Degradation of antibacterial 1-octylpyrrolidin-2-one by bacterial pairs isolated from river water and soil

Citation

RŮŽIČKA, Jan, Markéta JULINOVÁ, Michal ROUCHAL, Jan SALAČ, Ludmila VAŇHAROVÁ, Jaroslav URBAN, and Kateřina PANČOCHOVÁ. Degradation of antibacterial 1-octylpyrrolidin-2-one by bacterial pairs isolated from river water and soil. *Environmental Science and Pollution Research* [online]. vol. 29, Springer Science and Business Media Deutschland, 2022, p. 45292 - 45302 [cit. 2023-11-09]. ISSN 0944-1344. Available at https://link.springer.com/article/10.1007/s11356-022-19121-1

DOI

https://doi.org/10.1007/s11356-022-19121-1

Permanent link

Degradation of antibacterial 1-octylpyrrolidin-2-one by bacterial pairs isolated from river water and soil

Jan Ruzicka[1], Marketa Julinova[1], Michal Rouchal[2], Jan Salac[1], Ludmila Vanharova[1], Jaroslav Urban[1], Katerina Pancochova[1]

[1]Department of Environmental Protection Engineering, Faculty of Technology, Tomas Bata University in Zlin, Vavreckova 275, 760 01 Zlin, Czech Republic

[2]Department of Chemistry, Faculty of Technology, Tomas Bata University in Zlin, Vavreckova 275, 760 01 Zlin, Czech Republic

Jan Ruzicka ruzickaj@utb.cz, Marketa Julinova julinova@utb.cz, Michal Rouchal rouchal@utb.cz, Jan Salac janek.salac@gmail.com, Ludmila Vanharova l1_vanharova@utb.cz, Jaroslav Urban j aroslavurban.ml @ gmail.com, Katerina Pancochova k_p ancochova @ utb. cz

Abstract

The study of bacterial degradation of 1-octylpyrrolidin-2-one (NOP) by river water and soil bacteria was the main aim of the research. Although the compound demonstrated bacteriostatic as well as bactericidal effects against Gram-positive and certain Gram-negative bacteria at concentrations ranging from 100 to 1000 mg L^{-1}, its concentration of 100 mg L^{-1} was successfully degraded by microbial communities of both river water and alluvial soil; removal efficiencies reached 87.2 and 88.4% of dissolved organic carbon, respectively. Isolation of the strains responsible for the process showed that bacterial degradation was initiated by the octane-utilising bacteria of the genus Phenylobacterium, which used four carbon atoms of the NOP octyl chain and oxidised terminal carbon atom of the remaining chain. The structure of the intermediate produced by phenylobacteria was elucidated following the results obtained from the detailed electrospray mass spectrometry (ESI-MS) analysis; these experiments showed that it is a 4-(2-oxopyrrolidin-1-yl)butanoic acid. This intermediate was further degraded by other bacterial members of appropriate microbial communities, namely Bordetella petrii and Arthrobacter sp. Further tests proved that these bacteria were able to assimilate the nitrogen atom of the lactam ring and thus complete the degradation process.

Keywords: Bacteria, biodegradation, intermediate, Isolation, 1-Octylpyrrolidin-2-one

Introduction

1-Octylpyrrolidin-2-one or 1-octyl-2-pyrrolidone or N-octyl- 2- pyrrolidone (NOP) is a member of the N-alkylpyrroli-din-2-one group, which comprises important industrial compounds applicable to several spheres of human activity. NOP is considered a very weak base, proving to be a useful surfactant due to the chemical bonding of a nonpolar alkyl chain with a hydrophilic pyrrolidin-2-one head; its hydrophilic-lipophilic balance equals 6. It is stable to photolytic degradation in water and soil, and its logKow is 4.15 (**ECHA 2021**). NOP is soluble in other non-ionic surfactants, slightly soluble in water (1.2 g L^{-1}) and possesses both chemical and thermal stability (**Login 1995**). A further advantage is its high

solvency of hydrophobic molecules; hence, it supremely combines surface-active and solvent properties. It is applied as a solvent and wetting agent (**Login 1995**), a chemical intermediate, and a permeation enhancer for transdermal drug delivery (**Rajadhyaksha 1978; Yoneto et al. 1995**), a component of some pesticides (**Narayanan and Chaudhuri 1993; Narayanan et al. 2000**) and a cotton defoliant (**Wagner 2006**).

The antibacterial properties of NOP were partially described by **Kabra (2008)**, who demonstrated that a concentration of 500 mg L^{-1} supplemented with 18 g L^{-1} propylene glycol killed both Gram-negative (Pseudomonas aeruginosa and Escherichia coli) and Gram-positive (Staphylococcus aureus) bacteria in 6 h. Furthermore, according to the harmonised classification and labelling approved by the European Union, NOP is toxic to aquatic life. Its LC50 values for freshwater fish and invertebrates range between 17.8-22 mg L^{-1} and 7.59-12.17 mg L^{-1}, respectively, and the EC50 for freshwater algae is 16.6-19.0 mg L^{-1} (**ECHA 2021**).

Due to its properties and application extent, NOP may enter different environmental spheres, either as component of the several agrochemicals or as a result of improper handling during its storage, transport and actual use. Although it revealed to be biodegradable in screening water test (**ECHA 2021**), there has been no study describing NOP-degrading microorganisms from various environmental spheres so far. Hence, this study set out to obtain such bacterial members from two main environmental spheres, river water and alluvial soil and to describe their roles in the compound degradation.

Materials and methods

Chemicals and biological materials

NOP, pyrrolidin-2-one (PYR), A-methylpyrrolidin-2-one (NMP), A-ethylpyrrolidin-2-one (NEP) and all alkanes were purchased from Sigma-Aldrich, A-butylpyrrolidin-2-one (NBP) from TCI Chemicals and other chemicals from Penta, Czech Republic.

Bacterial strains from the Czech Collection of Microorganisms in Brno (CCM) were used to determine the minimum inhibitory concentrations (MIC) and the minimum bactericidal concentrations (MBC): Staphylococcus aureus CCM 3953, Enterococcus faecalis CCM 4224, Bacillus subtilis CCM 2216, Rhodococcus erythropolis CCM 7451, Escherichia coli CCM 3954, Klebsiella pneumoniae CCM 4415, Pseudomonas fluorescens CCM 4796 and Pseudomonas aeruginosa CCM 3955.

The sample of river water was taken from the River Drevnice, a medium-sized watercourse representing a common type of river in the Czech Republic. The sampling point was not affected by the effluent of a wastewater treatment plant. pH, dissolved organic carbon content and heterotrophic count of the river water were 8.2 ± 0.2, 4.7 mg L^{-1} and 8.8 X 10^5 CFU mL^{-1} (R2A agar, 25 °C, 3 weeks), respectively.

The sample of subsurface soil was taken from an alluvial meadow near the village of Hlubocky, Czech Republic; the site was not affected by industrial activity or intensive agriculture. The soil is light loam type, and its pH was 7.1 ± 0.2; the depth of the sampling was 15 cm. A 5-g portion of the soil was suspended in 100 mL of sterile mineral medium (see below) and shaken in a laboratory shaker for 15 min at 100 rpm; subsequently, coarse particles were separated by sedimentation for 30 s, and soil microbial suspension was taken from the surface layer of the mixture.

Growth media and nutrient agars

Tests for working out the MIC and MBC were performed in Tryptone soya broth (TSB, in g L^{-1}) as follows: tryptone 17, soya peptone 3, glucose 5, NaCl 5 and K_2HPO_4 2.

The compositions of the mineral medium (MM), nitrogen-free mineral medium (NFMM) and mineral agar (MA) were described previously (**Merkova et al. 2018**).

The nutrient agars employed throughout the study were Tryptone yeast extract agar (TYA) and R2A agar (Himedia). MEM vitamin solution was purchased from Biosera.

MIC and MBC determination of NOP

Ten portions of TSB were prepared, sterilised and amended with sterile NOP to reach later test concentrations of 0, 20, 50, 100, 175, 250, 375, 500, 750 and 1000 mg L^{-1}. A TSB portion of 180 pL combined with NOP was pipetted into three microplate wells; 20 µL of strain suspension in saline solution (2nd degree on the McFarland scale) was used as the inoculum. Blank tests with 20 µL of sterile saline solution were performed in parallel. After incubation at 37 °C for 48 h, the plates were visually checked and measured by a microplate reader (TECAN Sunrise) at 600 nm. The MIC was considered to be the lowest NOP concentration at which growth was fully inhibited in all replications, and no increase in optical density occurred. Each well that lacked any sign of strain growth was thoroughly mixed with a sterile loop and inoculated onto a fresh TYA plate. Following incubation at 37 °C for 3 days, the lowest NOP concentration with no exhibited strain growth was considered to be the minimum bactericidal concentration.

MIC and MBC values for the strains isolated in this study were determined in the same manner, but incubation at 30 °C for 5 days was applied.

NOP degradation by river and soil microorganisms

The NOP was applied at a concentration of 100 mg L^{-1}. A river water sample of 100 mL was amended with 0.5 mL of sterile MM and NOP. Incubation was performed in darkness at 25 °C on a rotary shaker for up to 3 weeks. The process was monitored by the use of dissolved organic carbon (DOC) determinations after cell removal by sterile filtration through a 0.2-µm ReliaPrep™ filter (Ahlstrom); an automatic carbon analyser (Shimadzu TOC-L) was used for carrying out such determinations. Concurrently, the same river water sample with MM (without NOP) was monitored for DOC level at the same periods of time; the obtained values were then subtracted. In addition, a blank test with a cell-free water sample was conducted in parallel; the cells were removed by the same sterile filtration.

In the test with soil microorganisms, 100 mL of MM was amended with NOP and inoculated with 2 mL of soil microbial suspension. Incubation and process monitoring were performed in the same manner as in the river water test. Both degradation tests were carried out in triplicate.

Isolation of bacterial strains

The bacterial strains were isolated from the final suspensions sampled at the end of the NOP degradation tests. At first, the parallel tests' suspensions were mixed, and the final river and soil suspensions (1 mL each) were individually enriched in 100 mL of fresh sterile mineral medium

amended with sterile NOP (100 mg L^{-1}) for 3 weeks at 25 °C on a rotary shaker in darkness. Afterwards, the isolation was performed according to the procedure given in **Merkova et al.** (**2018**) using R2A agar, MA and MA amended with NOP (100 mg L^{-1}).

Degradation tests using single isolates and their pairs

Tests to investigate NOP degradation were carried out in 500-mL bottles containing 120 mL of sterile MM amended with NOP at the concentration of 100 mg L^{-1} and MEM vitamins (120 µL). After inoculation with individual isolates (120 µL of suspension in saline solution; 1st degree on the McFarland scale) or binary consortiums, all the test bottles were incubated in the dark at 25 °C on a rotary shaker for 3 weeks. Substrate degradation was monitored by DOC determinations as mentioned above and the bacterial growth by optical density at 600 nm measurements (OD_{600}) using the TECAN Sunrise spectrophotometer, after pipetting a 200-µL sample into a microtitration well (in quadruple). Tests of NOP degradation in nitrogen-free mineral medium were performed in the same manner as mentioned above, with the use of NFMM medium instead of MM.

Moreover, the utilisation of octane, pyrrolidin-2-one, NMP, NEP and NBP by single isolates was tested in 100-mL bottles containing 20 mL of sterile MM and MEM vitamins, at the substrate concentrations of 50 and 100 mg L^{-1}. Bacterial growth was monitored by OD_{600} measurements; when OD_{600} values were gauged at approximately 0.02 to 0.06 or higher during the incubation, they were considered positive results. In some cases, DOC determinations at the beginning and the end of the tests were done as well. The growth of all isolates in the form of pure strain was confirmed by the test in which acetate and glycerol (100 mg L^{-1} each) were used as carbon and energy sources. In all cases, the purity and cell viability check was done after incubation by inoculation of the final bacterial suspension on R2A agar plates and subsequent incubation at 25 °C. All these tests were done in triplicates.

Identification of key bacteria and their description

All the key isolates were Gram-stained and identified by 16S rDNA sequence analysis. The DNAs of pure strains were isolated by the DNA Power Soil KIT (QIAGEN). PCR amplification of partial 16S rDNA was performed via a standard method employing the universal primers fD1 and rD1 (**Weisburg et al. 1991**). Each PCR reaction contained 10 μL of GoTaq Green Hot Start Master Mix (Promega), 1 μL of each primer solution (12.5 pmol), 7 μL of water for molecular biology and 1 μL of bacterial DNA. All the amplifications were carried out on a Piko Thermal Cycler (Finnzymes) with the following thermal profile: (a) initial denaturation at 95 °C for 3 min; (b) 10 cycles of Touch Down PCR: 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 90 s, followed by a 0.5 °C decrease of the annealing temperature for each cycle; (c) 25 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 90 s; and (d) final extension at 72 °C for 10 min. The amount of the amplified products was confirmed by agarose gel (2%) electrophoresis; the amplified products were then purified with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) and sent for sequencing (SEQme, Czech Rep.). Sequences obtained were compared by carrying out BLAST searches in the NCBI database (**http://www.ncbi.nlm.nih.gov/**).

Additionally, utilisations of hexane, decane, dodecane, tetradecane, hexadecane, toluene and phenol by certain key isolates were tested: 20 mL of MM was individually amended with sterile substrate (50 or 100 mg L^{-1}), and the bacterial growth was monitored by OD600 measurement.

To determine the maximum level of NOP concentration degraded by the isolates or pairs, NOP concentrations of 100 to 250 mg L^{-1} in MM were used (increment 25 mg L^{-1}); bacterial multiplication was detected by OD_{600} measurements.

Identification of intermediate produced by primary degraders

Sterile MM amended with NOP (100 mg L^{-1}) was inoculated with a single strain of presumable primary degrader and incubated in the dark at 25 °C for 1 week. Afterwards, the cells were removed by sterile filtration, as mentioned above, and the filtrate was used for mass spectrometry analysis. Electrospray mass spectra (ESI-MS) were recorded using an amazon X ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionisation source. All the experiments were conducted in both positive and negative-ion polarity mode. Individual samples (with a concentration of 0.5 μg mL^{-1}) were infused into the ESI source as methanol/water (1/1, v/v) solutions via a syringe pump with a constant flow rate of 3 μL min^{-1}. The other instrumental conditions were electrospray voltage of ± 4.2 kV, capillary exit voltage ± 140 V, drying gas temperature of 220 °C, drying gas flow of 6.0 dm^3 min^{-1} and nebuliser pressure of 55.16 kPa. Nitrogen was used as both nebulising and drying gas for all the experiments. Tandem mass spectra were collected using collision-induced dissociation (CID) with He as the collision gas after the isolation of the required ions.

Degradation of intermediate produced by primary degraders by isolated bacteria

The same filtrates as those used for the mass spectrometry analysis were applied for the assays of the intermediate degradation; a portion of 70 mL of the sterile filtrate was inoculated either by single isolate or by bacterial pair and incubated at 25 °C; the process was monitored through DOC determinations and OD_{600} measurement as mentioned above.

Results

Antibacterial effect of NOP on Gram-positive and Gram-negative bacteria

For all tests, 1000 mg L^{-1} of NOP was chosen as the highest concentration due to the limited solubility of NOP in water. The results of both MICs and MBCs for all test strains are given in **Table 1**.

The Gram-positive bacteria revealed considerably different sensitivities to NOP since G + rods were inhibited by an NOP concentration of 100 mg L^{-1}, while the growth of G+cocci ceased at 500 mg L^{-1} of NOP. The same tendency was found for the bactericidal effect of NOP, as its MBC figures for cocci were substantially higher than those for R. erythropolis and B. subtilis.

Concerning Gram-negative strains, MIC for two members of the Enterobacteriaceae family achieved values within the range found for Gram-positive bacteria; surprisingly, Kl. pneumoniae was revealed to be more sensitive than E. coli. Furthermore, NOP was unable to hinder both Pseudomonas species from growing. Generally, regarding the efficiency of NOP against bacteria, its impact appeared to be more species-dependent than fundamentally reliant on the structure of the cell wall.

Table 1 Figures for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of NOP

Test strain	MIC (mg L^{-1})	MBC (mg L^{-1})
Staphylococcus aureus CCM 3953	500	1000
Enterococcus faecalis CCM 4224	500	750
Rhodococcus erythropolis CCM 7451	100	100
Bacillus subtilis CCM 2216	100	100
Escherichia coli CCM 3954	375	500
Klebsiella pneumoniae CCM 4415	175	175
Pseudomonas aeruginosa CCM 3955	> 1000	> 1000
Pseudomonas fluorescens CCM 4796	> 1000	> 1000

NOP degradation by river water and soil microorganisms

The general consumption of 100 mg L^{-1} NOP by microorganisms of river water and alluvial soil was investigated, and the results of both tests are given in **Fig. 1**.

The data obtained showed that relatively fast degradations of NOP occurred, especially in the case of the river water test. Here, only 2 days of lag phase were sufficient for the beginning of NOP degradation, which occurred in the next 10 days. A slightly slower process was recorded in the soil suspension test, which required 16 days for substantial removal of the compound. Removal efficiencies reached 87.2 and 88.4% of DOC for water and soil tests, respectively. These reductions were accompanied by considerable microbial growth during both tests, proving that NOP and its soluble intermediates served as a carbon and energy source for certain species of river and soil bacteria; the involvement of other microbial groups, such as yeasts or microscopic fungi, was excluded by microscopic observation of several samples of each end suspension. Regarding the blank test, a comparison of DOC values at the beginning of the tests and on days 12 and 21 displayed quite negligible changes during incubation, proving no NOP sorption on cultivation bottles.

Obtaining bacterial isolates and determination of key strains

Microbial suspensions originating from the NOP degradation tests were used as the source of microorganisms. Further enrichment of each suspension on NOP was initially performed, followed by the isolation of bacterial members on three types of nutrient agars. After appropriate incubation of all plates, R2A agar showed good growth of several well-distinguished bacterial colonies, while NOP agar revealed smaller numbers of poorly differentiated colonies. All the bacterial types were re-inoculated on fresh R2A agars and subsequently examined for their basal features. A thorough comparison of all obtained bacteria showed that the isolates originated from NOP agars were identical to those primarily grown on R2A agar. Ultimately, five different river water bacterial isolates (designated as R1-R5) and seven soil suspension isolates (designated as S1- S7) were acquired.

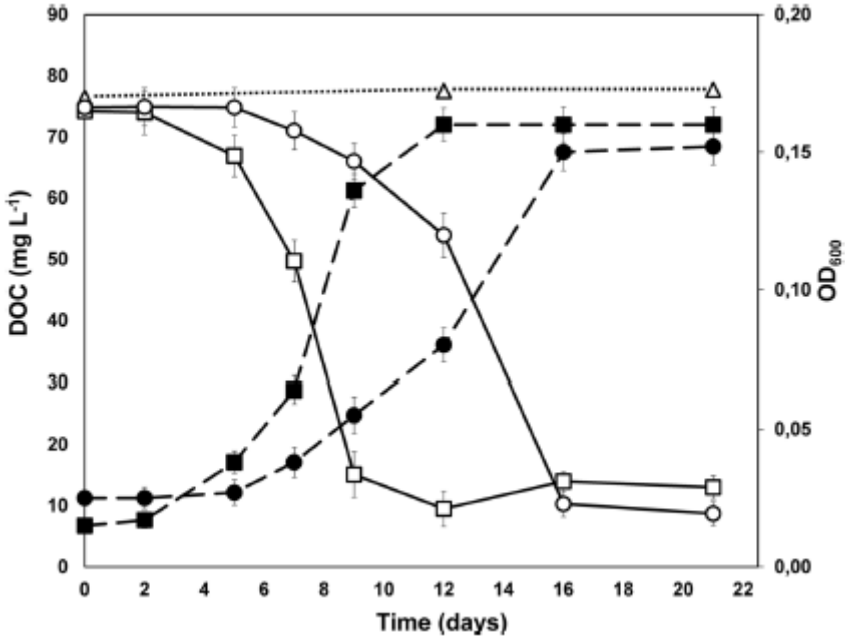

Fig. 1 The course of NOP degradation in river water and soil suspension; white symbols: DOC; black symbols: OD_{600}; squares: river water; circles: soil suspension; triangles: non-inoc-ulated blank; data are presented as mean and error bars denote standard deviations (for n = 3)

Afterwards, the capability of all isolates to use NOP as the only carbon and energy source was examined, including the determination of the DOC decrease during incubation. In addition, the growth of all bacteria on octane, pyrrolidin-2-one, NMP, NEP and NBP was monitored, thereby testing the capacity to utilise individual structural parts of the NOP molecule or several pyrrolidin-2-ones containing short alkyl chain.

The results, given in Online Resource, **Table SI1**, showed that only one isolate of each series was able to grow on NOP individually (R2 and S4). Additionally, the growth of these two bacteria was relatively poor, leading to a very limited decrease of organic carbon during the incubation, not exceeding 25% of the initial DOC values. However, as these isolates were capable of utilising octane, they were tentatively considered primary degraders of NOP that used a certain part of the NOP's octyl chain for their growth.

Furthermore, the results showed that none of the isolates was capable of using pyrrolidin-2-one, NMP, NEP and NBP as a carbon and energy source. These findings could indicate that the biodegradation of the pyrrolidin-2-one ring of NOP might be dependent on the action of two different isolates.

Hence, two new series of NOP degradation tests were subsequently conducted, in which assumed primary degraders were supplemented with other single isolates from the same sample. After these assays were conducted, it was found that only binary consortiums R2 + R1 and S4 + S3 were capable of noticeable growth on NOP associated with the significant removal of organic carbon. The results of both above-mentioned consortiums, along with the results of the growth of single primary degraders on NOP, are given in **Fig. 2** (data of all other binary consortiums not shown).

Furthermore, the bacterial pairs R2 + R1 and S4 + S3 were tested for their growth on pyrrolidin-2-one, NMP, NEP and NBP, as well as their growth on elevated concentrations of NOP. The degradation of NOP by primary degraders (R2 and S4) and by both consortiums in the absence of an external nitrogen

source was also studied. All the results are summarised in **Table 2**, including MIC and MBC figures of NOP for all isolates, and the course of NOP degradation in nitrogen-free media is given in **Fig. 3**.

Despite only using a culture-dependent method for clarifying NOP-degrading bacteria, the results given in **Fig. 2** proved that obtained isolates were responsible for the compound degradation, as the NOP-degradation curves in consortiums R2 + R1 and S4 + S3 were very similar to the curves given in **Fig. 1**. Furthermore, the activity of two different strains was confirmed as a crucial requirement for substantial NOP degradation for water and soil bacteria.

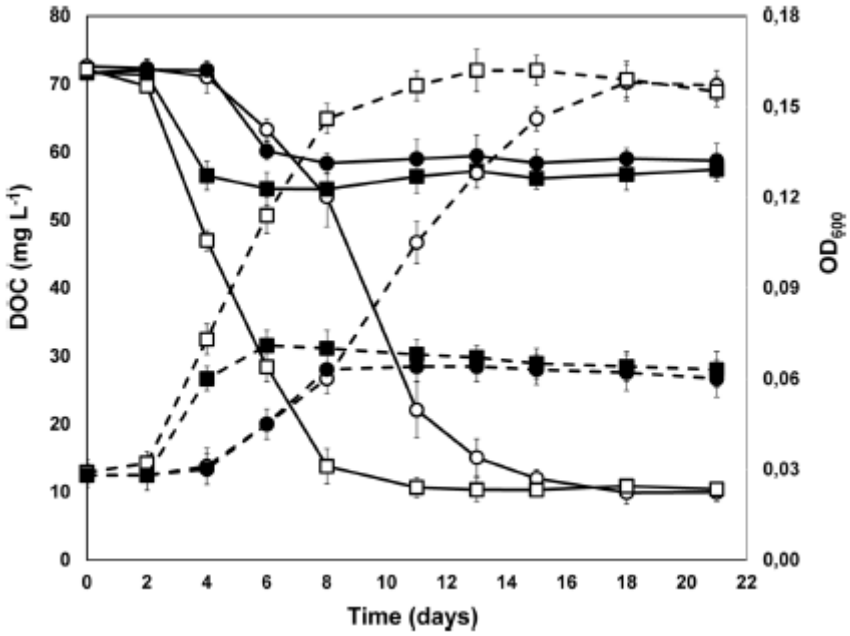

Fig. 2 The courses of NOP degradation by binary consortiums and primary degraders; full lines: DOC; dashed lines: OD600; white squares: R2 + R1; white circles: S4 + S3; black squares: isolate R2; black circles: isolate S4; data are presented as mean, and error bars denote standard deviations (for n = 3)

Table 2 Further properties of the individual strains and appropriate pairs

Strain or pair	Utilisation of				Max. NOP[a] (mg L^{-1})	MIC of NOP (mg L^{-1})	MBC of NOP (mg L^{-1})	Growth on NOP in NFMM[b]
	PYR	NMP	NEP	NBP				
R2	-	-	-	-	175	250	250	-
S4	-	-	-	-	175	250	250	-
R1	-	-	-	-	N	750	750	N
S3	-	-	-	-	N	250	250	N
R1+R2	-	-	-	-	175	N	N	+
S3+S4	-	-	-	-	175	N	N	+

(+) growth;

(—) no sign of growth (N) not tested

[a]Maximal growth concentration of NOP

[b]Growth on NOP in nitrogen-free mineral medium

In both cases, DOC removals reached approximately 86% of the beginning values during the assays with the consortiums, while individual isolates R2 and S4 ensured only 23.9% and 18.6% decreases of organic carbon, respectively.

Interestingly, despite NOP degradation by both consortiums, neither of them was capable of using pyrrolidin-2-one or its derivatives containing a short alkyl chain. Furthermore, as shown in **Fig. 3**, the R2 and S4 isolates were revealed to be fully unable to use NOP in the absence of an external nitrogen source, which demonstrated their inability to assimilate the nitrogen atom of NOP during primary degradation; this outcome supported the primal assumption that they only utilised certain carbons of NOP's octyl chain. Thus, all the above-mentioned findings might suggest that the partial degradation of NOP by R2 and S4 isolates could be crucial not only for their growth in complete mineral medium but also for making the remaining part of NOP accessible for the further degradation.

Therefore, the identification of the intermediate produced by R2 and S4 isolates after their growth on NOP was identified as a key step towards the elucidation of the course of bacterial NOP degradation. Moreover, the participation of obtained isolates in further biodegradation of the unknown intermediate was studied in the following test series: the samples containing the cell-free product of NOP degradation by primary degraders (R2, S4) were inoculated either by pairs R1 + R2 and S3 + S4 or by single isolates R1 and S3, respectively. The results of such assays, expressed as percentage values of DOC concentrations during the incubation, are given in **Table 3**.

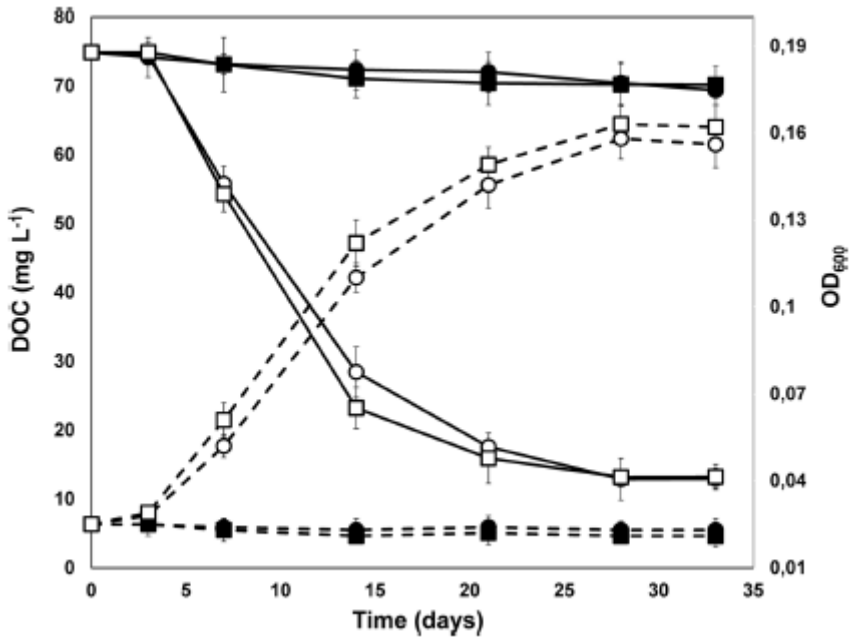

Fig. 3 The courses of NOP degradation by binary consortiums and primary degraders in nitrogen-free media; full lines: DOC; dashed lines: OD600; white squares: R2 + R1; white circles: S4 + S3; black squares: isolate R2; black circles: isolate S4; data are presented as mean and error bars denote standard deviations (for n = 3)

Table 3 DOC changes during the growth of single isolates and pairs on the intermediate

Incubation time (days)	DOC (%)			
	R1	R1+R2	S3	S3+S4
0	100	100	100	100
2	87.3±1.8	88.4±1.6	84.4±2.0	83.8±1.9
7	46.9±1.3	44.2±1.5	38.1±1.7	38.8±0.9
14	10.6±0.8	10.9±0.9	11.4±0.7	12.9±0.8

Obtained results showed that single bacteria R1 and S3 achieved practically the same results as appropriate pairs R1 + R2 and S3 + S4, respectively. Thus, it was evident that further degradation of the intermediate entirely ensured isolates R1 and S3, without any contribution from primary degraders. Additionally, DOC decreases of nearly 90% were recorded at the end of these tests, indicating complete or nearly complete mineralisation of the compound. The similar finding emerged from the assays of NOP degradation in nitrogen-free media, in which approximately 83% removals of organic carbon took place under the presence of isolates R1 and S3 together with primary degraders (**Fig. 3**).

Identification of intermediate produced by primary degraders

For the elucidation of the chemical structure of the intermediate produced by isolates R2 or S4, mass spectrometry was used. The analysis of commercially available NOP was initially performed. In the first-order mass spectra obtained in the positive-ion polarity mode, three singly charged ions at m/z 198, 220 and 236, respectively, were observed. These ions were assigned as protonated molecule (m/z 198), sodium adduct (m/z 220) and potassium (m/z 236) adduct of the molecule (Online Resource, **Fig. SI1**). Fragmentation of $[M + H^+]^+$ ion (m/z 198) under the CID conditions led to the formation of a singly charged ion at m/z 86 which was assigned as protonated pyrrolidin-2-one. This means that the neutral loss of the aliphatic chain occurred.

Afterwards, a series of ESI-MS analyses of cell-free samples obtained after the growth of isolates R2 or S4 on NOP was performed. In the first-order positive-ion mass spectra of all analysed samples, five singly charged signals (**Fig. 4A**) were observed. The ion at m/z 172 was assigned as a protonated molecule ($[M + H^+]^+$), while ions at m/z 194 and m/z 210 were referred to as sodium ($[M + Na^+]^+$) and potassium ($[M + K^+]^+$) adducts of the molecule, respectively. These signals were accompanied by a signal that was approximately twice as high (m/z 365), as determined by the sodium adduct of the dimer ($[2\text{-}M + Na^+]^+$). The structure of the last signal observed in + MS spectra (m/z 154) was assigned according to the tandem mass spektrometry experiment $[M + H^+]^+$ ion (m/z 172) of providing only one product ion at m/z 154 (**Fig. 4B**). It is assumed that a neutral loss of water molecule from $[M + H^+]^+$ ion occurred, so the structure was assigned as $[M + H^+ - H_2O]^+$. Since the ion at m/z 154 originates from a protonated molecule, its presence in the first-order mass spectra can be explained as a result of in-source fragmentation (**Klasek et al. 2020**). Furthermore, we studied the gas-phase behaviour of $[M + H^+]^+$ ion in more detail using the MS^3 experiment $[M + H^+ - H_2O]^+$ ion (**Fig. 4C**). In this case, neutral loss of carbon monoxide (28 m/z) led to the formation of the most abundant ion at m/z 126, which was accompanied by two other signals originating from the latter ion via neutral loss of ethene (m/z 98) and propyne (m/z 86).

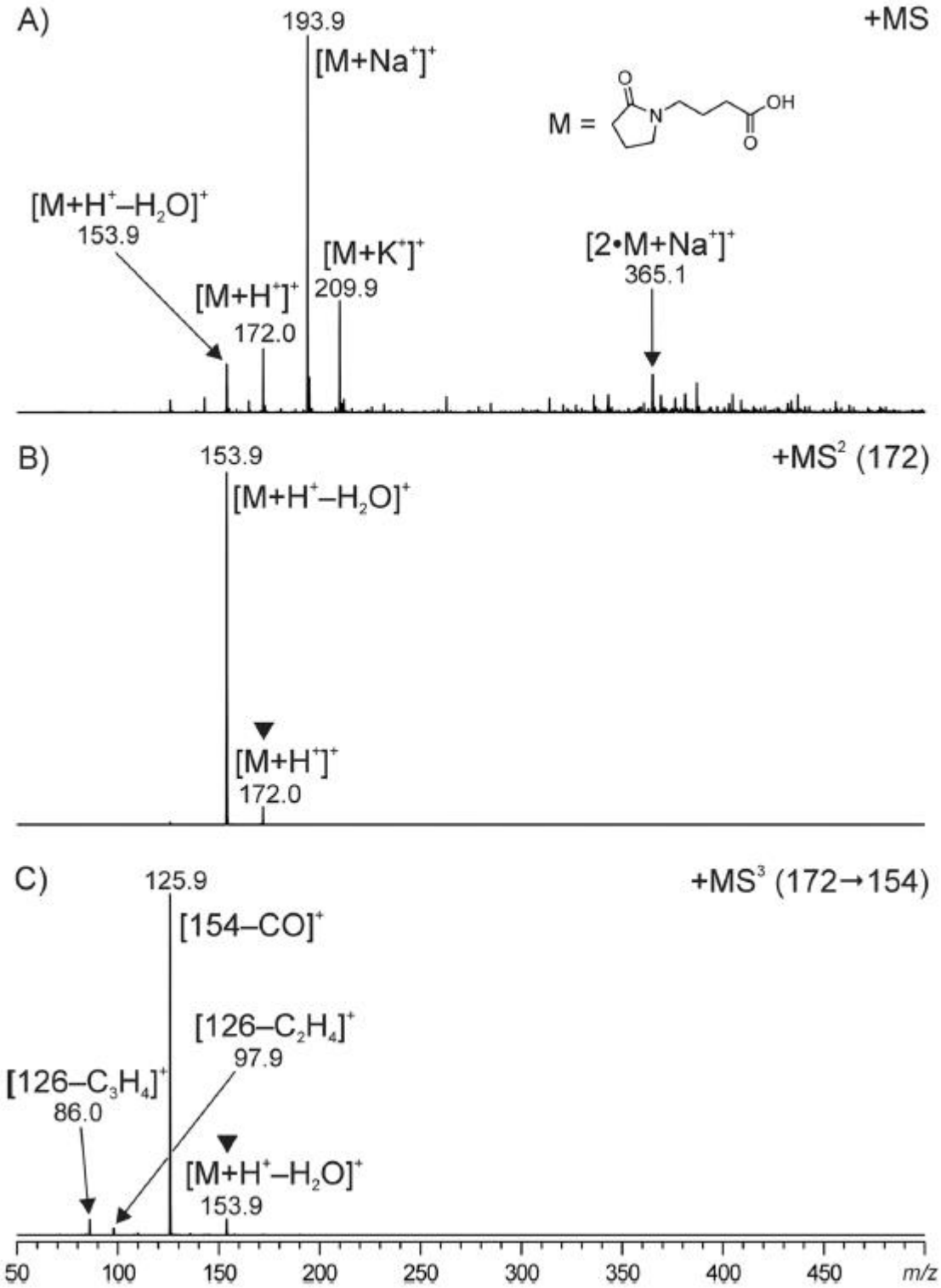

Fig. 4 The positive-ion ESI mass spectra of the product of NOP degradation by isolates R2 or S4 A first-order mass spectra, **B** MS^2, **C** MS^3; the assignments for observed signals are shown in brackets; the fragmented ion in tandem mass spectra is marked with a black downwardfacing triangle

In the negative-ion first-order mass spectra, we observed a signal with m/z 170, which we assigned as a deprotonated molecule. Fragmentation of this ion led to the formation of only one product ion at

m/z 84 originating via a simultaneous neutral loss of carbon dioxide and propene (for spectra, see Online Resource, **Fig. SI2**).

Based on the detailed mass spectrometry analysis, it may be supposed that there is only one product that originated from the degradation of NOP by primary degraders R2 or S4, with the exact molecular mass of m/z 171 and one nitrogen atom in the structure. In the positive-ion tandem mass spectra (MS^2 and MS^3), we observed consecutive neutral losses of water and carbon monoxide, respectively, which are typical for carboxylic acids (**Holcapek et al. 2010**). Moreover, the formation of product ion originating from a neutral loss of carbon dioxide during the fragmentation of $[M - H^+]^-$ ion ($[M - H^+ - CO_2]^-$) was not observed; thus, it can be assumed that the carboxylic acid has an aliphatic chain (**Levsen et al. 2007**). Based on a detailed tandem mass spectrometry analysis, it can be presumed that the product of NOP degradation by isolates R2 or S4 is aliphatic carboxylic acid, namely 4-(2-oxopyrrolidin-1-yl)butanoic acid (**Fig. 4A**); this is formed as a result of partial NOP degradation and concurrent oxidation of terminal carbon atom of the remaining chain.

Identification of the key isolates

Isolates R1, R2, S3 and S4 were identified by 16S rDNA sequence analysis and their base properties were determined. Furthermore, isolates R2 and S4 were tested for their capacity to utilise some aliphatic and aromatic hydrocarbons. The most significant results are given in **Table 4** and some additional information is provided in Online Resource, **Table SI2**.

Identification of primary degraders R2 and S4 as Pheny-lobacterium members indicated the crucial significance of the genus for NOP degradation both in river and soil ecosystems. Furthermore, the strains demonstrated the same capacity to utilise octane and some other medium-sized alkanes and the inability to grow on two common aromatic hydrocarbons (Online Resource, **Table SI2**). However, they slightly differed in their taxonomic position; while strain R2 was identified as Ph. koreense, the closest relative of strain S4 was Ph. haematophilum, with a similarity below 99%. Strain R2 displayed slightly faster growth on NOP than strain S4 (see **Fig. 2**). Concerning the bacteria finishing NOP degradation, strain R1 was indubitably identified as B. petrii, as it was well distinguished from related species; however, in the case of strain S3, it was impossible to properly determine one of the two closely related Arthrobacter species.

Table 4 Identification of key isolates

Isolate	Source	Sequence length (bp)	Identification	Similarity to type strain(s) (%)
R1	River	1058	*Bordetella petrii*	99.43
R2	River	1053	*Phenylobacterium koreense*	99.04
S3	Soil	1267	*Arthrobacter humicola* *Arthrobacter oryzae*	99.84 99.76
S4	Soil	1147	*Phenylobacterium* sp.	98.69[a]

[a]*See further in the text*

Ultimately, the four strains were deposited in the Czech Collection of Microorganisms (CCM, Masaryk University, Brno) under the accession numbers CCM 9070 (Bordetella petrii), CCM 9072 (Phenylobacterium koreense), CCM 9071 (Arthrobacter sp.) and CCM 9073 (Phenylobacterium sp.). Their partial 16S rDNA sequences were deposited in Gen-Bank, with accession numbers MW279309, MW279310, MW279311 and MW279312, respectively.

Discussion

NOP demonstrated bacteriostatic and bactericidal effects on test species of Gram-positive and Gram-negative bacteria except for pseudomonads. The comparison of the concentration range of antibacterial properties of NOP (100 - 1000 mg L^{-1}) and its both maximum growth concentration (175 mg L^{-1}) and MIC values for primary degraders (250 mg L^{-1}) does not indicate any special resistance of the strains against the compound. Only B. petrii R1 revealed significantly higher tolerance towards NOP. Thus, the capability of octane-utilising phenylobacteria to recognise the octyl chain of NOP seems to be a crucial agent for bacterial NOP degradation under aerobic condition. As detailed tandem mass spectrometry analysis showed that 4-(2-oxopyrrolidin-1-yl)butanoic acid is the intermediate released by primary degraders after their growth on NOP, it is evident that the obtained phenylobacteria use only four carbon atoms of NOP. This explains their poor growth on NOP and weak reductions in dissolved organic carbon depicted in Fig. 2. However, besides shortening the alkyl chain of NOP, the phenylobacteria oxidised the terminal carbon atom of the remaining part of the molecule, forming a carboxylic acid functional group. Such oxidation fully corresponds with the course of standard oxidation of medium- and long-chained alkanes, by either terminal or subterminal pathway (**Binazadeh et al. 2009**). This oxidation is quite essential for the degradation of the intermediate by B. petrii R1 and Arthrobacter sp. S3, as they are unable to use non-oxidised N-butylpyrro-lidine-2-one or other derivatives of pyrrolidine-2-one containing short alkyl chain, not even in the consortiums with appropriate primary degrader (**Table 2** and Online Resource, **Table SI1**). On the other hand, the inability of R1 and S3 strains to utilise pyrrolidine-2-one shows that the presence of certain hydrophilic chain bound to the pyrrolidine-2-one head is a crucial requirement for these strains to use the lactam ring. Considering the high levels of NOP mineralisation by both consortiums under nitrogen-rich and even nitrogen-free condition (**Figs. 2** and **3**) and high levels of the intermediate degradation by B. petrii R1 or Arthrobacter sp. S3 (**Table 3**), there is no doubt that these two strains are crucial for the assimilation of the nitrogen atom involved in NOP.

As indicated above, both primary degraders demonstrated the same effect on the NOP molecule. This was fundamentally proved by the discovery of the same intermediate after NOP degradation by these strains. In addition, two collateral assays showed that NOP degradation by river primary degrader could be completed by soil strain (i.e. R2 + S3) and vice versa (i.e. S4+R1) (data not shown). Thus, based on all the above-mentioned results and suggestions, the graphical scheme of bacterial NOP degradation and the role of individual strains may be depicted in Online Resource, **Fig. SI3**.

The key role of phenylobacteria for NOP biodegradation may be connected to their previously ascertained properties. The genus was described as chloridazon degrading soil microbe in 1985 (**Lingens et al. 1985**); further studies suggested its contribution to hydrocarbon utilisation (**Yang et al. 2014**), including polycyclic aromatic hydrocarbons (**Singleton et al. 2016**). Concerning bacteria finishing the degradation of NOP, B. petrii was firstly isolated from river sediment (von **Wintzingerode et al. 2001**) and several later works proved its capabilities to degrade various synthetic compounds such as poly(L-lactide) (**Kim and Park 2010**) or endosulfan (**Odukkathil and Vasudevan 2015**). Many members of the genus Arthrobacter can also degrade a vast array of natural organic compounds and

xenobiotics (Guo et al. 2019), including nitrogenous heterocyclic agents such as nicotine (**Fitzpatrick 2018**) or pyridine (**Khasaeva et al. 2011**). As typical soil microbes, they belong to several promising bacterial genera applicable for bioremediation of pesticide-polluted soils (**Cycon et al. 2017**).

Generally, there are perhaps hundreds of known degradation processes in which natural or synthetic organic compounds are degraded by consecutive action of two or more different bacteria. In the case of NOP, the primary degradation leads to partial degradation of the alkyl chain of the compound and, concurrently, to significant oxidation of the terminal carbon of the non-utilised moiety of the molecule. Hence, these structural changes executed by phenylobacteria fundamentally determine which bacterial species may continue subsequent degradation and terminate the compound mineralization. It is the description of degradation pathway of the found intermediate, 4-(2-oxopyrrolidin-1-yl)butanoic acid, that may mean an appeal for future research.

Conclusion

The results of this study prove that microbial aerobic degradation of NOP is ensured by bacterial pairs, in which water and soil members of genus Phenylobacterium use four carbon atoms of octyl chain for their growth. Such degradation is accompanied by the formation of a carboxylic group at the top of the shortened chain, which makes the produced intermediate available to other bacterial species present in an appropriate microbial ecosystem. Thus, the bacterial pairs are capable of accomplishing fast and virtually complete mineralisation of the compound, unless its concentration does not exceed the toxic level for the degraders.

References

Binazadeh M, Karimi IA, Li Z (2009) Fast biodegradation of long chain n-alkanes and crude oil at high concentrations with Rhodo-coccus sp. Moj-3449. Enzyme Microb Tech 45:195-202. **https://doi.org/10.1016/j.enzmictec.2009.06.001**

Cycon M, Mrozik A, Piotrowska-Seget Z (2017) Bioaugmentation as a strategy for the remediation of pesticide-polluted soil: A review. Chemosphere 172:52-71. **https://doi.org/10.1016/jxhemosphere.2016.12.129**

ECHA (2021)**https://echa.europa.eu/cs/brief-profile/-/briefprofile/100.100.541** Accessed 22 December 2021

Fitzpatrick FP (2018) The enzymes of microbial nicotine metabolism. Beilstein J Org Chem 14:2295-2307. **https://doi.org/10.3762/bjoc. 14.204**

Guo X, Xie C, Wang L, Li Q, Wang Y (2019) Biodegradation of persistent environmental pollutants by Arthrobacter sp. Environ Sci Pol-lut R 26:8429-8443. **https://doi.org/10.1007/s11356-019-04358-0**

Holcapek M, Jirasko R, Lisa M (2010) Basic rules for the interpretation of atmospheric pressure ionization mass spectra of small molecules. J Chromatogr A 1217:3908-3921. **https://doi.org/10.1016/j.chroma.2010.02.049**

Kabra BP (2008) 2-pyrrolidone derivatives for preservation of ophthalmic, otic and nasal compositions. US Patent application publication No. 2008/0095863 A1

Khasaeva F, Vasilyuk N, Terentyev P, Troshina M, Lebedev AT (2011) A novel soil bacterial strain degrading pyridines. Environ Chem Lett 9:439-445. **https://doi.org/10.1007/s10311-010-0299-6**

Kim MN, Park ST (2010) Degradation of poly(l-lactide) by a meso-philic bacterium. J Appl Polym Sci 117:67-74. **https://doi.org/ 10.1002/app.31950**

Klasek A, Lycka A, Rouchal M, Bartosik R (2020) Reaction of 1-sub-stituted 3-(2-hydroxyethylamino)quinolone-2,4-(1fl,3fl)-diones with isothiocyanic acid. Chem Heterocycl Comp 56:566-571. **https://doi.org/10.1007/s10593-020-02701-9**

Levsen K, Schiebel HM, Terlouw JK, Jobst KJ, Elend M, PreiB A, Thiele H, Ingendoh A (2007) Even-electron ions: a systematic study of the neutral species lost in the dissociation of quasimolecular ions. J Mass Spectrom 42:1024-1044. **https://doi.org/ 10.1002/jms.1234**

Lingens F, Blecher R, Blecher H, Blobel F, Eberspacher J, Frohner C, Gorisch H, Layh G (1985) Phenylobacterium immobile gen. nov., sp. nov., a Gram-negative bacterium that degrades the herbicide chloridazon. Int J Syst Bacteriol 35:26-39. **https://doi.org/10. 1099/00207713-35-1-26**

Login RB (1995) Pyrrolidone-based surfactants (a literature review). J Am Oil Chem Soc 72:759-771

Merkova M, Zalesak M, Ringlova E, Julinova M, Ruzicka J (2018) Degradation of the surfactant Cocamidopropyl betaine by two bacterial strains isolated from activated sludge. Int Biodeter Bio-degr 127:236-240. **https://doi.org/10.1016/j.ibiod.2017.12.006**

Narayanan KS, Chaudhuri RK (1993) N-alkyl pyrrolidone requirement for stable water based microemulsions. In: Devisetty BN, Chasin DG, Berger, PD (eds) Pesticide formulation and application systems: 12th volume. American Society for Testing and Materials, Special Technical Publication 1146:85-104

Narayanan KS, Jon D, Prettypaul D (2000) Emulsion concentrates of fungicides, and aqueous use formulations thereof for wood preservation. US Patent No. 6,033,681.US Patent No. 6,033,681

Odukkathil G, Vasudevan N (2015) Biodegradation of endosulfan isomers and its metabolite endosulfate by two biosurfactant producing bacterial strains of Bordetella petrii. J Environ Sci Heal B 50:81-89. **https://doi.org/10.1080/03601234.2015.975596**

Rajadhyaksha VJ (1978) Physiological vehicle compositions containing higher alkyl substituted azacyclopentan-2-ones. US Patent No. 4,122,170

Singleton D, Adrion AC, Aitken MD (2016) Surfactant-induced bacterial community changes correlated with increased polycyclic aromatic hydrocarbon degradation in contaminated soil. Appl Microbiol Biot 100:10165-10177. **https://doi.org/10.1007/ s00253-016-7867-z**

von Wintzingerode F, Schattke A, Siddiqui RA, Rosick U, Gobel U, Gross R (2001) Bordetella petrii sp. nov., isolated from an anaerobic bioreactor, and amended description of the genus Bordetella. Int J Syst Evol Micr 51:1257-1265. **https://doi.org/10.1099/00207 713-51-4-1257**

Wagner P (2006) Inert Reassessments: Two Exemptions from the Requirement of a Tolerance for N-(n-dodecyl)-2-pyrrolidone (CAS Reg. No. 2687-96-9) and N-(n-octyl)-2-pyrrolidone (CAS Reg. No. 2687-94-7). Action memorandum, US EPA. **http://www. epa.gov/sites/production/files/2015-04/documents/pyrrolidone. pdf.** Accessed 9 Dec 2020

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S Ribo-somal DNA amplification for phylogenetic study. J Bacteriol 173:697-703. **https://doi.org/10.1128/jb.173.2.697-703.1991**

Yang S, Wen X, Zhao L, Shi Y, Jin H (2014) Crude oil treatment leads to shift of bacterial communities in soils from the deep active layer and upper permafrost along the China-Russia crude oil pipeline route. PLoS ONE 9:e96552. **https://doi.org/10.1371/journal. pone.0096552**

Yoneto K, Ghanem AH, Higuchi WI, Peck KD, Li SK (1995) Mechanistic studies of the 1-alkyl-2-pyrrolidones as skin permeation enhancers. J Pharm Sci 84:312-317. **https://doi.org/10.1002/jps. 2600840310**