Research Article

Cell Adaptations of Rhodococci to Pharmaceutical Pollutants

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Abstract. Against the background of atense environmental situation, the risk of drug pollution in the natural environment is steadily increasing. Pharmaceuticals entering open ecosystems can cause toxic effects in wildlife from molecular to population levels. The aim of this research was to examine the impact of pharmaceutical pollutants on rhodococci, which are typical representatives of soil actinobacteria and active biodegraders of these compounds. The pharmaceutical products used in this research werediclofenac sodium and ibuprofen, which are non-steroidal anti-inflammatory drugs (NSAIDs) that are widely used and frequently found in the environment. The most common cell adaptations of rhodococci to the effects of NSAIDs were changes in zeta potential, catalase activity, morphometric parameters and degree of hydrophobicity; elevated contents of total cellular lipids; and the formation of cell conglomerates. The findings demonstrated the adaptation mechanisms of rhodococci and their increased resistance to the toxic effects of the pharmaceutical pollutants.

Keywords: pharmaceutical pollutants, NSAIDs, diclofenac, ibuprofen, cell responses, *Rhodococcus*

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1. Introduction

Currently, one of the challenging problems of terrestrial and aquatic ecosystems is their drug contaminationby a large number of pharmaceutical substances and their metabolites [1]. Most often and in relatively low concentrations (from ng/L to μg/L),those are massively consumed antibiotics, anti-inflammatory drugs (NSAIDs), hormones, antispasmodics, antihypertensives, anticancer agents, anticonvulsants and antidepressants;and also statins and antidiabetic drugs increasingly usedowing to the spread of sedentary lifestyles associated with urbanization[1]. As reported in a number of studies,pharmaceutical pollutants with pronounced biological activity and high chemical resistance, have negative impacts on invertebrates [2–4], vertebrates [5, 6], plants [7, 8], and also disrupt the structure and functioning of ecosystems [9, 10]. However, the nature of such impacts of pharmaceutical compounds on natural microorganisms, which represent a native system of "primary response" to xenobiotic load in open ecosystems, is still insufficiently studied. Only in recent years the attention

of researchers has been drawn to the study of physiological status and morphological changesof bacteria exposed to the most frequently detected pharmaceuticals [11, 12].

Some of these are diclofenac ($C_{14}H_{10}Cl_2NNaO_2$, CAS 15307-86-5, 2-(2-[2',6'-dichlorophenyl]amino)phenylacetic acid as a sodium salt) and ibuprofen ($C_{13}H_{18}O_2$; CAS 15687-27-1; (RS)-2-[4-(2-methylpropyl)phenyl]propanoic acid), widely available and often used in human medicine NSAIDs. The concentrations of diclofenac and ibuprofen detected in groundwater [13], surface(including marine) water [14], wastewater [15], and even drinking water [16]vary from a few ng/L to tens of mg/L. Being constantly released into the environment, they may accumulate inhigh long-term concentrations and stimulate negative effects on living organisms and humans [17, 18].

We have previously showed for the first time the ability of strains *Rhodococcus ruber* IEGM 346 and *R. cercidiphylli* IEGM 1184 to complete biodegradation of diclofenac and ibuprofen, respectively [19, 20]. The aim of this work is to compare the interaction of rhodococci with the selected pharma pollutants and evaluate the effectiveness of cell adaptations of rhodococciexposed topharmaceuticals.

2. Materials and methods

In this work, strains *Rhodococcus cercidiphylli* IEGM 1184 and *R. ruber* IEGM 346 deposited in the Regional Specialised Collection of Alkanotrophic Microorganisms (acronym IEGM, Large-Scale Research Facilities#73559, World Federation for Culture Collections # 285, http://www.iegmcol.ru) were used. Bacteria pre-grown in a nutrient broth for three days were cultured in RS mineral medium [21] in the presence of 50 mg/L diclofenac (Glentham Life Sciences, UK) or 100 mg/L ibuprofen (Sigma-Aldrich, USA). Either D-glucose (0.5 %) or *n*-hexadecane (0.1 % v/v) wasused as an additional source of carbon and energy.

Morphological changes in cells under the influence of NSAIDs were studied by a combined scanning system consisting of Olympus FV1000 confocal laser scanning microscope (CLSM) (Olympus Corporation, Japan) and Asylum MFP-3D atomic force microscope (AFM) (Asylum Research, USA). To differentiate living and dead cells, bacteria were stained with LIVE/DEAD®*Bac*Light™ Bacterial Viability Kit (Invitrogen, USA). AFM scanning in a semi-contact mode at 0.2 Hz was performed using a silicon AC240TS cantilever with resonant frequency of 50–90 kHz,spring constant of 0.5–4.4 N/m, and a tip curvature radius of 9 nm. Cell volume and surface area were calculated using the formulas given in Ref.[22].

The catalase activity of rhodococci was determined according to the method [23] using a Lambda EZ201 spectrophotometer (Perkin-Elmer, USA). The zeta potential of bacterial cells was measured bya Zetasizer Nano ZS analyzer (Malvern Instruments, UK) [19]. The Salt Aggregation Test was applied to assess thehydrophobicity of rhodococci [24]. When analyzing the degree of hydrophobicity, a scale was used: high hydrophobicity – the salinity index of an ammonium sulfate solution ranging from 0 to 0.8 M; moderate – from 1.0 to 2.0 M; weak – from 2.2 to 3.8 M [25]. The total cellular lipids weredetermined gravimetrically [19]. Rhodococci grown in the mineral medium in the presence of glucose or *n*-hexadecane without NSAIDs were used as controls. All experiments were performed in triplicates.

3. Results and discussion

According to our findings, the most typical reaction of rhodococci to diclofenac and ibuprofen was the formation of separate cell aggregates of different size (10–70 μm) and irregular shape (Figure 1). Apparently, the aggregation-based cooperation ensures the coordinated functioning of associated cells and allows the population to adapt and grow under pharmaceutical impact, in which individual cells are not able to reproduce and biodegrade the pharmaceuticals studied[19, 26, 27].

In the presence of diclofenac,aggregation of rhodococci was accompanied by changes in the morphological structure of cells: a change in shape (Figure 2), an increase in cell length and width (Table 1).

Diclofenac-exposed, individual cells often suffered damage to the integrity of cell walls accompanied by the release of cytoplasm into the external environment (Figure 3), as well as the accumulation of dead cells in the sample (see Figure 2). In addition, the destructive effect of diclofenac at the morphological level was manifestedin a significant ($p<0.05$) decrease in area-to-volume ratio (S/V) of bacterial cells (Table 1). An increase in the cell size and a concomitant decrease in surface-to-volume ratio appears to be an adaptive response of rhodococci to the membrane-active toxic effect of diclofenac[22].

A different picture was observed in the presence of ibuprofen: the membrane area of rhodococci increased for better uptake and transformation of the abovementioned NSAID[28].

The study of nanogeometry of the surface of living cells revealed an increase in root mean square roughness and microrelief amplitude in the presence of both substrates (Table 1). However, changes in the surface roughness were expressed to different

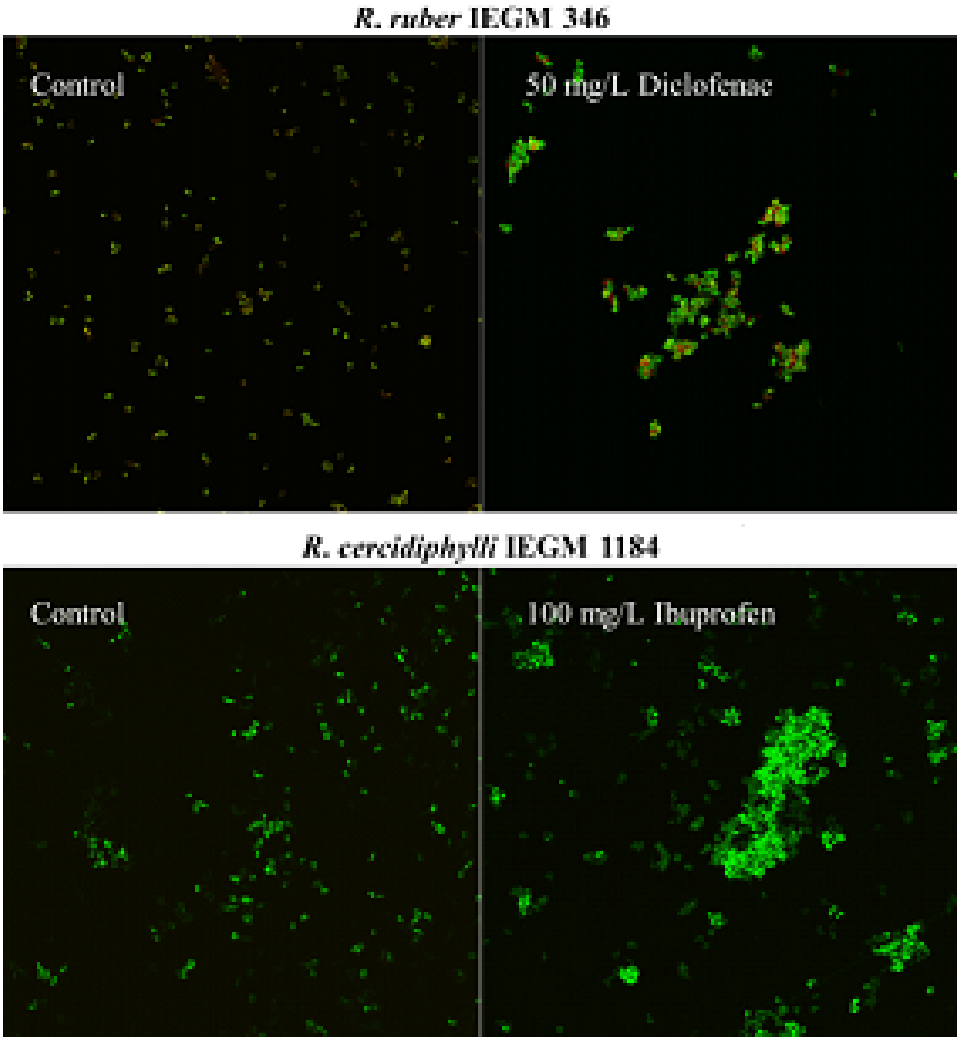

Figure 1: CLSM images of *R. ruber* IEGM 346 и *R. cercidiphylli* IEGM 1184. The cells were grown for 10 days. Green fluorescence indicates living cells.

degrees: the increase in cell surface roughness was 20 % (Figure 4) and 65 % (Figure 5) in the presence of diclofenac and ibuprofen, respectively.

The effect of the pharmaceuticals on rhodococci alsoresultedintheir changed electrokinetic properties (Table 2). In the presence of ibuprofen, there was an increase in the “negativity” of the zeta potential values compared to those of the control samples. The reverse trend was observed in the presence of diclofenac: the electrokinetic potential shifted to more positive values (Figure 6).

It is known that the shift of the zeta potential towards neutral values might lead to destabilization of the cell membrane (see Figure 3) [29]. Another confirmation of the presence of cells in a suppressed state weremarkers of oxidative stress, in particular, the documented change in catalase activity (Figure 7).

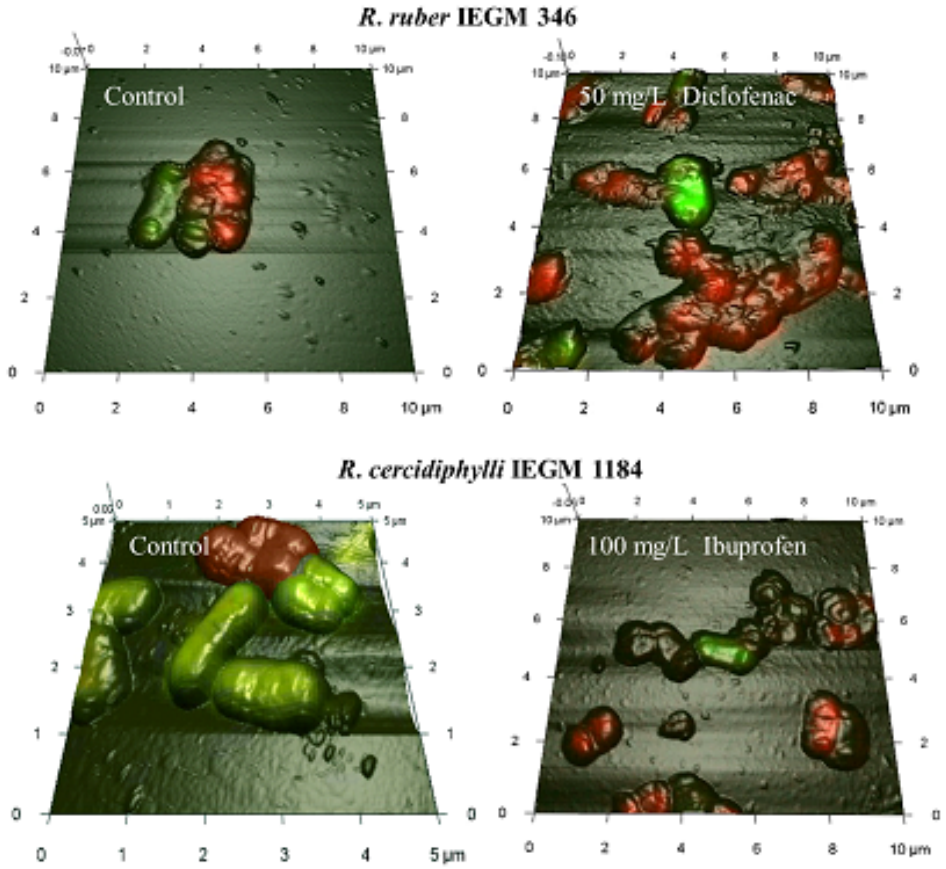

Figure 2: Combined 3D AFM-CLSM images of *R. ruber* IEGM 346 and *R. cercidiphylli* IEGM 1184. Cells were grown for 10 days. Red fluorescence indicates damaged cells.

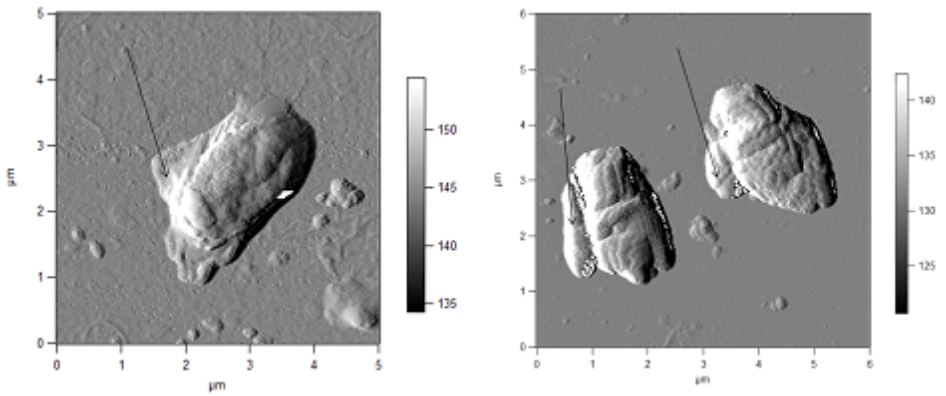

Figure 3: AFM images of *R. ruber* IEGM 346 cells grown for 10 days in the presence of 50 mg/L diclofenac. The arrows indicate areas of cell damage and release of cellular contents.

The initial values of catalase activity of *R. ruber* IEGM 346 cells were 4.5±0.15 μm/min×OD. On the 10th day of the experiment, a significant increase (up to 6.6±0.21 μm/min×OD) in catalase activity was registered. The increased catalase activity is probably due to the fact that under oxidative stress, bacteria apply theirenergy toprotective

Table 1: Morphometric parameters of rhodococci in the presence of NSAIDs.

Treatment	Length, μm	Width, μm	Volume,V, μm^3	Surface, S, μm^2	S/V, μm^{-1}
R. cercidiphylli IEGM 1184					
Control	3.3±0.14	1.0±0.27	2.6±0.07	6.8±0.17	2.6±0.12
Ibuprofen (100 mg/L)	1.3±0.11	1.1±0.06	1.1±0.00	3.7±0.02	3.4±0.05
R. ruber IEGM 346					
Control	3.0 ± 0.02	0.9 ± 0.05	1.9 ± 0.03	5.5 ± 0.05	2.9 ± 0.02
Diclofenac (50 mg/L)	3.5 ± 0.13	1.1 ± 0.02	3.3 ± 0.05	7.9 ± 0.10	2.4 ± 0.08
Note: Here and in Table 2, the cells were grown for 10 days.					

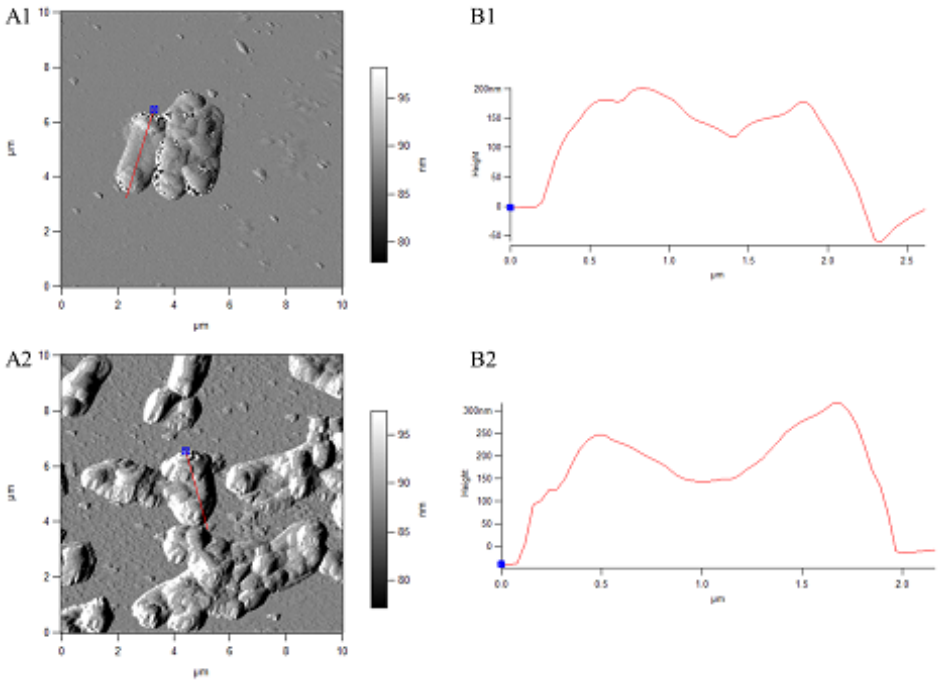

Figure 4: AFMimages (A) and profile (B) of *R. ruber* IEGM 346 cells grown for 10 days in the presence of glucose (1); 50 mg/L diclofenac and glucose (2).

enzymatic reactions, since non-enzymatic antioxidant systems (glycogen, polysaccharides, trehalose mycolates) do not provide effective protection from reactive oxygen species associated with destabilization of cell membranes and damageof the membrane integrity (see Figure 3) [23]. A slight decrease in catalase activity was registered in the controls. The decrease in catalase activity of rhodococci was observed on 20–30 days of the experiment (Figure 7). Amidst the detected changes in catalase activity, a sequential shift of the zeta potential towards more negative values(-37.47±2.33 and -47.81±2.57 mV) was registered (see Figure 6).

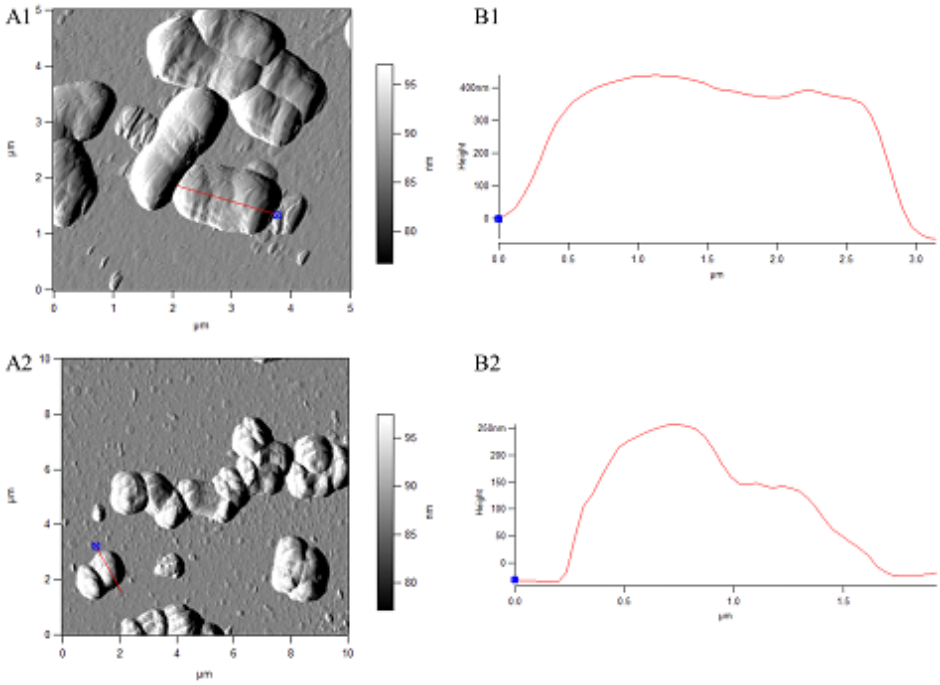

Figure 5: AFM images (A) and profiles (B) of *R. cercidiphylli* IEGM 1184 cells grown for 10 days in the presence of *n*-hexadecane (1);100 mg/L ibuprofen and *n*-hexadecane (2).

Table 2: Changes in the properties of the cell surface in the presence of NSAIDs.

Treatment	Mean square roughness, nm	Zeta potential, mV
R. cercidiphylli IEGM 1184		
Control	163.9±5.20	-17.1±1.3
Ibuprofen (100 mg/L)	465.3±16.26	-25.4±0.8
R. ruber IEGM 346		
Control	197.8 ± 2.30	-35.2±2.33
Diclofenac (50 mg/L)	216.1 ± 5.51	-31.3±0.83

The presence of diclofenac and ibuprofen induced an increase in the percentage of total cellular lipids from 43.4±1.72 to 70.8±4.46 % and from 23.5±2.67 to 45.1±1.82 % of dry biomass weight, respectively. A rise in the lipid content of rhodococci cultured in the presence of diclofenac obviously allows maintaining the integrity of the cell membrane and enhancingits stability. Stabilization of the cell membrane was also proved by a decrease in the catalase activity of rhodococci (see Figure 7), indicating the priority of the non-enzymatic antioxidant system (cell wall) overthe fermentative one.

As the zeta potential shifted in the presence of diclofenac, an intensive formation of cell aggregates was observedthatcorrelated with an increase in the degree of hydrophobicity of rhodococcal cells. Figure 8shows that rhodococci produced stable

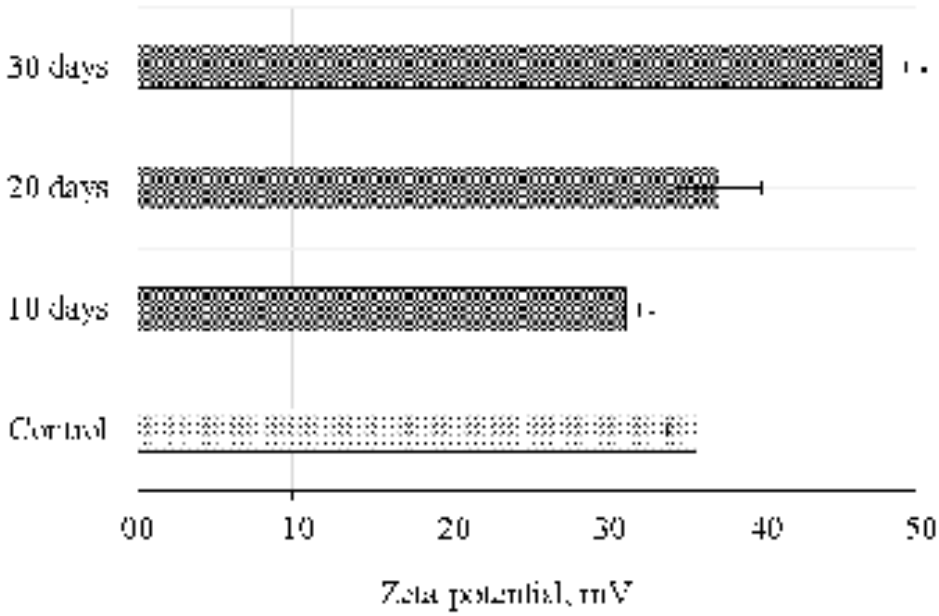

Figure 6: Change in zeta potential of *R. ruber* IEGM 346. Cells were grown in the presence of glucose (, control) or 50 mg/L diclofenac and glucose (). *Values are significantly different from the control ones ($p<0.05$).

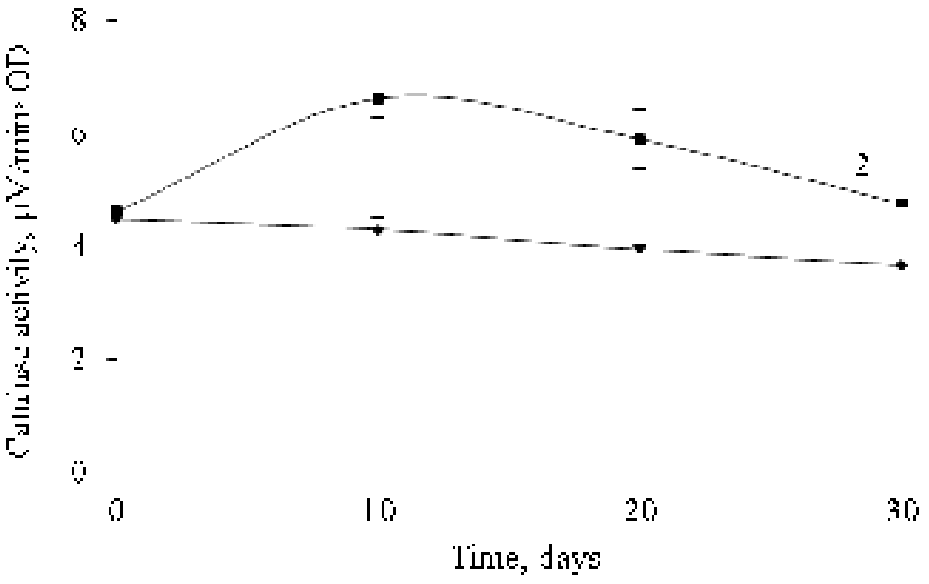

Figure 7: Catalase activity of *R. ruber* IEGM 346 cells in the presence of glucose (1); 50 mg/L diclofenac and glucose (2).

microaggregates when exposed to a relatively low (0.6 M) concentration of ammonium sulfate, indicating the high hydrophobicity of their cell surface [25].

Table 3: Total lipid content in *Rhodococcus*cells grown in the presence of NSAIDs.

Cultivation conditions	Dry biomass, g/L	Total lipids, % of dry weight
R. ruber IEGM 346		
Control	2.1±0.08	43.4±1.72
Ibuprofen (100 mg/L)	0.4±0.02	70.8±4.46
R. cercidiphylli IEGM 1184		
Control	1.5±0.24	23.5±2.67
Diclofenac (50 mg/L)	0.8±0.04	45.1±1.82
Note: The cells were grown for 15 days.		

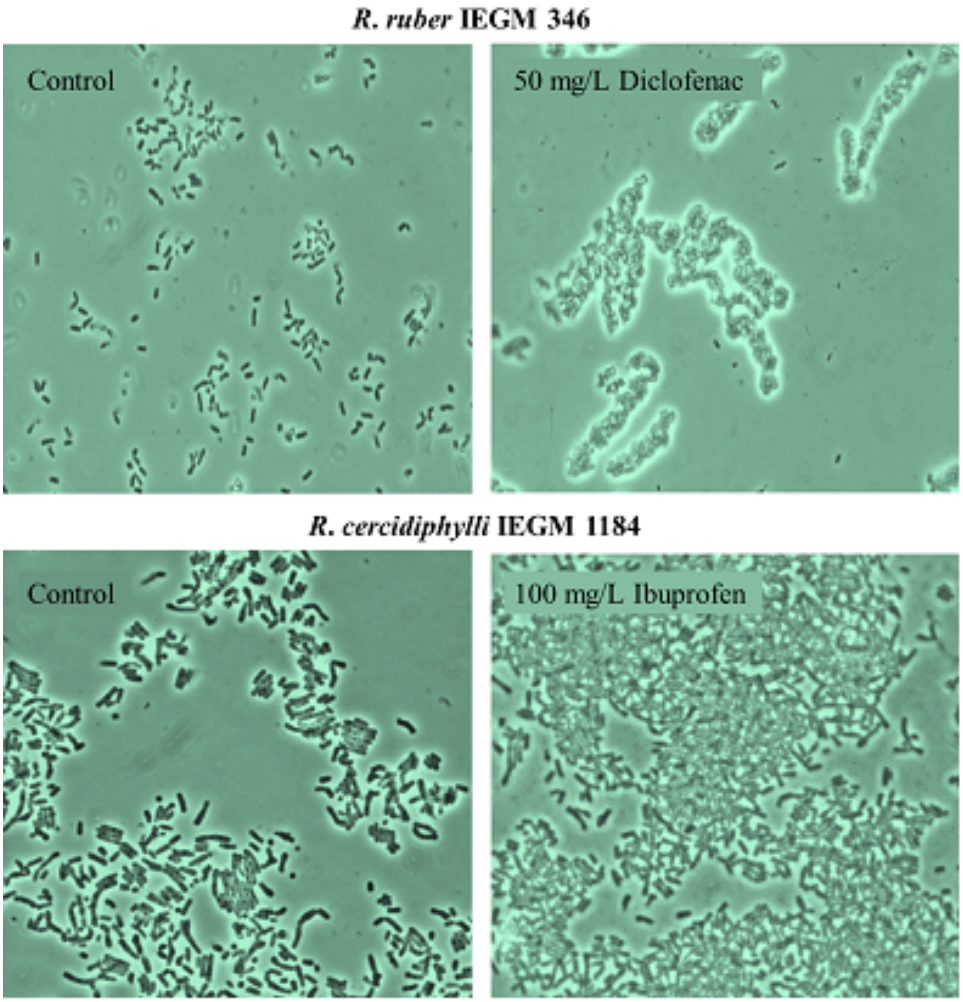

Figure 8: Micrographs of *R. ruber* IEGM 346 and *R. cercidiphylli* IEGM 1184 cells. Cells were grown for 15 days. Microscopy was performed in the presence of 0.6 M $(NH_4)_2SO_4$. (x1000).

4. Conclusion

Microorganisms are able to decompose a wide range of chemically stable compounds, thereby returning essential nutrients to global cycles and preventing the accumulation

of “dead” residues on the Earth’s surface. Under the xenobiotic load of the environment, microorganisms implement a set of survival strategies that ensure their effective adaptation to harsh environmental conditions, as well as their ability to biodegrade complex organic compounds. Knowing the protective mechanisms triggered in bacteria exposed to pharmaceutical pollutants is extremely important in understanding andsubsequentfacing the challenge of pharmaceutical pollution. On the example of actinobacteria of the genus *Rhodococcus*,ubiquitous inhabitants of pristine and polluted environments, which realizea wide range of adaptive capabilities and oxidizea large number of complex organic pollutants, characteristic responses of bacteria exposed to diclofenac and ibuprofen often detected in the environmentwere revealed. The most typical reactions of rhodococci on the effects of the NSAIDs were the formation of multicellular conglomerates, the increase in the content of total cellular lipids and the degree of hydrophobicity of the cell wall, changes of morphometric parameters and zeta potential. The observed regularities indicate that the structure of adeveloping *Rhodococcus*population pliably changes towards more stable forms, and we consider themas adaptation mechanisms of rhodococci resultingintheir increased resistance to toxic effects of the pharmaceutical pollutants tested.

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7. Ethics policies

The authors declare that they have no conflict of interest.

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