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DIFFERENCE IN INFLUENCE OF COMMERCIAL INDUSTRIAL PAINTS ON MICROBIAL BIOFILMS AND PLANKTONIC CELLS

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ABSTRACT. This study compares the effect of commercial industrial paints on the *Escherichia coli* PMFKG-F2, *Proteus mirabilis* PMFKG-F4 and *Saccharomyces cerevisiae* PMFKG-F6 planktonic cells and biofilms. A MBEC^{TM}-HTP assay and standard 96 microtiter plate assay were used to test the levels of resistance of planktonic cells and biofilms. The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of the tested substances, which affects planktonic cells and biofilms, were determined and the results were confirmed by fluorescence microscopy.

Results obtained for planktonic cells were compared between them and with the results obtained for biofilms. Noticeable difference in the resistance between the biofilms and the planktonic cells on paints, was observed. The *E. coli* PMFKG-F2 planktonic cells showed the highest resistance in the presence of the tested substance 2 (MICp 2.5 µl/ml), while the *P. mirabilis* PMFKG-F4 planktonic cells showed the highest resistance in the presence of the tested substance 2 (MICp 5 µl/ml). The *S. cerevisiae* PMFKG-F6 planktonic cells showed the same level of resistance in the presence of the tested substances 1, 2 and 5 (MICp 0.62 µl/ml). The *E. coli* PMFKG-F2, *P. mirabilis* PMFKG-F4 and *S. cerevisiae* PMFKG-6 biofilms showed the highest resistance in the presence of the tested substance 5 (MICb 125 µl/ml, MICb 125 µl/ml and MICb 62.5 µl/ml).

The obtained results suggest that the biofilm may have a potential to be used in bioremediation of wastewater contaminated with industrial paints.

Key words: planktonic cells, biofilm, industrial paint, resistance.

INTRODUCTION

Industrial production process results in production of different types of waste. Main source of dangerous waste matters in production facility, emerges during process of painting vehicles (GEFFEN and ROTHENBERG, 2000). PAPASAVVA *et al.* (2001) quote that industrial paints have the biggest impact on environment, whereby around 80% of all environmental problems comes directly from an automotive assembly plant (paint shop) and similar operational units (LOWELL *et al.,* 1993). The major environmental impacts of the automotive assembly plant (paint shop) are air emissions of regulated chemicals, including volatile organic compounds (VOCs) and hazardous pollutants. Paint and coating impact was reported

by ZORPAS and INGLEZAKIS (2012). The main problem is paint sludge, which occurs during precipitation of colors and paints, which is classified by EU code for waste management 080113, and shows that paint sludge is treated as waste with dangerous characteristics (SALIHOGLU and SALIHOGLU, 2016). According to EU law regulation, paint sludge cannot be stored on landfills as it contains high levels of organic carbon (SALIHOGLU and SALIHOGLU, 2016). For that reason, much research in area of impact and reduction of colors and paints in industry are needed.

Increasing demand in industrial paints leads to general concern because of the dangerous organic compounds in paints (CHANG *et al.,* 2002). During paint process, different types of thinners are added to maintain viscosity of paint, which contain very toxic substances. Toluene, xylene, ethyl acetate, n-butanol are among the most dangerous compounds (DEAN, 1985). CHANG *et al.* (2002) conducted research in which they determined quantity and composition of exhaust gases from 5 facilities in Taiwan. It was determined that organic compounds from paint (primer and thinner) are lead sources of pollution. In order to satisfy needs of existing and future laws on environmental protection, manufacturers are obliged to reduce the percentage of these compounds to a minimum. Volatile organic compounds (VOCs) removal is tested using bioreactor with activated sludge, with and without activated carbon (KIM *et al.,* 2000). Based on these results it was reported that in comparison with adsorption/thermal oxidation process, biological process proved to be cheaper method for VOC removal. Removal and degradation of components of hydrophilic paints were efficient. Toluene removal was also efficient and the range of removal was from 74-91% without and 86-93% in the presence of activated carbon.

In order to improve paint and thinner removal process, next to chemical treatment, it is necessary to take into account different, live organisms and their degradation potential. Therefore, the possibility of using microorganisms for biological treatment of waste waters, attracted researchers' attention (FU and WANG, 2011; PENNAFIRME *et al.,* 2015). Until now, microbiologists were focused on bacteria in suspension (planktonic form), grown as pure laboratory cultures. Bacterial growth dynamic was tested from wastewater sample of a car painting facility as well as their degradation potential in the presence of aromatic compound mixture (STOFFELS *et al.,* 1998). For removal and biodegradation of a wide-range organic compounds from industrial plants, usage of fungi was tested. Also, some research was already performed to examine the impact of VOC within paints on planktonic bacteria (STOFFELS *et al.,* 1998) and filamentous fungi (QI and KINNEY, 2005). QI and KINNEY, (2005) tested efficiency of the pure culture of *Cladosporium sphaerospermum* in removing the mixture of *n*-butyl acetate, methyl ethyl ketone, methyl propyl ketone, and toluene. Experimental results showed that fungi, as biofilters, can succesfully remove mixture of tested substances within paint ingredients.

In order to improve biotechnological processes in removal of paint sludge waste, further research will be based on discovering new applicable techniques, which will prevent, or reduce the generation of paint sludge. According to the available literature, no study on the impact of industrial paints and thinners on microbiological biofilms, was found. Therefore, the main goal of our research was to test the resilience of different biofilms, as well as of planktonic cells in the treatment with commercial industrial paints.

MATERIALS AND METHODS

Organisms and growth conditions

Microorganisms *Escherichia coli* PMFKG-F2, *Proteus mirabilis* PMFKG- F4 and *Saccharomyces cerevisiae* PMFKG-F6 (University of Kragujevac, Faculty of Science

collection) were used to test the resistance of two life forms of microorganisms (planktonic cells and biofilm) in parallel. The Tryptic Soy Broth (TSB, MossHeMoss) medium was used as a nutritious medium for culturing of planktonic cells and biofilms of bacteria and yeasts (HARRISON *et al.*, 2006). The suspension of microorganisms has been designed to match the McFarland 1.0.

Preparation of test substances

The resistance of planktonic cells and biofilms was tested in the presence of the test substances shown in Table 1. Stock solutions were made in sterile glass bottles. Work solutions were prepared in TSB medium on the day of the experiment setup. Given the fact that so far, there were no studies conducted which examined the effect of these test substances (Table 1) on planktonic cells and biofilms, preliminary testings followed. Concentration range of the tested substances is selected, so that in the final test the lowest applied concentration in relation to the positive control does not lead to a significant response (in comparison with growth control). However, the highest concentration brings 100% a response of test organism.

Table 1. Test substances used as antimicrobial agents.

Commercial industrial paint-Product Code	Test substances (mark in text)
A-F107486-FH	1
A-F107137-MF	2
A-F107119-CN	3
A-F107107-FM	4
A-F107117- CP	5

The ranges of the concentrations of the applied test substances are shown in the Table 2.

Table 2. Concentration ranges of tested substances.

	The range of tested concentrations (µl/ml)						
Planktonic cells	10	5	2.5	1.25	0.62	0.31	0.15
Biofilm	1000	500	250	125	62.5	31.25	15.62

Biofilms cultivation

The biofilms were formed in the MBEC^{TM}-HTP device (MBEC BioProducts) according to the method described by CERI *et al.* (1999). 200 µl of the cell suspension was added to a 96 well peg-lid plate. After a period of incubation (24 h for bacteria and 48 h for yeast) at 26^{o}C, the biofilm formed on the pegs was used to simultaneously examine the resistance of biofilms and planktonic cells in the treatment with test substances.

To confirm that the biofilm was formed, the lid with inoculated pegs was transferred to a new plate containing fresh medium. The plate together with the lid was sonicated (Aquasonic 250 HT Ultrasonic Cleaner, VWR International, Radnor, PA, USA) to detach biofilms from the pegs. The optical density of each well was determined by spectrophotometric reading at 650 nm (OD_{650}) on an ELISA reader (Rayito, China) to confirm the formation of a biofilm.

Treatment of biofilms and planktonic cells

The resistance of formed biofilms and planktonic cells was examined using the MBEC^{TM}-HTP device (MBEC BioProducts) according to the method described by CERI *et al.* (1999). The test substances were applied in concentrations corresponding to those presented in Table 2, for planktonic cells.

After period of exposure (24 h and 48 h for bacteria; 48 h and 72 h for yeast), plastic peg lid was removed and washed twice with sterile 0.9% saline. Plastic lid with pegs, was transferred to a new plate with fresh TSB (200 µl per well). The plate together with the lid was sonicated (Aquasonic 250 HT Ultrasonic Cleaner, VWR International, Radnor, PA, USA) to detach biofilms from the pegs. After incubation, the minimum inhibitory concentration (MICb) and the minimum lethal concentration (MLCb) was obtained by spectrophotometric reading on an ELISA plate reader (Rayito, China) at 650 nm (OD_{650}).

Experiments with planktonic cells were prepared according to the method described by Ceri *et al.* (1999), also. Plates with the tested substances were prepared as described above. 20 µl aliquots of planktonic cultures were placed into the 96 well microplate with fresh TSB. Survival of planktonic cells was presented trough turbidity. During incubation period, some of planktonic cells will not get in the process of biofilm formation. These cells maintain their planktonic phenotype and are being exposed to the impact of tested substances. The minimum inhibitory concentration (MICp) which inhibits the growth of 50% of cells, and the minimum lethal concentration (MLCp) which kills 90-100% of cells (detected by the absence of turbidity) were determined in each plate by spectrophotometric reading on an ELISA plate reader (Rayito, China) at 650 nm (OD_{650}).

All tests were performed three times and the mean value was calculated.

Biofilms resistance in the presence of test substances

E. coli PMFKG-F2, *P. mirabilis* PMFKG- F4 and *S. cerevisiae* PMFKG-F6 biofilm formation was repeated in polystyrene 96-well microtiter plate (SARSTEDT, Belgrade) according to the method described by ADAM *et al.* (2002) with certain modifications. In each polystyrene microtiter plate, 100 µl of suspension was added. The plates were then placed in an incubator at 26°C for 24 h for bacteria and 48 h for yeast. After period of incubation, the test substances were applied in concentrations corresponding to those presented in Table 2 (24 h and 48 h for bacterial biofilms; 48 h and 72 h for yeast biofilm).

Quantification was determined by crystal violet (CV) test according to the method of ALMEIDA *et al.* (2013) with certain modifications as follows: upon the incubation, the content of the plates was removed and 50 µl of 98% methanol (vol / vol) was added. After 15 minutes, the methanol was removed and the plate was left to dry at a room temperature. After that, 50 µl CV was added (5 min). Plate was rinsed three times with sterile distilled water and 100 µl of glacial acetic acid 33% (vol / vol) was added. OD_{650} was measured by using microtiter plate reader (Rayito, China). All tests were performed three times and the mean value was calculated.

Fluorescence microscopy analysis

Fluorescence microscopy was used to evaluate the impact of the test substances on biofilms according to the method of HARRISON *et al.* (2006). Biofilms were treated with tested substances as described above. The liquid content of the plates in which the biofilm was formed and treated with tested substances was removed. Plate was rinsed with sterile physiological solution, in order to remove the non-adherent cells. Biofilm fixing is carried out with methanol (30 minutes to 1 hour at 30°C). After that, plates were rinsed with sterile

physiological solution, treated with a suitable fluorescent dye and then analyzed under Olympus BX51 fluorescent microscope (Olympus, Shinjuku, Tokyo, Japan) and analyzed by using Cytovision 3.1 software package (Applied Imaging Corporation, Santa Clara, California, USA). Biofilms were colored with SYTO 9 (viable cells were colored green) and ConA-Texas Red which the extracellular polymer substance (EPS) was colored red. An overview of used dyes is provided in Table 3.

Table 3. Dyes used for visualization of biofilms by using fluorescence microscopy.

Dye I	Dye II	Excitation (nm)		Collected emissions (nm)		Time of incubation (min)	
		λ1	λ 2	λ1	λ 2	Dye I	Dye II
[a]ConA-Texas Red (50 µg ml^{-1})	[b]Syto-9 (6.7 µM)	543	488	555 – 615	510 – 540	60	5

Abbreviation for dyes: [a]ConA–Texas Red = Concanavalin A, Texas Red Conjugate; [b] Syto-9 = 500 times diluted in compared to initial concentration stipulated by the manufacturer ((Molecular Probes).

RESULTS AND DISCUSSION

The resistance of planktonic cells and biofilms in the presence of test substances

By determining the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC), the level of resistance of planktonic cells and biofilms on the presence of 5 test substances was tested. By testing the level of resistance of planktonic cells and biofilms within the MBEC^{TM}-HTP device, results showed that the biofilm was more resistant against impact test substances than planktonic cells. The lowest concentration of tested substances for biofilm treatment had high impact on planktonic cells. For this reason, testing the level of resistance of biofilms was repeated in polystyrene microtiter plates with 96 well and the obtained results are shown in Tables 4, 5, 6, 7 and 8.

Table 4. Resistance of planktonic cells and biofilms on the presence of test substance 1.

	Test substance 1			
Species	**[a]MICp**	**[b]MLCp**	**[c]MICb**	**[d]MLCb**
Escherichia coli PMFKG-F2	1.25	5	31.25	250
Proteus mirabilis PMFKG- F4	0.31	5	62.5	125
Saccharomyces cerevisiae PMFKG-F6	0.62	5	31.25	500

The values in the table are given in µl/ml. [a] MICp-minimum inhibitory concentration of planktonic cells, [b] MLCp- minimum lethal concentration of planktonic cells, [c] MICb- minimum inhibitory concentration of a biofilm, [d] MLCb- minimum lethal concentration of a biofilm.

In the group of planktonic forms, the highest resistance on the test substance 1 was obtained by *E. coli* PMFKG-F2 (MICp 1.25 µl/ml). The highest sensitivity was noticed in *P. mirabilis* PMFKG-F4 (MICp 0.31 µl/ml). In biofilm resistance assays, the highest resistance was obtained by *P. mirabilis* PMFKG-F4 *(*MICb 62.5 µl/ml). The highest sensitivity was noticed in biofilms of *E. coli* PMFKG-F2 and *S. cerevisiae* PMFKG-F6 (MICb 31.25 µl/ml) (Table 4).

The *P. mirabilis* PMFKG-F4 planktonic cells showed the greatest sensitivity in the presence of test substance 1, while the biofilm of the same species was with the most resistance. Based on this result it can be assumed that the biofilm is more resistant to the

impact of test substance 1 than its planktonic cells due to metabolic differences or the existence of „persister“ cells in biofilm such as in the case in study of HARRISON *et al.* (2005). The main function of EPS is to secure protection. EPS hydrated layer prevents dehydration of biofilm, increases biofilm resistance. EPS also functions as a barrier to toxins and protects from predators (ROMEO, 2008).

Table 5. Resistance of planktonic cells and biofilms in the presence of test substance 2.

	Test substance 2			
Species	**[a]MICp**	**[b]MLCp**	**[c]MICb**	**[d]MLCb**
Escherichia coli PMFKG- F2	2.5	10	7.81	31.25
Proteus mirabilis PMFKG- F4	5	10	15.62	15.62
Saccharomyces cerevisiae PMFKG – F6	0.62	10	7.81	15.62

The values in the table are given in µl/ml. [a] MICp-minimum inhibitory concentration of planktonic cells, [b] MLCp- minimum lethal concentration of planktonic cells, [c] MICb- minimum inhibitory concentration of a biofilm, [d] MLCb- minimum lethal concentration of a biofilm.

In the group of planktonic forms, *P. mirabilis* PMFKG-F4 (MICp 5 µl/ml) showed the highest resistance on the test substance 2. The highest sensitivity was noticed in *S. cerevisiae* PMFKG–F6 (MICp 0.62 µl/ml). In biofilm resistance assays, the *P. mirabilis* PMFKG-F4 (MICb 15.62 µl/ml) was distinguished. Both, planktonic cells as well as biofilm of *P. mirabilis* PMFKG-F4, showed relatively high resistance (Table 5). The obtained results show that the *P. mirabilis* PMFKG-F4 biofilm had the same MIC and MLC values.

Table 6. Resistance of planktonic cells and biofilms in the presence of test substance 3.

	Test substance 3			
Species	**[a]MICp**	**[b]MLCp**	**[c]MICb**	**[d]MLCb**
Escherichia coli PMFKG-F2	0.08	5	<7.81	15.62
Proteus mirabilis PMFKG-F4	0.08	10	31.25	62.5
Saccharomyces cerevisiae PMFKG-F6	0.08	5	31.25	62.5

The values in the table are given in µl/ml. [a] MICp-minimum inhibitory concentration of planktonic cells, [b] MLCp- minimum lethal concentration of planktonic cells, [c] MICb- minimum inhibitory concentration of a biofilm, [d] MLCb- minimum lethal concentration of a biofilm.

In the test with the substance 3, there was no difference in sensitivity between planktonic cells of the tested species (MICp 0.08 µl/ml). Contrary to planktonic cells, the biofilm of *P. mirabilis* PMFKG–F4 and *S. cerevisiae* PMFKG–F6 was distinguished with MICb of 31.25 µl/ml (Table 6). Significant difference in resistance of planktonic cells and biofilm can be explained by difference in their life forms. In the case of biofilms, the EPS was identified as the main component responsible for the genesis of exceptional characteristics of biofilm (CORNING, 2002), and due to the production of matrix within the biofilm, the protection against antimicrobial agents is provided. Also, this significant difference in resistance can be explained by the fact that EPS represents the primary structural component of microbiological microenvironment, which has an impact on the physical characteristics of the biofilm (stabilization and protection of the microenvironment). The activities of cells within the biofilm can be enhanced with the production of EPS (DECHO, 2000).

In the test with substance 4, planktonic cells of the tested species showed the highest sensitivity (MICp 0.08 µl/ml), except *P. mirabilis* PMFKG – F4 (MICp 0.31 µl/ml). The biofilm of *E. coli* PMFKG-F2 was the most resistant on the presence of test substance 4 (MICb 31.25 µl/ml) as opposite to biofilms of *P. mirabilis* PMFKG-F4 and *S. cerevisiae*

PMFKG-F6 that showed the greatest sensitivity (MICb 15.62 µl/ml) (Table 7). Based on the fact that the *E. coli* PMFKG-F2 planktonic cells was the most sensitive of all, while on the other hand, the biofilm of *E. coli* PMFKG-F2 was the most resistant one, we can try to explain, based on FLEMMING assertion (2016), main function of EPS is to secure protection. Based on this statement, we can assume that similar situation has happened during the exposure of *E. coli* PMFKG-F2 biofilm to test substance 4, in which case biofilm increases resistance on the paint.

Table 7. Resistance of planktonic cells and biofilms in the presence of test substance 4.

	Test substance 4			
Species	**[a]MICp**	**[b]MLCp**	**[c]MICb**	**[d]MLCb**
Escherichia coli PMFKG – F2	0.08	5	31.25	125
Proteus mirabilis PMFKG – F4	0.31	10	15.62	62.5
Saccharomyces cerevisiae PMFKG – F6	0.08	2.5	15.62	250

The values in the table are given in µl/ml. [a] MICp-minimum inhibitory concentration of planktonic cells, [b] MLCp- minimum lethal concentration of planktonic cells, [c] MICb- minimum inhibitory concentration of a biofilm, [d] MLCb- minimum lethal concentration of a biofilm.

Table 8. Resistance of planktonic cells and biofilms in the presence of test substance 5.

	Test substance 5			
Species	**[a]MICp**	**[b]MLCp**	**[c]MICb**	**[d]MLCb**
Escherichia coli – PMFKG – F2	0.15	10	125	125
Proteus mirabilis – PMFKG – F4	0.31	10	125	125
Saccharomyces cerevisiae PMFKG - F6	0.62	10	62.5	125

The values in the table are given in µl/ml. [a] MICp-minimum inhibitory concentration of planktonic cells, [b] MLCp- minimum lethal concentration of planktonic cells, [c] MICb- minimum inhibitory concentration of a biofilm, [d] MLCb- minimum lethal concentration of a biofilm.

In the presence of test substance 5, a high sensitivity was noticed in all planktonic cells. Within the biofilms, two tested species had the high resistance (MICb 125 µl/ml) (Table 5).

Within the biofilm, different physical, physiological and genetic processes take place, which enable microorganisms to develop mechanisms of tolerance that will provide the resistance to the presence of antimicrobial agents (HARRISON *et al.*, 2007).

Results showed noticeable difference in the resistance between the biofilms and the planktonic cells on paints, was observed. The *E. coli* PMFKG-F2 planktonic cells showed the highest resistance in the presence of the tested substance 2 (MICp 2.5 µl/ml), while the *P. mirabilis* PMFKG-F4 planktonic cells showed the highest resistance in the presence of the tested substance 2 (MICp 5 µl/ml). The *S. cerevisiae* PMFKG-F6 planktonic cells showed the same resistance in the presence of the tested substances 1, 2 and 5 (MICp 0.62 µl/ml). The *E. coli* PMFKG-F2, *P. mirabilis* PMFKG-F4 and *S. cerevisiae* PMFKG-6 biofilms showed the highest resistance in the presence of the tested substance 5 (MICb 125 µl/ml, MICb 125 µl/ml and MICb 62.5 µl/ml).

Fluorescence microscopy

The influence of test substances on biofilms was determined after 48 hours for bacterial, and after 72 hours for yeast biofilm, respectively. The results are presented in Figures 1, 2, 3, 4 and 5.

The results of fluorescence microscopy correspond with the obtained MLCb values (Tables 4, 5, 6, 7 and 8). In the figures, the MLCb is estimated as the concentration that causes a lethal effect on the test organism (Andrews, 2001). The results of fluorescence microscopy also show that the color ConA-Texas Red, which colors extracellular matrix in red, was not visible in Figure 1h, so that it can be assumed that the biofilm of *S. cerevisiae* PMFKG-F6 does not produce the EPS in the presence of the test substance 1 (Figure 1). The same results were obtained for biofilms of *S. cerevisiae* PMFKG-F6 in the presence of the test substance 5 (Figure 5h).

Based on the current knowledge on the use of microorganisms in reduction of industrial paint, it is possible to develop efficient and environmentally friendly (bio) technologies for remediation of paint in wastewater treatment plants. The obtained results showed a significant difference in paint resistance between the biofilm and their corresponding planktonic cells. The biofilms showed much higher resistance comparing to planktonic cells, which suggests that biofilm should be used in development of biotechnologies suitable for remediation of environment contaminated with industrial paint.

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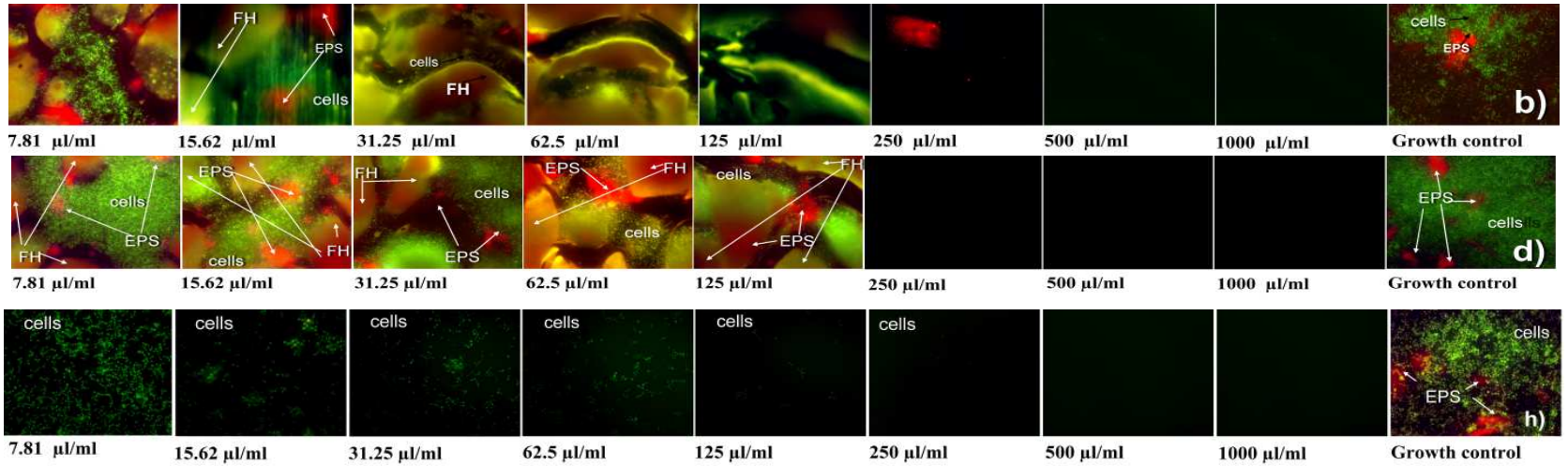

Figure 1. Influence of the test substance 1 on biofilms b) b) *E. coli* PMFKG-F2, d) P. *mirabilis* PMFKG-F4, h) *S. cerevisiae* PMFKG-F6. EPS – Extracellular polymer substance, FH – product code of the A-F107486-FH commercial industrial paint (Table 1).

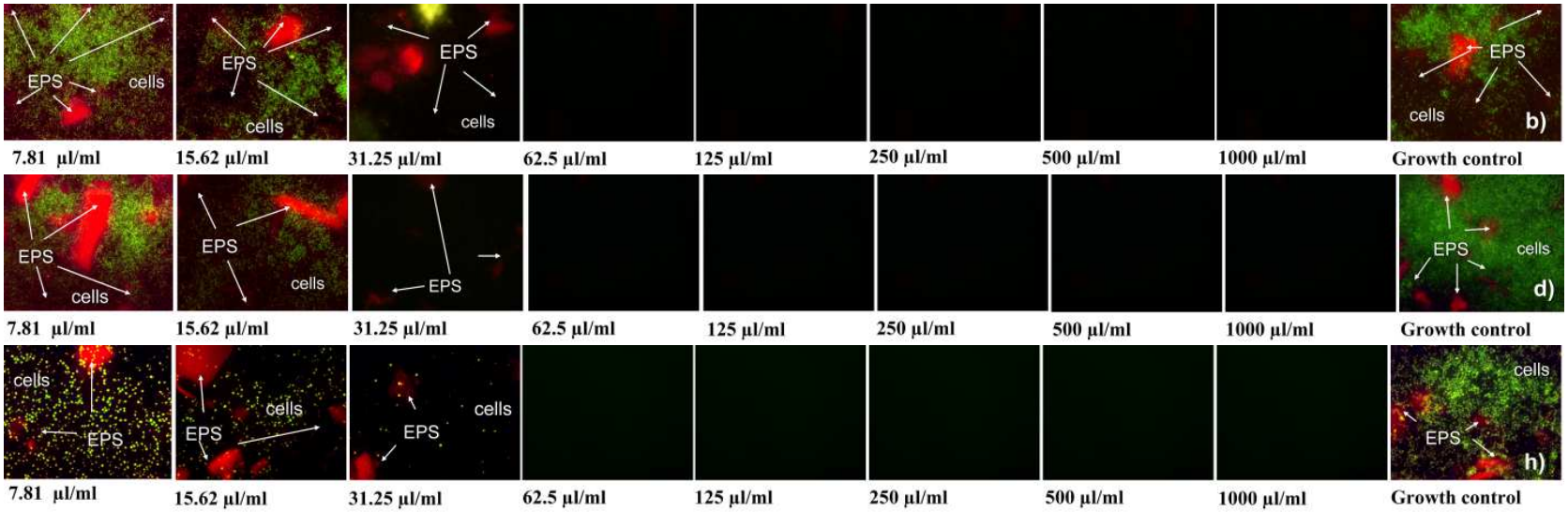

Figure 2. Influence of the test substance 2 on biofilms b) b) *E. coli* PMFKG-F2, d) P. *mirabilis* PMFKG-F4, h) *S. cerevisiae* PMFKG-F6. EPS – Extracellular polymer substance.

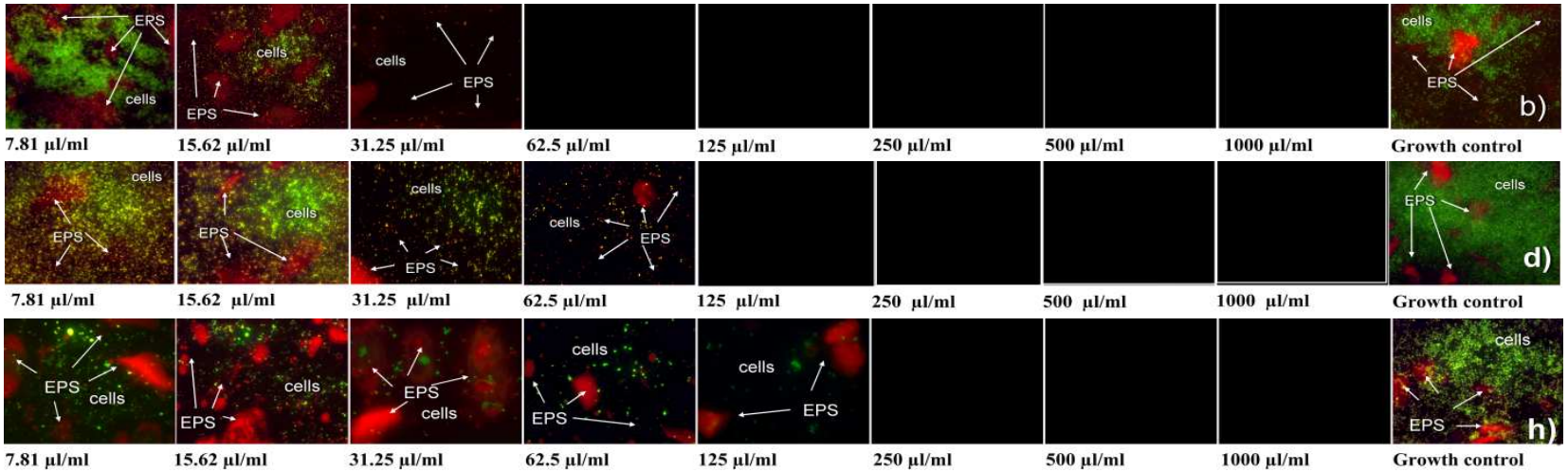

Figure 3. Influence of the test substance 3 on biofilms b) b) *E. coli* PMFKG-F2, d) P. *mirabilis* PMFKG-F4, h) *S. cerevisiae* PMFKG-F6. EPS – Extracellular polymer substance.

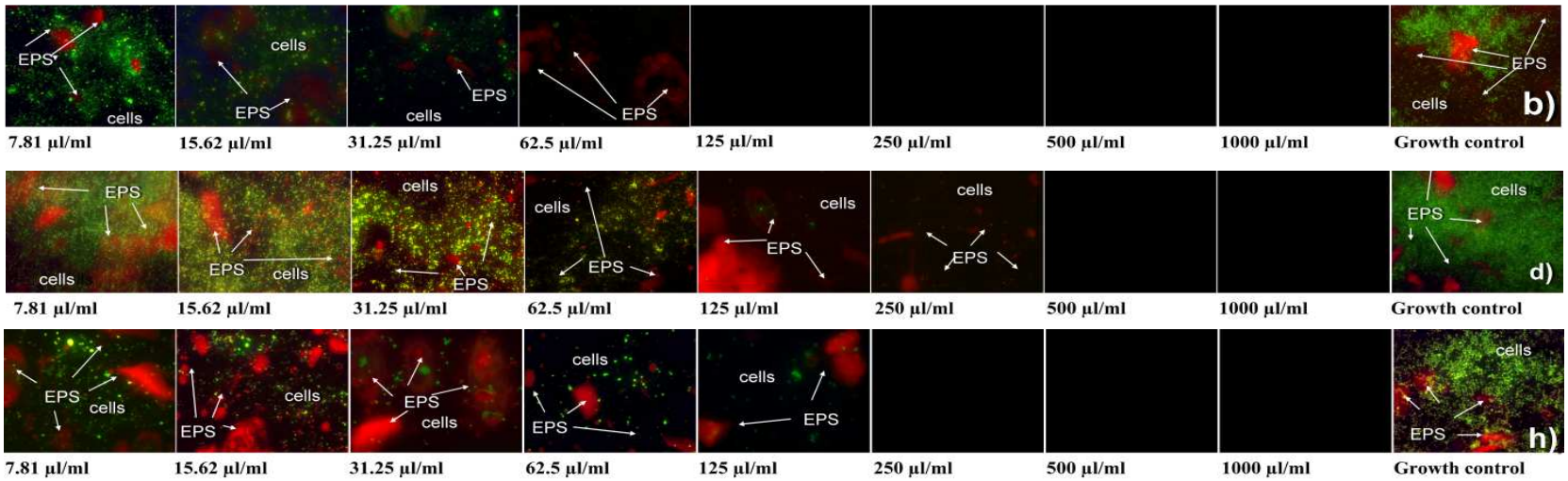

Figure 4. Influence of the test substance 4 on biofilms b) b) *E. coli* PMFKG-F2, d) P. *mirabilis* PMFKG-F4, h) *S. cerevisiae* PMFKG-F6. EPS – Extracellular polymer substance.

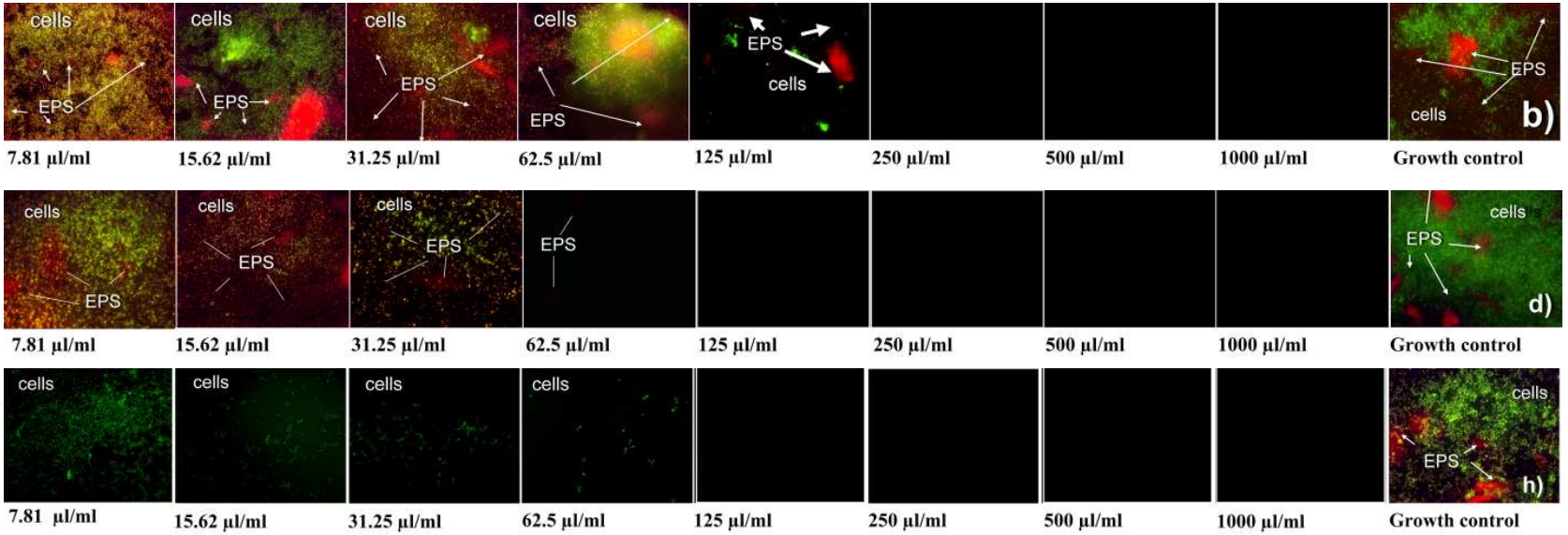

Figure 5. Influence of the test substance 5 on biofilms b) *E. coli* PMFKG-F2, d) P. *mirabilis* PMFKG-F4, h) *S. cerevisiae* PMFKG-F6. EPS – Extracellular polymer substance.