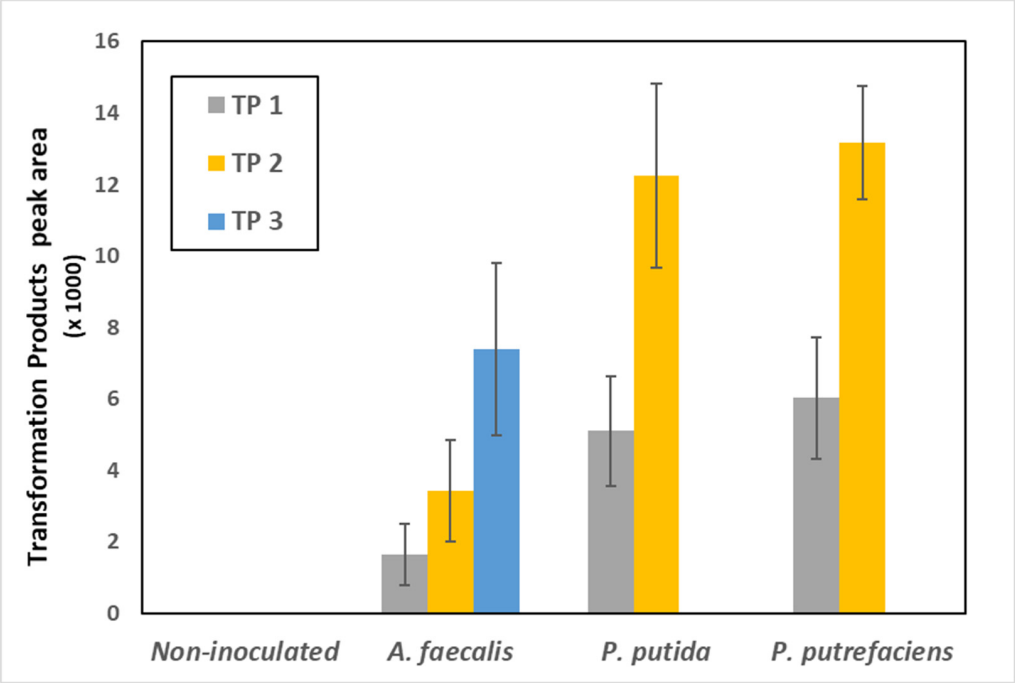

Figure 2. Transformation products (TPs) detected in solution after 28 days of degradation of 4-n-NP by various bacterial strains.

On the contrary, TPs 2 and 3 were detected previously by Gabriel et al. [22,23] and more recently by López-Pacheco et al. [39], who also commented that their toxicity had not been discussed in previous studies. According to Gabriel et al. [22,23], TP3 was generated from TP2 and served as the substrate, but only in the case of *A. faecalis* such a reaction takes place, since in the case of *S. putrefaciens* CN17 and *P. putida* CN14, TP2, as well as TP1, is accumulated in the system. Regarding the increased toxicity observed, these results seem to indicate that the TPs generated and/or other metabolites that we have not been able to detect are more toxic than the parent compound. The observed increase in toxicity highlights the importance of considering TPs in ecological risk assessments, as focusing solely on the parent compound may lead to an underestimation of the actual environmental risk.

Therefore, the bacterial strains tested in the present study can be used to degrade 4-n-NP, but degradation should be improved using consortia with other bacterial strains capable of degrading the toxic TPs produced, since the limited metabolic functions of these pure strains resulted in the incomplete degradation of 4-n-NP or its degradation products.

3.3. In Silico Analysis of Bacillus safensis CN12 Genome Sequence

Among all NP-degrading bacterial strains isolated from sewage sludge, our *B. safensis* CN12 strain was identified as exhibiting the highest degradation efficiency for 4-n-NP. Although no transformation products indicative of aromatic ring cleavage were detected during 4-n-NP degradation, genomic analysis of *B. safensis* CN12 was conducted to investigate the presence of genes associated with aromatic compound metabolism. The assembled genome comprises 24 contigs with a total length of 3,673,492 bp and a GC (guanine–cytosine) content of 41.6%. The draft genome contains 3688 coding sequences, including 3 5S rRNAs, 4 16S rRNAs, 5 23S rRNAs, and 64 tRNAs. The taxonomic classification of the strain was confirmed through comparative BLAST analysis of the 16S rRNA gene, which exhibited 100% sequence identity to the 16S rRNA gene of *Bacillus safensis* (GenBank accession: OP143733.1). The genome harbors six genes implicated in aromatic compound metabolism: three associated with quinate degradation, one with biphenyl degradation, one with gentisate degradation, and one linked to salicylate and gentisate catabolism (Supplementary Figure S3). Salicylate and gentisate, key intermediates in aromatic compound degradation, are metabolized via the gentisate pathway.

This pathway also participates in the catabolism of benzoates, phenolic compounds, and polycyclic aromatic hydrocarbons (PAHs) [38,52]. Additionally, 44 genes (Figure S3). related to motility and chemotaxis were identified, which may enhance aromatic compound removal by promoting bacterial proximity to contaminants, thereby improving degradation efficiency [53].

To date, there is limited genetic information available in the literature regarding the genes involved in the degradation pathway of 4-n-NP. In general, it is known that the catechol 1,2-dioxygenase (*C12O*) and catechol 2,3-dioxygenase (*C23O*) genes are crucial for the ortho- and meta-cleavage pathways of aromatic rings, respectively, and play a significant role in the biodegradation of aromatic compounds [54]. Specifically, in the case of 4-n-NP, Zhang et al. [55] investigated the presence of the *C12O* and *C23O* genes in natural water microcosms from an urban river using the most-probable-number-polymerase chain reaction (MPN-PCR) method and terminal restriction fragment length polymorphism (T-RFLP). Their study found a notable increase in the number of *C23O* gene copies during the biodegradation of 4-n-NP, representing the first genetic evidence of a potential aromatic ring meta-cleaving pathway for NP. On the other hand, the alkane hydroxylases (*alk*) gene and single-component monooxygenase (*sMO*) are also involved in the degradation of 4-n-NP. Both were studied in *Stenotrophomonas* strain Y1 and *Sphingobium* strain Y2

using quantitative PCR (qPCR) [56]. The *alkB* gene acts via the oxidation of the alkyl chain, while the *sMO* gene is responsible for converting NP to hydroquinone via the ipso substitution pathway [57]. The relative abundance of the *alkB* gene increased in the 4-n-NP biodegradation test in the presence of *Stenotrophomonas* strain Y1. However, when *Sphingobium* strain Y2 was inoculated, the proportion of the *sMO* gene increased instead of the *alkB* gene. Also, Takeo et al. [58] identified a nonylphenol monooxygenase gene (*nmoA*) responsible for ipso-hydroxylation in the NP biodegradation conducted by *Pseudomonas putida*. In another previous study, the *alk*, *C12O*, *C23O*, *sMO*, and multi-component phenol hydroxylase (α-subunit) (*mPH*) gene were investigated through PCR in a 4-n-NP-degrading bacterial consortium primarily composed of members from the genera *Sphingomonas*, *Pseudomonas*, *Alicycliphilus*, and *Acidovorax*. In this consortium, the genes *sMO*, *mPH* (it is responsible for monohydroxylating the carbon atom adjacent to the pre-existing hydroxyl group, producing alcohols and catechols in short- and/or medium-chain alkylphenols), and *C12O* were detected, whereas *alk* and *C23O* were not identified [29].

According to the literature reported previously, various sequences of the C23O gene (Accession numbers: WP_095432302.1, WP_048623272.1, and NP_388705.2) and nonyphenol monooxygenase (Accession number: BAI43694.1) retrieved from databases (NCBI) were compared with the complete genome of *B. safensis* CN12. However, the results did not show the presence of these genes in its genome. Hence, they would not be responsible for NP degradation in this strain.

Only Zhang et al. [59] and Guo et al. [60] have described genes responsible for 4-n-NP degradation in species of the *Bacillus* genus (*cotA* and *mhqO*); for this reason, they were studied in the genome of our bacteria of interest. The surface-expressed multicopper oxidase, CotA, and the ring-cleaving dioxygenase, MhqO, were identified with a BLAST search [61]. A 99% identity score was achieved when aligning the sequence of the CotA protein (Accession number: AEX93437.1) described for *Bacillus pumilus* strain A56 and the assembled genome of strain CN12, confirming its presence in the genetic material of the study bacteria. Zhang et al. [59] described the CotA enzyme in the *Bacillus* genus as responsible for 4-n-NP degradation and mineralization. This oxidation process happens on the cell surface, facilitating 4-n-NP immobilization on the bacterial cells and increasing the reaction speed. Moreover, the in silico study of the genomic annotation of *B. safensis* CN12 demonstrated the presence of a gene annotated as encoding the ring-cleaving dioxygenase MhqO protein. This protein showed 64% identity with the MhqO protein (Accession number: AGE62380.1) described for *Bacillus subtilis* XF-1 by Guo et al. [60]. Thus, it could play an important role in the degradation and/or mineralization pathway of 4-n-NP by *B. safensis* CN12. MhqO with dioxygenase function can oxidize a substrate, transferring an atom of oxygen from molecular oxygen (O_2) to the substrate, and according to Zhang et al. [55], dioxygenase enzymes have a relevant role in 4-n-NP biodegradation, as they can break the aromatic ring of the molecule, leading to its degradation into simpler compounds or complete mineralization. These authors were indeed the first to report genetic evidence of a possible aromatic ring-cleaving pathway of 4-n-NP in an aquatic environment.

Based on the biodegradation results and the genetic information provided by the whole genome sequencing results, we can conclude that strain CN12 is a potential candidate for the degradation of aromatic compounds and even for their mineralization, including 4-n-NP.

3.4. Analysis of cotA and mhqO Gene Expression in B. safensis CN12 in the Presence of 4-n-NP

Gene expression analysis is a widely known method to demonstrate the specific induction of genes; for this reason, many authors employ RT-qPCR to detect and quantify the expression of bacterial genes involved in the degradation pathways of organic pollutants in the environment [62,63].

In this study, genomic, transcriptional, and quantitative approaches were employed to demonstrate the specific induction of two functional genes, *cotA* and *mhqO*, which may play a key role in the 4-n-NP biodegradation pathway. The initial RT-qPCR amplification tests revealed unique fragments for both genes, confirming the primers' specificity. The tests also revealed that *cotA* and *mhqO* needed 10 ng of cDNA for effective amplification, while 16S rRNA could be amplified with as little as 0.003 ng of cDNA, resulting in CT values close to 20 for these genes. Regarding the qPCR amplifications conducted for the expression study, the slopes observed in the RT-PCR standard represented as regression line plots of the CT value vs. the log of the input cDNA were different for the studied genes (−4.36, −3.62, and −3.49 for the *cotA*, *mhqO*, and *16S rRNA* genes, respectively), which implies variable PCR efficiencies and excludes the applicability of the comparative CT method (ΔΔ CT method) [64]. This was confirmed by the validation test, which showed that both the target genes and the reference gene had different qPCR efficiencies, as the absolute value of the slope of the regression line between the log input RNA amount (ng) and ΔCT was more than 0.1 in both cases. Therefore, both gene expressions were examined according to the Relative Quantitation of the Gene Expression approach by the standard curve method (Figure 3 and Table S3).

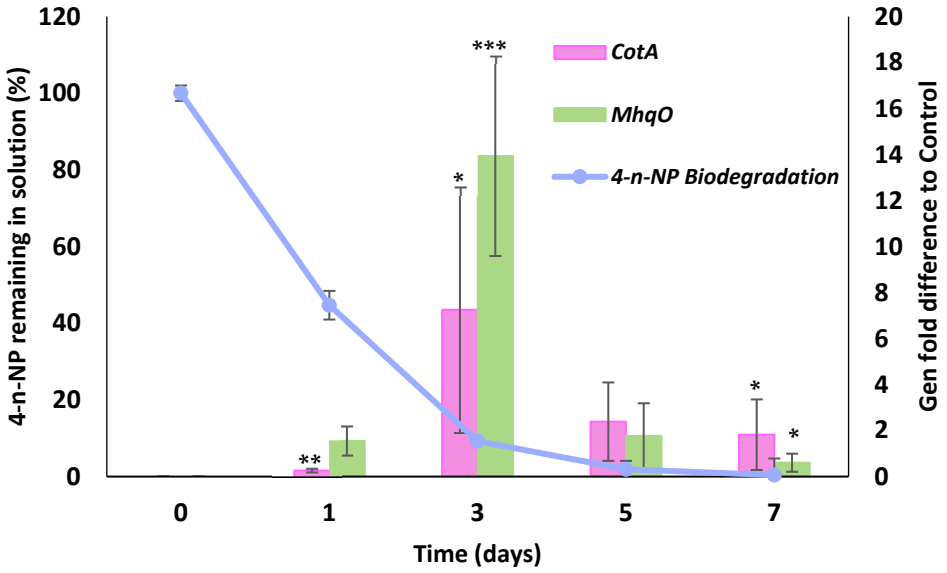

Figure 3. Biodegradation of 4-n-NP in solution *by Bacillus safensis* CN12 in the presence of glucose and the corresponding relative expression of *cotA* and *mhqO* (fold change compared to control). ANOVA: Comparison of *cotA* and *MhqO* gene expression between the control treatment (without 4-n-NP) and the treatment in the presence of 4-n-NP. Expression values are normalized to the 16S rRNA gene. Asterisks indicate statistically significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

The *cotA* gene responds to 4-n-NP presence in *B. safensis* CN12 and encodes a protein that shares similarities to the CotA protein of *B. pumilus* A56. Gene induction corresponding to a transcription increase of ~7.2 times after 3 days of treatment was observed, coinciding with the point of maximum decrease in 4-n-NP concentration (Figure 3). From that moment on, higher expression continues to be observed at 5 and 7 days, but at lower values of ~2.4 and ~1.8 times, respectively, compared to the control (without 4-n-NP).

In the case of the *mhqO* gene, induction by 4-n-NP contact compared to samples in the absence of 4-n-NP was even more evident. Like the *cotA* gene, the highest transcription rate of *mhqO* was observed at 3 days, reaching values ~13.9 times higher compared to the control. Five days after inoculation, gene expression remained above the control (~1.8 times). However, after 7 days, induction was no longer observed. (The level of transcription was ~0.4 lower than in the control.)

The *cotA* gene was reported to code for a protein that belongs to the outer spore coat of *B. pumilus* strain A56. This protein, known as CotA laccase, is a copper-dependent enzyme. It participates in the oxidation of a variety of substrates, including phenolic and non-phenolic compounds [59]. Based on the protein sequence and the transcription studies conducted, we can suggest that in the presence of 4-n-NP, this protein's expression in *B. safensis* CN12 increases, enhancing the immobilization of 4-n-NP and subsequent oxidation on the bacterial cells.

The MhqO enzyme is known to break the aromatic ring in organic compounds. For example, this gene has been predicted to exhibit similarities with *linE*, which encodes chlorohydroquinone/hydroquinone 1,2-dioxygenase found in *S. paucimobilis*. This enzyme is responsible for cleaving substrates resembling hydroquinone [65,66]. Gabriel et al. [67] described a degradation pathway of 4-n-NP where hydroquinone appears as an intermediate transformation product in the presence of *Sphingobium xenophagum*. Later, this route was also observed for *Sphingomonas* sp. strain NP5 [58]. However, the *mhqO* gene has never been specifically described in the degradation of 4-n-NP. There are few studies in the literature on the genes involved in the degradation pathway of 4-n-NP. Therefore, by suggesting a relation between the transcription of *mhqO* and the decay of 4-n-NP concentration in CN12 cultures (Figure 3), these results represent an advancement in the field of genetic research on 4-n-NP bacterial degradation.

In addition to the *cotA* and *mhqO* genes, which have shown a significant correlation with nonylphenol (NP) degradation, other genes, such as *sMO* (single-component monooxygenase), *alkB* (alkane monooxygenase), *mPH* (multi-component phenol hydroxylase), and *C12O* and *C23O* (catechol 1,2- and 2,3-dioxygenases), also play key roles in NP degradation pathways. The involvement of these genes has been reported in various studies. For example, Yang et al. [68] identified the role of *sMO* in the degradation of 4-*n*-NP by *Aspergillus* strains, highlighting its function in initiating the breakdown of the aromatic ring. Similarly, Tuan et al. [69] demonstrated that *Pseudomonas putida* TX2 and *Pseudomonas* sp. TX1 employ *mPH*, *C12O*, and *C23O* to degrade long-chain alkylphenols, including NP, via hydroxylation followed by ring cleavage. Despite these insights, the literature on the genetic basis of NP degradation remains limited. Therefore, adopting a broader genetic perspective in future investigations could provide a more comprehensive understanding of microbial catabolic potential and significantly contribute to the development of more effective bioremediation strategies for NP-contaminated environments.

3.5. 4-n-NP Mineralization by Bacillus safensis CN12

Based on the gene expression results of *mhqO*, there are strong indications that *B. safensis* CN12 could be a 4-n-NP-mineralizing bacterium since this gene encodes an enzyme that breaks the aromatic ring of some compounds known to be TPs of 4-n-NP. To explore the possibility of 4-n-NP mineralization by *B. safensis* CN12, mineralization tests in a solution were conducted, although no mineralization was observed. The main reason to explain this fact could be the lack of specific enzymes for certain intermediate compounds generated. Although a bacterium may possess genes encoding certain enzymes that break the aromatic ring, it may lack other enzymes necessary to fully degrade the resulting intermediate products [70]. Mineralization tests using *B. safensis* CN12 were also carried out when 4-n-NP was previously adsorbed on sewage sludge to observe if the presence of other specific enzymes provided by the endogenous microbial community in sludge could help to obtain its mineralization. Several tests were carried out, and the results are shown in Figure 4. When 4-n-NP was added to sewage sludge without any other treatment or bacterial inoculation (Sludge treatment), only a low mineralization rate of about 7% was observed due to the activity of its endogenous microbial community. However, our

previous studies carried out under the same conditions [36] showed a high percentage of NP degradation (>80%) after 40 days of incubation, but the present study shows that, nevertheless, mineralization does not occur. This indicates that the sludge endogenous microbiota was able to degrade adsorbed 4-n-NP, generating various TPs. However, it was only capable of mineralizing a very small fraction of these TPs into CO_2. It is important to highlight that some of these non-mineralized TPs are even more toxic than the parent compound, thereby increasing the overall toxicity of sewage sludge.

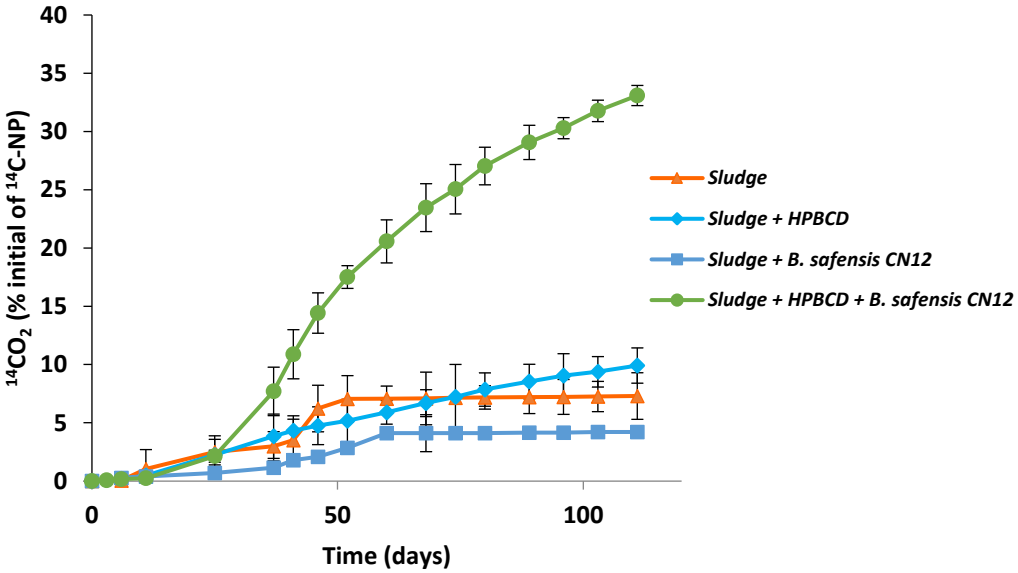

Figure 4. Mineralization of 4-n-NP in sewage sludge under slurry conditions after the application of the different treatments studied.

Inoculation with *B. safensis* CN12 (sludge + *B. safensis* treatment) produced a similar low mineralization rate (4.2%) (Figure 4). It is probably due to the great tendency to remain adsorbed on the hydrophobic surface of sludge organic matter that presents not only 4-n-NP not degraded by the sludge endogenous microbiota (with log Kow 5.76) but also its TPs, which maintain their aromatic moiety due to their high hydrophobic character. To overcome recalcitrant adsorption onto sewage sludge, cyclodextrins have been previously used as availability enhancers for a wide variety of hydrophobic contaminants [71–73], as well as for 4-n-NP [74,75], to improve their extraction from different matrices, facilitating their degradation by the strains selected for each contaminant. Moreover, CDs exhibit minimal adsorption onto environmental matrices, demonstrate very low toxicity to microbial populations, and possess high biodegradability due to their composition of glucose units, which significantly reduces their residues in the environment [37]. The previous study by Lara-Moreno et al. [36] showed that the use of hydroxypropyl-β-cyclodextrin (HPBCD) as an availability enhancer allowed for the extraction of 4-n-NP strongly adsorbed on sewage sludge, facilitating its complete degradation by the endogenous microbiota in only 30 days. But, despite this, its mineralization is very low, as shown in Figure 4 (only 9.9% after almost 4 months, sludge + HPBCD treatment), indicating that only the indigenous microbiota present in sewage sludge was not able to mineralize 4-n-NP even if it was made bioavailable using HPBCD to extract it from sludge. On the contrary, when *B. safensis* CN12 was inoculated in the presence of HPBCD, an increased mineralization rate of >33% was achieved (Figure 4, sludge + HPBCD + *B. safensis* treatment), demonstrating the suitability of HPBCD as an availability enhancer to facilitate not only the degradation of 4-n-NP but also its mineralization, but only if *B. safensis* CN12 is present, and therefore, the capacity of this bacterial strain to not only degrade but also mineralize 4-n-NP is demonstrated.

However, it needs the presence of other specific enzymes provided, in this case, by the indigenous microbiota from sewage sludge. Although *B. safensis* CN12 has genes for certain enzymes that break the aromatic ring, other enzymes provided by the endogenous microbiota of sewage sludge are necessary to fully mineralize 4-n-NP and its TPs.

When microbial consortia are applied, as could be the case with *B. safensis* CN12 and indigenous microorganisms in sewage sludge, the specific functions of each species are not clear. For instance, Bai et al. [76] used a consortium formed by *P. stutzeri* N2 and *R. quingshengii* FF for degrading a mixture of phenolic pollutants from wastewater; their metabolic division labor together with that from indigenous microorganisms in the wastewater resulted in the phenolic pollutants undergoing hydroxylation, decarboxylation, oxidation, and ring cleavage, obtaining a complete degradation in 5 days. Similarly, Wittich et al. [77] isolated three bacterial strains from a municipal WWTP, namely *Pseudomonas citronellolis* RW422, RW423, and RW424, which, as a consortium, were capable of mineralizing ibuprofen. Two of them, RW422 and RW423, were shown to contain the catabolic *ipf* operon responsible for the first steps of ibuprofen mineralization, but they need the addition of the third one, RW424, to the consortia to permit ibuprofen mineralization. The capacity provided by RW424 to reach ibuprofen mineralization remains unclear.

4. Conclusions

This study demonstrated that among the eight bacterial strains isolated from sewage sludge through NP enrichment cultures, *S. putrefaciens* CN17, *A. faecalis* CN8, and *B. safensis* CN12 showed the highest degradation rates of 4-n-NP, with *B. safensis* CN12 achieving complete degradation (100%) and *S. putrefaciens* CN17 and *A. faecalis* CN8 achieving 74% and 75%, respectively. Despite these high degradation rates, the TPs produced during the process, namely 4-(6-oxo-1,1-dimethylheptyl)phenol, 4-nonyl-4-hydroxycyclohexan-2,5-dienone, and 4-nonyl-4-hydroxycyclohex-2-enone, were found to be more toxic than the parent compound. Genetic analysis of *B. safensis* CN12 revealed the presence of key genes, such as multicopper oxidase *cotA* and ring-cleaving dioxygenase *mhqO*, which are involved in the degradation and mineralization of aromatic compounds. These genes were significantly upregulated during 4-n-NP biodegradation, underscoring their essential role in the process.

B. safensis CN12 was able to degrade 4-n-NP in solution, but it was not able to achieve mineralization. However, mineralization was achieved when 4-n-NP was adsorbed onto sewage sludge and treated in the presence of the bioavailability enhancer HPBCD (which facilitated the extraction of 4-n-NP from the sludge), achieving over 33% mineralization. Through mineralization, a large number of the TPs produced were removed from the sewage sludge, thereby reducing its toxicity.

In conclusion, while bioaugmentation strategies offer promise for bioremediation, it is crucial to consider the possible formation of toxic TPs. However, as demonstrated in this study, the interaction between the degrading bacterium and the indigenous microbiota in contaminated environments can lead to a synergistic effect that enhances contaminant mineralization and promotes more effective environmental remediation. Future studies should be carried out to determine the presence of such TPs as well as the toxicity of the final system, since only a few studies have paid attention to the toxicity changes due to their presence resulting from metabolic pathways. To deepen the understanding of the mechanisms underlying the synergistic promotion of NP mineralization by microbial consortia, studies about the effects of bioaugmentation on the indigenous microbial community should be carried out by integrating metagenomic sequencing approaches. This would allow for a detailed characterization of the endogenous microbial community dynamics during the degradation process, providing direct evidence of shifts in microbiota composition and

the potential establishment of functional synergies among microbial groups. Furthermore, such analyses could offer insights into the ecological impacts of the biodegradation process, particularly regarding the observed increase in toxicity, and help assess whether additional post-treatment steps are necessary to mitigate environmental risks.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app15126408/s1, Figure S1. Percentages of 4-n-NP degraded by the isolated bacterial strains after 30 days of incubation in the absence and presence of glucose (1 g L-1); Figure S2. Chemical structures of 4-n-NP and the transformation products (TPs) detected during its degradation in solution by various bacterial strains isolated from sewage sludge; Figure S3. Subsystem category distribution of major protein-coding genes of B. safensis CN12 as annotated by the RAST annotation server. The bar chart shows the subsystem coverage in percentage (the blue bar corresponds to the percentage of proteins included). The pie chart shows the percentage distribution of the 26 most abundant subsystem categories; Table S1. Physico-chemical properties of sludge sample used (mean of four replicates). Standard deviations are in parentheses; Table S2. Optimized MS/MS parameters for Multiple Reaction Monitoring (MRM) analysis for 4-n-NP and transformation products*; Table S3. The relative standard curve method calculates the relative expression of cotA and mhqO in B. safensis CN12.

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