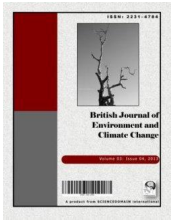

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Investigating the Functional and Structural Adaptation Changes of Biofilm Communities Toward Better Azo-dye Wastewater Treatment

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Authors' contributions

This work was carried out in collaboration between both authors. Author MB provided valuable suggestions for the study design, ran the model, performed the data analyses and wrote the manuscript. Author YT designed the study, supervised the model runs and the data analyses and reviewed the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to investigate the functional and structural adaptation changes in three biofilm communities purifying azo-dye contaminated wastewater.
Study Design: Three lab-scale sand biofilters were constructed for treating wastewater with azo-dye. The dye amaranth was chosen as model toxicant and its concentration was gradually increased in the wastewater from 10 mg/L up to 55 mg/L. The biofilters functioned for 26 days.
Place and Duration of Study: Laboratory of Environmental biotechnology, Sofia University "St. Kliment Ohridski", 2011-2013 year.
Methodology: The residual amaranth concentration, efficiency and rate of its removal were monitored. The diagnostics of the functional structure of the biofilms is based on a cross analysis of data from culturable, culture-independent (fluorescent *in-situ* hybridization - FISH) and digital (digital image processing) techniques.
Results: The efficiency of the biofilters varied from 88.35% up to 95.97%. Three phases of the azo-detoxification process were distinguished.
In the early phase of functioning (0-191 h) culturable *Pseudomonas sp*. had key role in azo-degradation as their part of the community was about 70% for the three biofilters.
In the late phase of functioning (191-455 h) the biofilters eliminated 2 times higher concentration of amaranth. The mean value for the part of the microorganisms from g. *Pseudomonas*, calculated on the base of FISH, remained unchanged (42%). Simultaneously the cultivation techniques showed

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significantly decreased part of *Pseudomonas sp*. (4-10%). This suggest an important role of the unculturable *Pseudomonas sp*.
In the ending period (455-623 h) the rate of amaranth removal was increased with 20%. Well-formed zones with high concentration of bacteria from g. *Pseudomonas* were found in the biofilms which indicated formation of cooperative relationships.
Conclusion: This study shows three-stage mechanism of development of amaranth degradation potential. It includes successive importance of culurable, unculturable bacteria and cooperation in the g. *Pseudomonas*.

Keywords: Biofilm; FISH; digital analysis; azo-dye; unculturable; cooperative relationships.

1. INTRODUCTION

The hydrosphere, lithosphere, atmosphere and biosphere are the essential parts of our planet. They are our environment and their protection from pollution and deterioration is the main global problem and responsibility of the human society, science and technologies. In 21 century the humankind is facing many ecological problems related with the depletion of resources and pollution of the environment. Although the wastewater treatment technologies aroused in order to preserve the health of the people from the big cities, the goals of the contemporary biotechnologies are much broader and reached far beyond the human's health. They are also a solution for many problems concerning the "health" of ecosystems, water purification and minimization of the human impact.

The protection of water against pollution more and more are being recognized as main topics in the context of climate change. On the 21 Conference of the Parties (COP21), taking place in 2016 in Paris, new water related initiative "Climate is water" was announced [1]. With this the international community officially had recognized the sustainable water use as one of the most important factors affecting climate change. Most of the considered measures are related with water reuse, development of more efficient and sustainable use of water resources by better controlling the demand and increasing water availability [2]. In the same time among the most vulnerable industries is the textile processing industry because the use of large amounts of water and recalcitrant compounds. They are highly colored and difficult to eliminate in the same time [3,4]. Because of this special attention should be paid on the efficient treatment of dye-contaminated wastewater.

The annual production of the synthetic dyes is estimated at 280 000 t [3]. One of the most widely used synthetic dyes are the azo-dyes whose use globally increased from 28 000 t in 1992 year [5] up to 53 500 t in 2002 year. These are the second most used synthetic dyes for 2002 year [6].

In the textile industry the azo-dyes are the most widely used types of dyes [4]. One of the characteristics of the textile coloring technologies is that much of the dye can't be absorbed from the fibers (between 2% and 50%). As result the wastewater becomes highly colored [7]. Some of the azo-dyes are visible in concentrations below 1 mg/L. Often the wastewater from textile processing contains dyes in concentration between 10-200 mg/L which makes them highly colored [7]. When such effluent is discharged in the surface waters, not only aesthetical problems occur but also the light penetration is reduced. This inhibits the photosynthesis and decreases the oxygen concentration in the receiving water body. The whole ecosystem becomes negatively affected [4,8,9,10]. Also because of their toxicity and carcinogenic properties many of the azo-dyes are hazardous for the human health [4,11,12,13,14,15,16].

Because of their adaptation potential and enzymatic diversity, the bacteria are most often used as azo-dyes biodegrading organisms. Some of the genera with high azo-degradation capacity are *Shewanella, Proteus, Bacillus, Klebsiella, Micrococcus* and *Pseudomonas* [17,18,19,20,21,22,23,24,25]. Single species of microorganisms may have a high decolorizing activity, and are suitable for studying the mechanisms of azo-dyes biodegradation but in practice wastewater treatment with single-species technology is impossible [17,26,27,28, 29,30,31,32]. The communities containing diverse microorganisms have advantages related with cooperation in pollutants biodegradation, availability of wide variety of enzymes ensuring complete mineralization [33], development of synergistic and co-metabolic relationships between biodegraders. For example one species

can reduce the azo-bond and another species can metabolize the resulted aromatic amines [32]. Another important advantage of the multi-species communities is that the biodegradation processes are carried out with higher rate and efficiency (because of the established microbial relationships) [34,35,36,37].

In lab-scale processes the microorganisms are able to eliminate azo-dyes with concentrations about 50-200 mg/L for period of time from 2 h. up to 48 h. [38]. In the real practice the most often used technologies for treating textile wastewater are the classical bioreactor with activated sludge or SBR bioreactors [39]. Yet, in the scientific field, many technological designs have been proposed in order to achieve more efficient dye treatment. For example biological aerated filters [40,41], up-flow anaerobic sludge blanket (UASB) bioreactors [42,43], membrane bioreactors [44,45,46], immobilized microorganisms [47,48], combination of biological treatment and nanofiltration or adsorption [49,50,51,52], constructed wetlands [53,54].

Generally the scientific investigations are concentrated in finding effective working biodegraders or bioreactors for efficient purification of the textile wastewater. Outside of the focus remains the adaptation of microbial biofilm communities as a tool for optimal azo-dye wastewater treatment. That is why in the center of this study is put on the development of azo-degradation capacity of three microbial biofilm communities. The adaptation of the communities is carried out under conditions of stepwise increase of the azo-dye concentration. The key technological and microbiological parameters are monitored. Special attention is paid on the data obtained with two non-culturable methods – FISH (fluorescent *in-situ* hybridization), combined with digital image processing.

2. MATERIALS AND METHODS

2.1 Design of the Experiment

Three model sand biofilters with volume 171 cm^3 and depth of 3 cm functioned in the Laboratory of Environmental biotechnology in Sofia University for 26 days. The biofilters treated wastewater (520-790 mL/day) polluted with toxicant - the azo-dye amaranth. The concentration of the xenobiotic was stepwise increased from 10 mg/L up to 55 mg/L. The technological parameters (residual amaranth concentration, decolorization efficiency, rate of amaranth removal) were monitored 2-3 times per day. Microbiological, FISH and digital analysis were made in three key points in the experiment: 191 h (early phase of functioning), 455 h (late phase of functioning), 623 h (end of the experiment).

2.2 Biological System

The biological systems are biofilms formed on the sand carrier. Activated sludge (AS) from WWTP (wastewater treatment plant) of Sofia city was used as inoculation material. Before immobilization AS was disintegrated by an ultrasonic disintegrator (3 × 10 s). In this way the floculas in AS were destroyed and homogenous microbial suspension, suitable for immobilization of microbial cells, was obtained.

2.3 Synthetic Wastewater

The used wastewater was synthetic and contained 1/ salt solution (NaH_2PO_4 – 3.5 g/L, K_2HPO_4 – 5.0 g/L, $(NH_4)_2SO_4$ – 2.5 g/L, $MgSO_4.7H_2O$ – 0.3 g/L, $FeSO_4$ – 0.05 mg/L, $CuSO_4$ – 0.01, $ZnSO_4$ – 0.005 mg/L, $CoCl_2$ – 0.005 mg/L, $MgCl_2$ – 0.005 mg/L, $CaCl_2$ – 0.005 mg/L, Na_2MoO_4 – 0.005 mg/L), 2/ 3% nutritious solution (NaCl 5 g/L; peptone – 10 g/L; yeast extract 5 g/L) and 3/ amaranth (from 10 to 55 mg/L).

2.4 Quartz Sand

As inert carrier for biofilters quartz sand was used. The sand particles size was between 0.08 and 0.16 cm and it was kindly provided by the Bistritza Drinking Water Treatment Plant (Sofia City, Bulgaria).

2.5 Amaranth

The model xenobiotic was supplied by Fluka Chemical Corp.

2.6 Technological Parameters

Residual amaranth concentration was determined spectrophotometrically (Utrospec 3000, PharmaciaBiotech), λ = 520 nm.

The efficiency of elimination of amaranth is calculated with the formula:

$$Eff = \frac{C_{in} - C_{eff}}{C_{in}} \cdot 100 \quad , \qquad (1)$$

where Eff is the efficiency of amaranth removal in %, C_{in} is concentration of amaranth in the influent in mg/L, C_{eff} is the concentration of amaranth in the effluent in mg/L.

The elimination rate of amaranth is calculated with the following formula:

$$V = (C_{in} – C_{eff}).Q, \qquad (2)$$

where V is the rate of amaranth elimination in mg/h, C_{in} is concentration of amaranth in the influent in mg/mL, C_{eff} is the concentration of amaranth in the effluent in mg/mL, Q is the flow in mL/h.

2.7 Microbiological Analysis

The key microbial groups were studied by the plate count techniques [55]. The aerobic heterotrophs were cultivated on Nutrient Agar and bacteria from genus *Pseudomonas* were cultivated on Glutamate Starch *Pseudomonas* Agar. (Note: The samples are collected from 3 layers of the biofilters (upper, middle and bottom) and are analysed separately. Here only mean values are presented). *Pseudomonas* sp. ratio for culturable microorganisms was calculated on the base of aerobic heterotrophs.

2.8 FISH Analysis

The sample fixation, immobilization, dehydration and permeabilization were made according [56]. Oligonucleotide probe for microorganisms from genus *Pseudomonas* [57] was used to investigate the abundance and the spatial distribution of the target bacteria, key participants of amaranth biodegradation. The used probe was Cy3 5'-labelled 15-mer oligonucleotide with sequence 5'-GCT GGC CTA GCC TTC-3' [58]. As a control non-sense probe was used – NON338 (5'-ACT CCT ACG GGA GGC AGC-3') [57]. The hybridization was performed with 20% formamide. After the hybridization the samples were counterstained with DAPI (4',6-diamidino-2-phenylindole) (AppliChem GmbH). The pictures were taken with fluorescent microscope Leica Microsystems DFC310FX, at 400x magnification.

2.9 Digital Image Analysis

The digital processing of the pictures was made by using the computer program *daime* [59]. The pictures are first segmented by using custom threshold criteria and then data for the area of interest was collected. *Pseudomonas* spp. ratio to the total quantity of the microorganisms was calculated on the basis of the pictures with DAPI and the pictures with the specific probe.

3. RESULTS AND DISCUSSION

Based on the received data for the three biofilters, the azo-degradation process can be divided on three time periods: early phase (0-191 h), late phase (191-455 h) and ending period (455-623 h). In the early phase the amaranth concentration was low (10-30 mg/L) and the decolourization efficiency was high, almost without fluctuations (Table 1). The biofilm community was in its early phase of development and adaptation toward amaranth biodegradation. In the late phase the amaranth concentration was raised from 30 mg/L up to 45 mg/L. The biofilters were well adapted and they reached highest efficiency (Table 1). In the ending period (455-623 h.) the xenobiotic concentration got close to the critical one (from 45 mg/L up to 55 mg/L) which destabilized the functioning of the biofilters. This reflected as decreased efficiency of amaranth removal for two of the three biofilters (Table 1).

Several analyses were made on the biofilm community from the biofilters in order to study the adaptation changes during the detoxification processes. As it is described in "Materials and methods", the cultivation analyses were interpreted together with culture independent techniques as FISH and digital image analysis. Microorganisms of g. *Pseudomonas* were chosen as key amaranth biodegraders [60]. The data for these microorganisms as part of the biofilm community is shown on Fig. 1.

In the early azo-detoxification phase (0-191 h) main role played the culturable *Pseudomonas sp*., which was between 67% and 70% for the three biofilters. For this period digital image analysis showed 37% of microorganisms of g. *Pseudomonas* for biofilters 1 and 3, and 53% for the biofilter 2. The FISH pictures from this period of time suggested a low abundance of the target bacteria for the three biofilters (Table 2, pink-red fluorescent signal).

Table 1. Key technological parameters for amaranth removal in the three biofilters

	Early phase			Late phase			Ending period		
	Residual concentration, mg/L	Rate of removal, mg/h	Efficiency of removal, %	Residual concentration, mg/L	Rate of removal, mg/h	Efficiency of removal, %	Residual concentration, mg/L	Rate of removal, mg/h	Efficiency of removal, %
Biofilter 1	1.75	0.41	88.35%	4.04	0.99	90.60%	4.91	1.14	89.76%
Biofilter 2	1.33	0.42	90.92%	1.72	0.91	95.73%	2.90	1.09	94.92%
Biofilter 3	1.23	0.43	92.40%	2.83	0.94	93.35%		1.21	93.71%

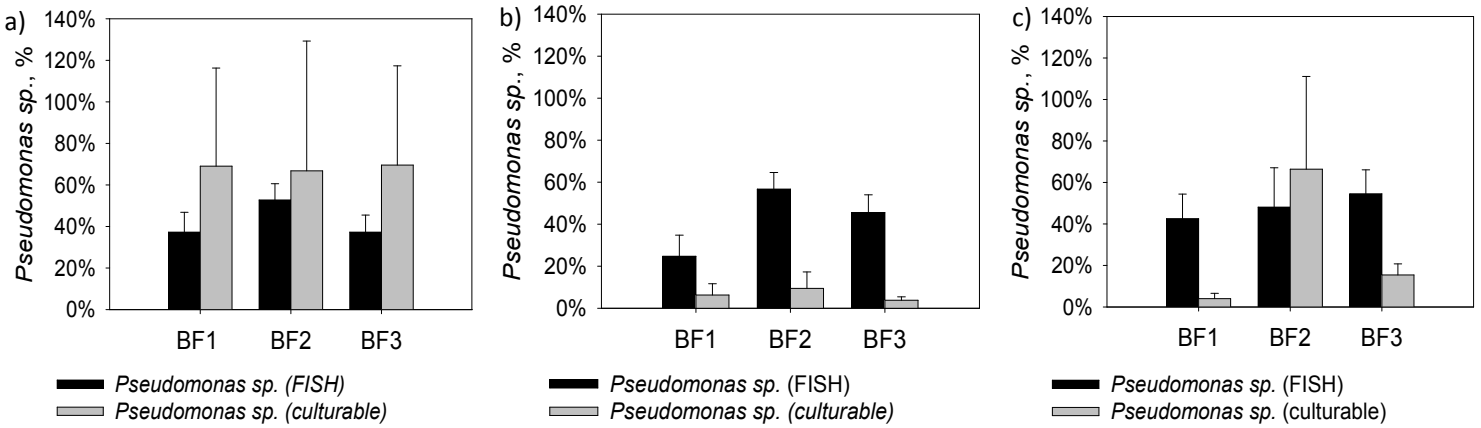
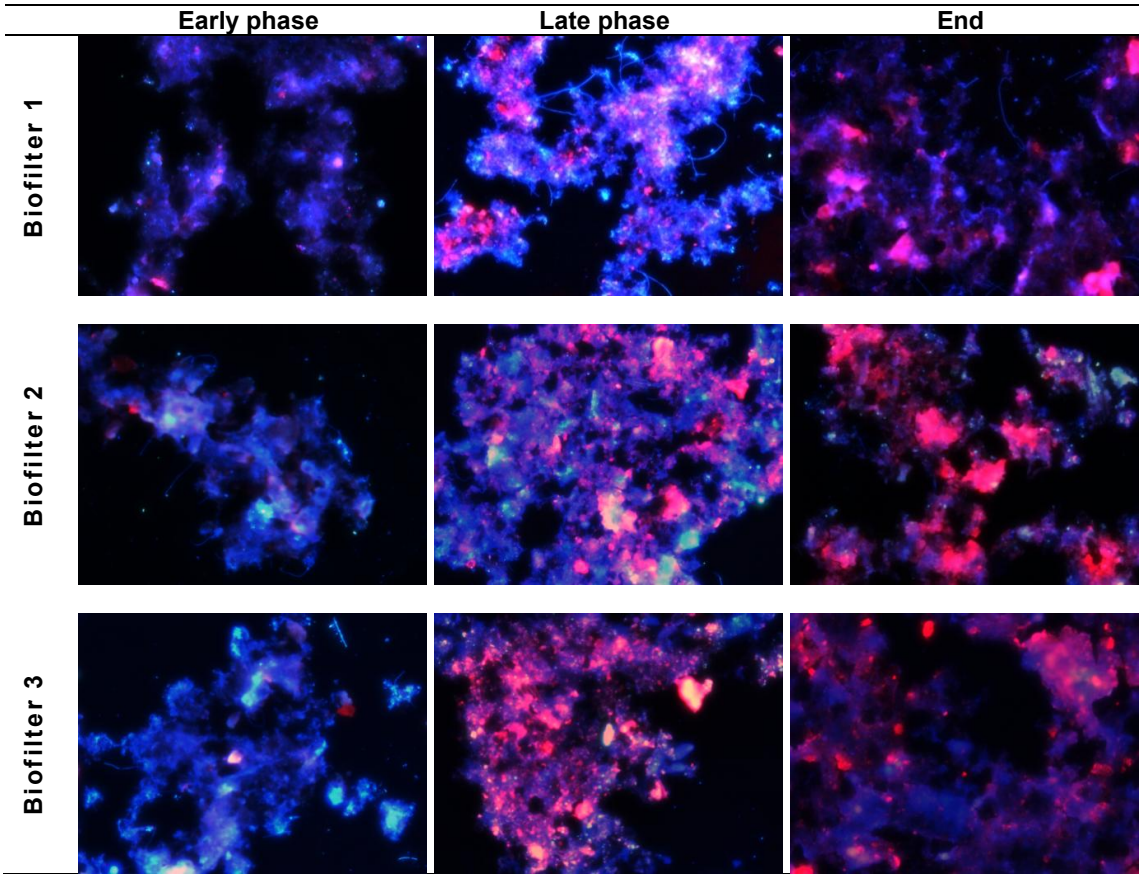


Fig. 1. *Pseudomonassp.* ratio for the three lab-scale biofilters (BF), calculated on the base of FISH pictures (*Pseudomonas sp.* (FISH)) and on the base of aerobic heterotrophs (*Pseudomonas sp.* (culturable)) for: a) Early phase; b) Late phase; c) Ending period of the detoxification processes

Table 2. FISH images from the early, late phase and the end of the process for the three biofilters. Blue fluorescent signal is from DAPI and the pink-red signal is obtained from the *Pseudomonas*-specific oligonucleotide probe



In the late phase of functioning of biofilters the inflow amaranth concentration was raised 2 times. That is why the residual concentrations in the three biofilters were slightly increased. Nevertheless the efficiency of amaranth removal was even higher in the all three biofilters. These results suggested development and adaptation of the biofilm community toward azo-dye biodegradation. The FISH images confirmed this. The fluorescent signal from g. *Pseudomonas* in the pictures in Table 2 (late phase) is significantly increased. In the same time the culture techniques showed significantly decreased part of the microorganisms from g. *Pseudomonas* (from 7 up to 18 times) (Fig. 1b). Because FISH gives information not only for culturable but also for unculturable target bacteria it can be suggested that unculturable microorganisms from g. *Pseudomonas* play significant role in azo-degradation in the conditions of the increased toxicity.

In the ending period the amaranth concentration and the rate of its removal were additionally increased from 45 mg/L up to 55 mg/L for the amaranth concentration and from 0.91-0.99 mg/h up to 1.09-1.21 mg/h for the rate of amaranth removal. In this period of time the biofilm communities carried out azo-degradation process under strong toxic pressure but their efficiency hasn't been decreased significantly (Table 1). The cultivation techniques showed an increased part of *Pseudomonas sp*. for the biofilter 2 with 56.95% and for the biofilter 3 with 11.62%. The digital image analysis demonstrates an increased part of the target *Pseudomonas sp.* in biofilter 1 (with 17.82%) and biofilter 3 (with 8.99%). These results haven't been conclusive for the role of cultural and unculturable part of the microorganism from g. *Pseudomonas*. However the mean values for the three biofilters (culture technique – 22.11% increase; digital image analysis – 6.07% increase) suggested that the biofilm community additionally developed. FISH

technique demonstrated structural changes in the communities adapted towards amaranth biodegradation in these unfavorable conditions. From the pictures in Table 2 it is seen that the key biodegraders (*Pseudomonas sp.*) were found grouped in zones with high metabolic activities. Most probably the high toxicity stimulated microorganisms to the adaptation and concentration in "hot spots" for high azo-degradation. These zones are indirect proof for established cooperative microbial relations such as synergistic, symbiotic and co-metabolic relationships. This represents adaptation on higher level, where the microorganisms aren't just raised their numbers but they become interdependent in the common process – the azo-degradation of the xenobiotic Amaranth.

4. CONCLUSION

The presented study showed that a cross analysis of data from plate count, culture independent and digital techniques could reveal some of the specific mechanisms for adaptation of azo-degrading microbial community. It was established that a biofilm adaptation process with stepwise amaranth increase for 26 days consisted of three stages with specific features: 1) early phase when the culturable *Pseudomonas sp*. had dominant role and increase their amount in the course of adaptation; 2) late phase when the community developed and the unculurable microorganisms from g. *Pseudomonas* had key importance; 3) end stage, when under the high toxicity pressure, zones with high concentration and high metabolic activity of the target bacteria were formed. From these results it is seen that the microorganisms from g. *Pseudomonas* developed cooperative relationships for maintain the high efficiency of azo-dye removal under the strong xenobiotic inhibition.

Obtained adaptation algorithm and mechanisms of the biological control will contribute to the increase the effectiveness and applicability of the wastewater treatment technologies. All these reveal the potential for acceleration of the detoxification technologies as well as to improve the quality of water in the water receivers – natural water bodies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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