CHARACTERIZATION OF BIOFILMS FROM SELECTED SYNTHETIC MATERIALS USED IN WATER DISTRIBUTION SYSTEM

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ABSTRACT

Materials like polyvinyl chloride (PVC), polypropylene (PP), ultra high molecular weight polyethylene (UHMW-PE) are used for the construction of drinking water supply systems. It was found that regardless of the type of material the distribution network is built of, microorganisms formed biofilms on every available surface. The pipes material plays a key role in terms of biofilm formation. Important factors are the surface roughness, adhesives, plasticizers, stabilizers, which can be a source of nutrients for bacteria. The metabolic activity of microorganisms on polymer materials, induces migration of compounds from the material into water. The aim of this study was to present the differences in the structure and the metabolic profile of biofilm formed on the technical materials.

Keywords: biofilm, metabolic profile, drinking water, bacteria consortia, phenotypic microarray (PM)

INTRODUCTION

Drinking water distribution systems are considered as an oligotrophic environment with low content of carbon, nitrogen and phosphorus sources (Chandy *et al.,* 2001; Hu *et al.,* 2005). Microorganisms in water supply systems form a biofilm, adapting to grow in this specific environments (Rodriguez *et al.,* 2013).

Formation of biofilm in water distribution system consists of several stages: surface conditioning, irreversible attachment, colonization, detachment. They are regulated by physical, chemical and biological processes (Chavant *et al.,* 2002, Nikolaev *et al.,* 2007). The first phase is a nonspecific and reversible binding the bacteria cell to the surface. In the next phase, the bacteria secrete the insoluble extracellular polymeric substances (EPS) and form the hydrogel matrix in which cells are embedded (Manuel *et al.,* 2007). With the increasing amount of EPS a mature biofilm is developing. The main components of EPS are polysaccharides, proteins, glycolipids, phospholipids, as well as nucleic acids and enzymes. The extracellular matrix, beside maintaining a stable microconsortia of different species, is used to uptake nutrients from water, protect against harmful chemicals (antibiotics, disinfectants), facilitate horizontal gene transfer and facilitate intercellular communication, which allows the regulation of the gene expression associated with the temporary adaptation to environmental conditions (phenotypic variation) (Boe-Hansen *et al.,* 2002; Leroy *et al.,* 2007; Wotton, 2011).

Among the materials used for the construction of water supply systems plastics are used increasingly often (PVC, PP, UHMW-PE). It was found that regardless of the type of material the distribution network is built of, microorganisms colonize every available space (Parizzi *et al.,* 2004). The pipes material plays a key role in terms of biofilm formation. Important factors include the surface roughness, adhesives, plasticizers, stabilizers, which can be a source of nutrients

(Batte *et al.,* 2003, Manuel *et al.,* 2007; Sitarska *et al.,* 2009, Chavant *et al.,* 2002, Djordjevic *et al.,* 2002, Momba and Makala, 2004). It was also found that there is a phylogenetic diversity of biofilm microorganisms depending on the technical material (Schwartz *et al.,* 2003).

The metabolic activity of microorganisms on polymer materials, induces migration of compounds from the material into water (van der Kooij, *et al.,* 1995). Compounds migrating from the high density polyethylene can be 2,4-ditertiary-butylphenol, esters, aldehydes, ketones, aromatic hydrocarbons and terpene. Some unidentified compounds which were detected as well may result from the oxidation of secreted substances. Such compounds as hexanal, octanal, nonanal and decanal can migrate from PVC (Skjevrak *et al,* 2003; Skjevrak *et al.,* 2005). This allows to see how important is the selection of appropriate materials for the construction of the water installation and ensuring the microbiological stability of water. The results of research about biofilm formation can be used in the selection of technical materials for the construction of various types of water installations, as well as in the design of technical processes.

The aim of this study was to present the differences in the structure and the metabolic profile of biofilm formed on the technical materials.

EXPERIMENTAL PROTOCOLS

Biological material and biofilm formation

Bacterial strains were isolated from the biofilm of drinking water supply network in Wrocław. Selection of microorganisms was based on the identification of high ability to adhere and presenting resistance against antibiotics and chemotherapeutics. Selected 11 bacterial strains were accepted as a model group for biofilm formation in water distribution system. Among the selected strains, there was no antagonistic effects. The biofilm culture system consisted of a 6000 ml flask of sterile synthetic tap water, 10000 ml outflow flask, a peristaltic pump (Cole Palmer) and magnetic stirrer and CDC biofilm reactor (BioSurface Technologies Corp) with holders for analysed materials (PVC – polyvinyl chloride, PP – polypropylene, UHMW-PE – ultra high molecular weight polyethylene). Reactor contains disc coupons on coupon holder rods suspended from the reactor lid. This construction allows time-course studies of biofilm formation on the materials used to construction of water supply network in the reactor. Materials were analysed as disc coupons (12.7 mm diameter x~ 3.8 mm thickness) provided by the BioSurface Technologies Corp and dedicated for the CDC reactor (Fig. 1). The reactor content was inoculated with selected strains (1 ml of strains culture OD_{540} = 1) and operated under continuous stirring for 45 days. Next stage was a continuous flow of synthetic tap water (1.2 mmol/l $NaHCO_3$, 0.54 mmol/l $MgSO_4 \times 7H_2O$, 0.2 mmol/l $CaSO_4 \times 2H_2O$, 0.004 mmol/l K_2HPO_4, 0.002 mmol/l KH_2PO_4, 0.08 mmol/l $(NH_4)_2SO_4$, 0.17 mmol/l NaCl, 36 10^6 mmol/l $FeSO_4 \times 7H_2O$, 0.011 mmol/l $NaNO_3$, 0.2 mmol/l $CaCO_3$, pH 8.2) (Morrow and Almeida, *et al.,* 2008). The continuous flow experiment was carried out for 30 days. This is the sufficient time to the formation of the young biofilm structure.

Metabolic fingerprint

Biolog microplates PM1-PM4 were used to analyse substrate utilization patterns of biofilms. Biolog PM1 and PM2 plates contain 190 wells with different carbon sources, PM3 plate contain nitrogen sources and PM4 contain sulfur and phosphorus sources. Each of these plates contained blank wells with no substrate. Every well contains the redox dye tetrazolium which was reduced by NADH produced by microbial metabolic pathways. The rate of colour development correlated with bacterial rate of metabolism.

Biofilm final technical materials sterile swab was taken and suspended in a solution of 0-IF to obtain a transmittance of 42%. Then 8 ml of a suspension in a solution of 0-IF poured into 40 ml IF-0 + dye (39.52 ml IF-0 and 0.48 Biolog Dye Redox MixD 100X). In the prepared mixture was poured into 24 ml of sterile trough and added to a microplate PM1 and PM2, and 100µl per well. The remaining 24 mL was added 0.24 ml of a solution of iron citrate 100x, the mixture was pipetted into microplate PM3 and PM4 after 100µl per well. Plates were placed in an incubator with the temperature set at 22^0C. Following the incubation at 22 °C, data were collected every 15 minutes with BIOLOG OmniLog.

Biofilm structure analysis

Scanning electron microscopy (SEM) allows to evaluate the area of biofilm surface, as well as the technical material. Analysis conducted with
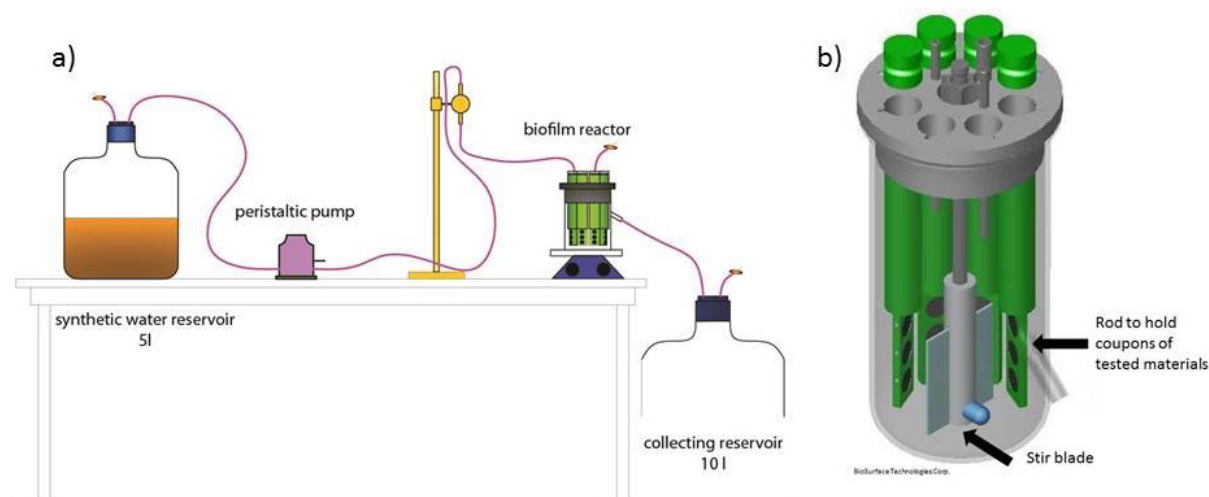
Figure 1. Scheme of biofilm culture system (a) and biofilm reactor (b).

confocal laser scanning microscopy (CLSM), through the optical cross-sections, allowed to assess the shape and structure of the biofilm. These mutually complementing microscopic methods give a broad view on the spatial structure of the biofilm on the tested materials.

Biofilms were dried and sputter-coated with a gold thin film in a sputter Scancoat 6 (Oxford). The samples were examined using SEM Leo Zeiss 435 Vp (Oberkohen).

The viability of biofilms was determined using the Live / Dead BacLight Bacterial Viability Kit from Invitrogen. Samples were prepared in accordance with the manufacturer instruction. A mixture of dye (propidium iodide, SYTO 9 and 1: 1) in the amount of 50 μl was applied on biofilm samples. The entire surface of the material was covered with the solution. The prepared samples were incubated in the dark for 1 hour. Biofilm samples were gently rinsed with 0.9% saline solution. After rinsing, images were obtained using a CLSM Nikon. The number of optical sections in the horizontal plane was uniquely matched to each sample depending on the thickness of the biofilm, with maintaining optimum optical resolution of the z axis.

Data and statistical analysis

The data obtained from microarray system PM OmniLog have been converted from the csv format to xls, and then imported into MATLAB software. The matrix data for each substrate were transformed with non-parametric method based on regression curve fitting (Local Area robust loess), which was resistant to the outlying objects.

Collected data were divided by type of substrate to form 4 matrix named: carbon, nitrogen, sulfur and phosphorus.

In each of the prepared matrix operation was performed subtracting the value of the blank sample value in a given time interval. The next stage included the logic conditioning, where each negative value had been changed to 0. Data Visualization included preparation:

- the appointment of one substrate from each group, which were characterized by the highest level of consumption through the use of the "Quick sort" algorithm in ascending order.
- consumption of substrates with the "jet" scale – the scale of colours (called colour map) was selected for easier results interpretation.

The PCA algorithm has been used to compare three different polymers. Implemented function of PCA in MATLAB computing software (MathWorks Inc., Natick, MA, USA) has been applied to receive first three principal components. After all calculations were finished, data points have been plotted in three-dimensional space using principal components as axis.

RESULTS

The data obtained from the BIOLOG OmniLog system were analysed as catabolic activity of biofilms. It was found that microorganisms of all samples mostly used carbon compounds as a source of main nutrients. The highest level of consumption of carbon sources was found in the sample of biofilm from polypropylene

(65.79%). Analysis of 95 of nitrogen compounds as a source of nutrients indicated that the highest level of consumption shown also in biofilm bacteria from polypropylene (28.42%), bacteria from UHMW-PE utilized 16.84% and PVC utilized 12.63% available nitrogen sources. Only 2 substrates from 59 of phosphorus compounds and also 2 organic sources of sulfur were utilized in biofilm from and ultra-high molecular weight polyethylene (Fig. 2).

The best metabolized compounds in each group (C, N, P, S) for the biofilm microorganisms on the polymeric materials were determined. Among the 190 carbon substrates, dihydroxy acetone for PP and PVC biofilms, and the microorganisms forming a biofilm on UHMW-PE had the highest catabolic activity relative to α-D-glucose (Fig. 3). Moreover, 95 nitrogenous compounds were analysed. The microorganisms forming biofilm on the PVC and PP showed the highest affinity for L-cysteine, but the most used source of nitrogen for the consortium on UHMW-PE was N-acetyl-D-galactosamine (Fig. 4).

The use of phosphorus and sulfur substrates was very low. Among the analysed phosphorus compounds, carbamyl phosphate catabolized by bacteria from PP and UHMW-PE and thiophosphate catabolized by microorganism from PVC biofilm were determined. Bacteria forming biofilm on the PVC, PP and UHMW-PE showed the highest affinity for tetramethylene sulfone as a sulfur source.
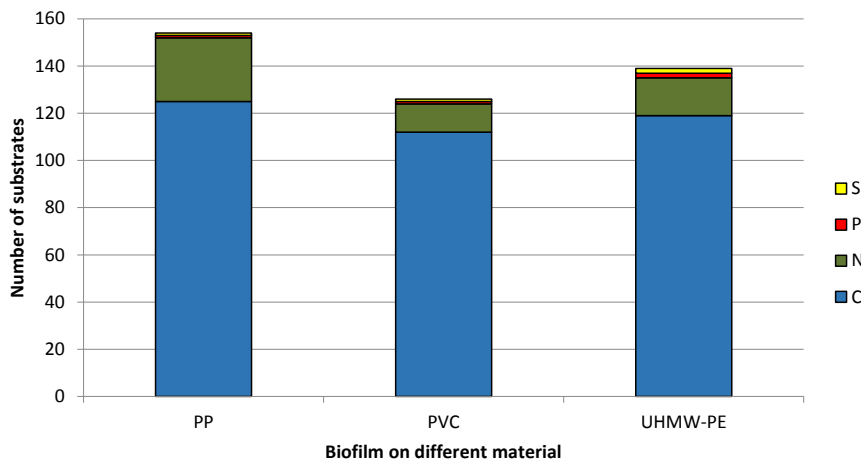

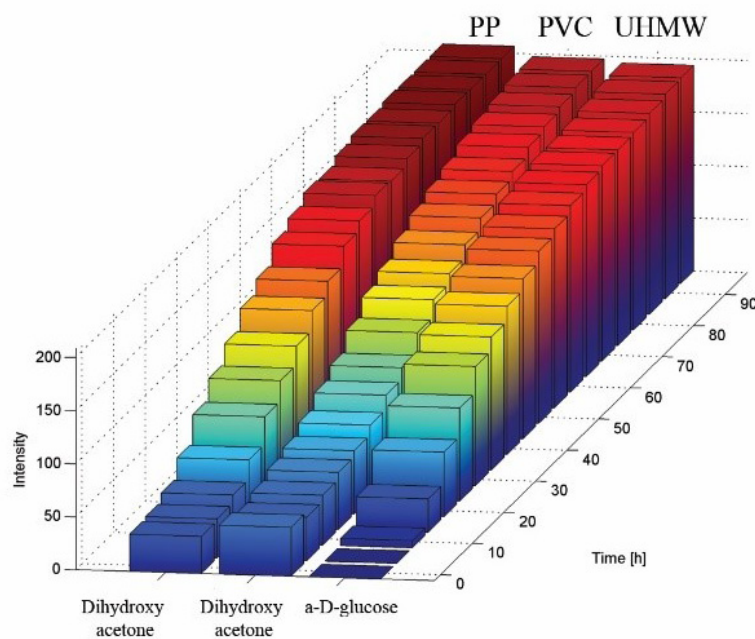**Figure 2.** Consumption of carbon, nitrogen, sulfur and phosphorus sources

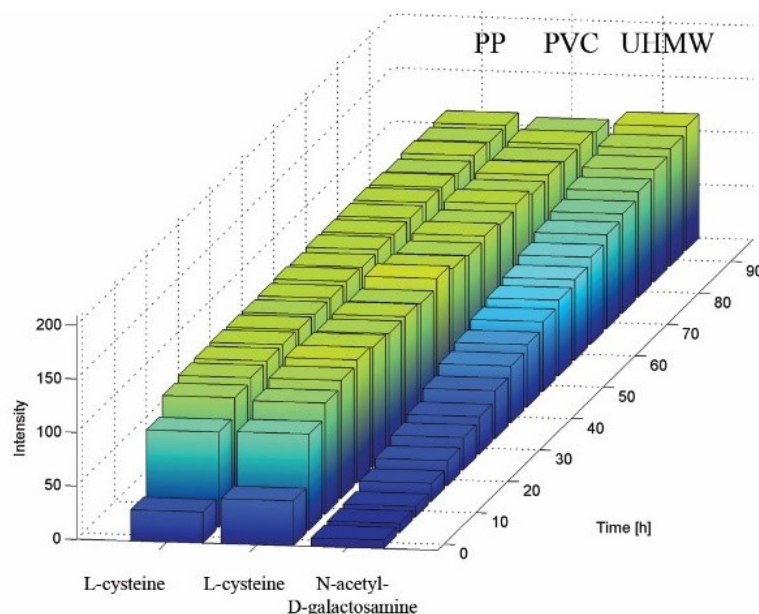**Figure 3.** The best metabolized carbon compounds.

Figure 4. The best metabolized nitrogen compounds

On the basis of the data of metabolic activity, it was found that microorganisms of the biofilms formed on the each of the three materials catabolized 100 carbon compounds and 12 nitrogen sources. There was no phosphorus substrate catabolized by three analysed consortia, but one sulfur compound was reported (tetramethylene sulfone). Unique compounds catabolized by biofilm microorganisms were also identified. Characteristic biofilm metabolic profile was determined for each material (Table 1).

On the basis of the analysis of consumption of all tested substrates, PCA was made over normalized value of all substrates utilization. The first 3 principal components described the 94.49% of explained variance (78.93%, 12.33% and 3.23%) and clustered apart samples of biofilm coming from the different materials. Shapes of plotted results in projection on PC1 and PC3 (Fig. 5c) shows that the materials used in experiment have

similar behaviour to each other, while on projection on PC1 and PC2 there is a significant difference between PP and PVC with UHMW-PE (Fig. 5b). Figure 5 shows similarity of PVC and UHMW-PE. Biofilm formed on the PP, despite a comparable shape of the graph shape, significantly stands out from the analyzed materials. Probably the key role played unique compounds catalysed by biofilm bacteria on PP.

Microscopic characterization of biofilm included an assessment of the size and shape by scanning electron microscope (SEM), thickness and viability by confocal laser scanning microscopy (CLSM).

A layer of living cells coated by a layer of dead cells formed on the biofilm based on polypropylene. Microorganisms on this surface formed clusters of microcolonies with broad channels (Fig. 6c, d, e). The biofilm thickness did not exceed 25 microns. On the basis of SEM

Table 1. The list of the unique substrates catabolized by biofilm bacteria.

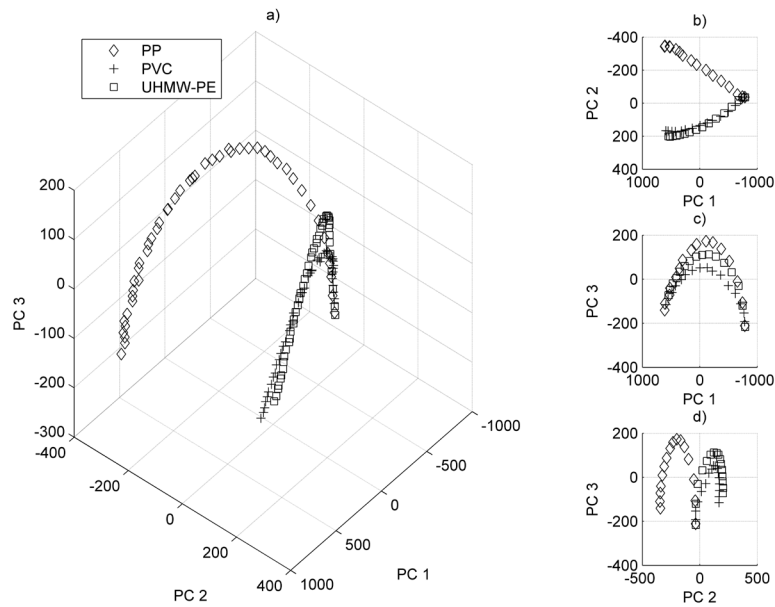| Source	PVC	PP	UHMW-PE
C	Sorbic acid	D-Glucosaminic acid, a-Hydroxybutyric acid, m-Inositol, D-Arabitol, g-Amino-N-Butyric acid, Capric acid, 4-Hydroxybenzoic acid, Quinic acid, L-Histidine, Hydroxy-L-Proline, L-Isoleucine, L-Valine, D,L-Carnitine, Putrescine	D-Aspartic acid a-Hydroxyglutaric acid-g-Lactone Tricarballylic acid 3-Methylglucose L-Tartaric acid L-Ornithine
N		L-Alanine, L-Histidine, L-Isoleucine, L-Lysine, Ethanolamine, Putrescine, Agmatine, Xanthine, g-Amino-N-Butyric acid, DL-a-Amino-Caprylic acid, a-Amino-N-Valeric acid, Ala-His, Gly-Gln	Ala-Leu
S			L-Methionine Sulfoxide
P	Thiophosphate		Carbamyl Phosphate

Figure 5. PCA analysis results. A) Score plots of PC1 vs. PC2 vs. PC3. B) Score plots of PC1 vs. PC2.
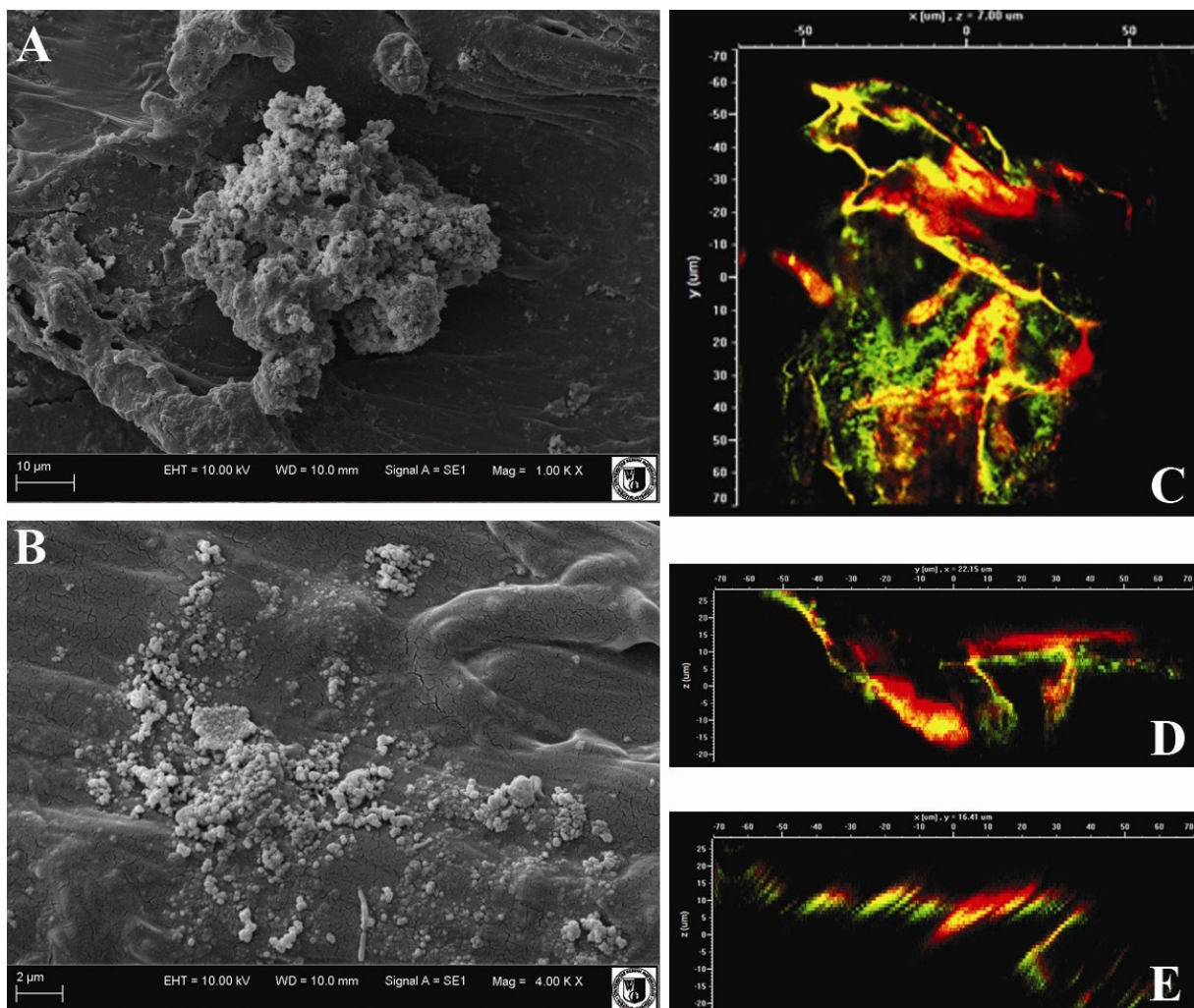C) Score plots of PC1 vs. PC3. D) Score plots of PC2 vs. PC3

Figure 6. Biofilm structure on polypropylene. A) SEM analysis of biofilm, ×1000 magnificence.
B) SEM analysis of biofilm, ×4000 magnificence. C) CLSM analysis of biofilm, xy axis. D) CLSM analysis
of biofilm, zy axis. E) CLSM analysis of biofilm, zx axis. Green – living cells, red – dead cells

analysis it may be considered that a layer of dead cells detected by CLSM analysis were a mineralization of biofilm structure (Fig. 6a). While not mineralized, microcolonies, are surrounded by a large amount of EPS (Fig. 6b). Polyvinyl chloride was the material where microbes have formed the most complex structure of biofilm. There were numerous channels between microcolonies and the thickness of biofilm was 30 microns. Live and dead cells form intertwining aggregates (Fig 7c, d, e). SEM analysis showed the microorganisms adhered on the material surface and formed a continuous layer and colonized all cracks in PVC (Fig. 7a, b).

Biofilm on polyethylene characterized with the smallest thickness (4 microns) formed by layers of living cells among which were aggregates of dead cells (Fig. 8c, d, e). Polyethylene surface on the SEM images shows a relatively small colonization by microorganisms. Biofilm lacks the continuous structure, and was present as aggregates of microcolonies with densely packed cells and a relative low amount of EPS (Fig. 8a, b).

DISCUSSION

Biofilm formed on PVC was thicker and had a very complex structure with numerous channels. Moreover, in his research Djodjevic concluded that on polyvinyl chloride there was no positive correlation between cell motility and biofilm formation (Djordjevic *et al.,* 2002). The adhesion on PVC is closely related to the hydrophobicity of the cells (Takahashi *et al.,* 2010). On the basis of the results results, it was found that the metabolic profile of a biofilm on PVC indicated the ability
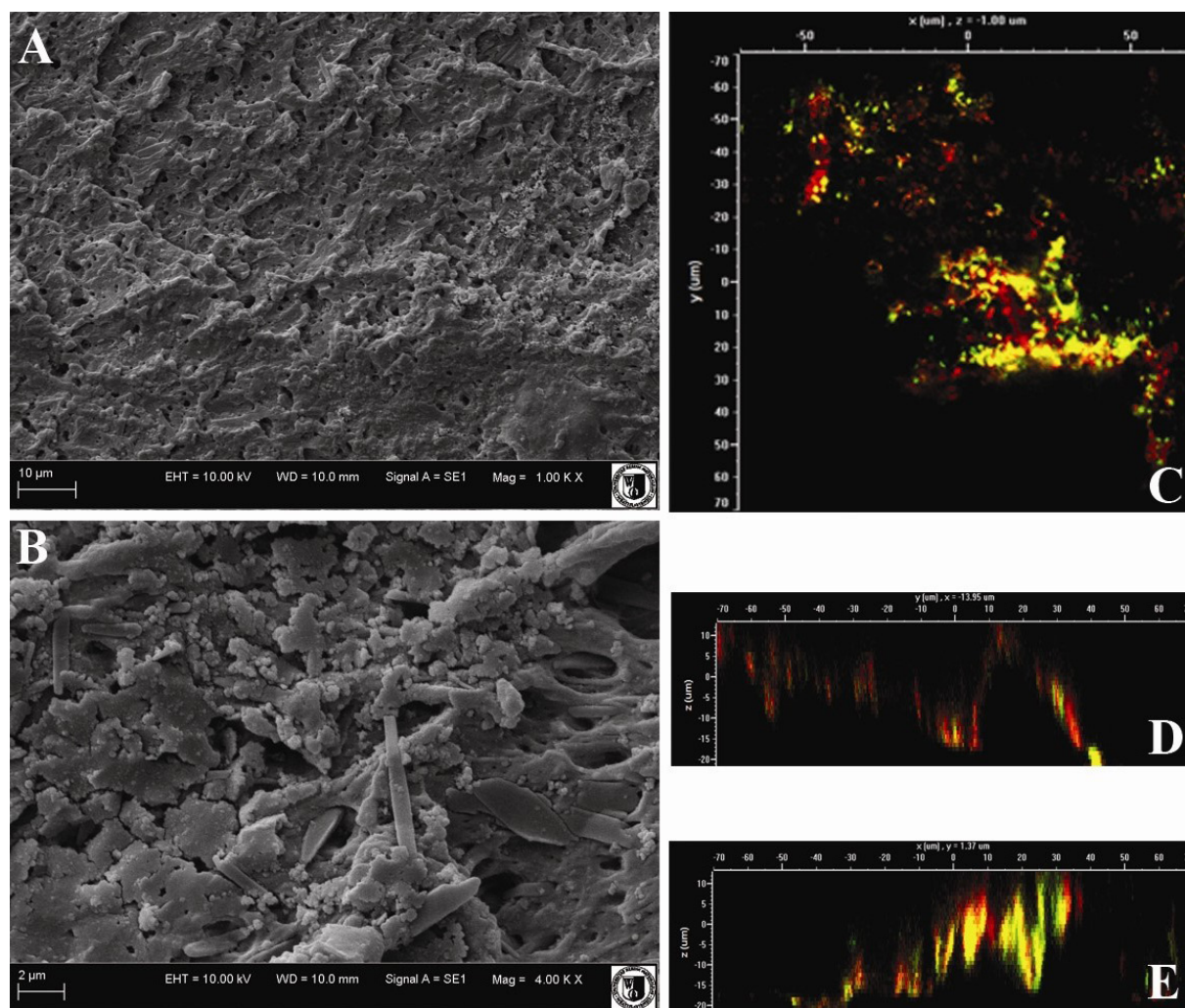

Figure 7. Biofilm structure on polyvinyl chloride. A) SEM analysis of biofilm, ×1000 magnificence. B) SEM analysis of biofilm, ×4000 magnificence. C) CLSM analysis of biofilm, xy axis. D) CLSM analysis of biofilm, zy axis. E) CLSM analysis of biofilm, zx axis. Green –living cells, red – dead cells

Figure 8. Biofilm structure on UHMW-PE polyethylene. A) SEM analysis of biofilm, ×1000 magnificence. B) SEM analysis of biofilm, ×4000 magnificence. C) CLSM analysis of biofilm, xy axis. D) CLSM analysis of biofilm, zy axis. E) CLSM analysis of biofilm, zx axis. Green – living cells, red – dead cells
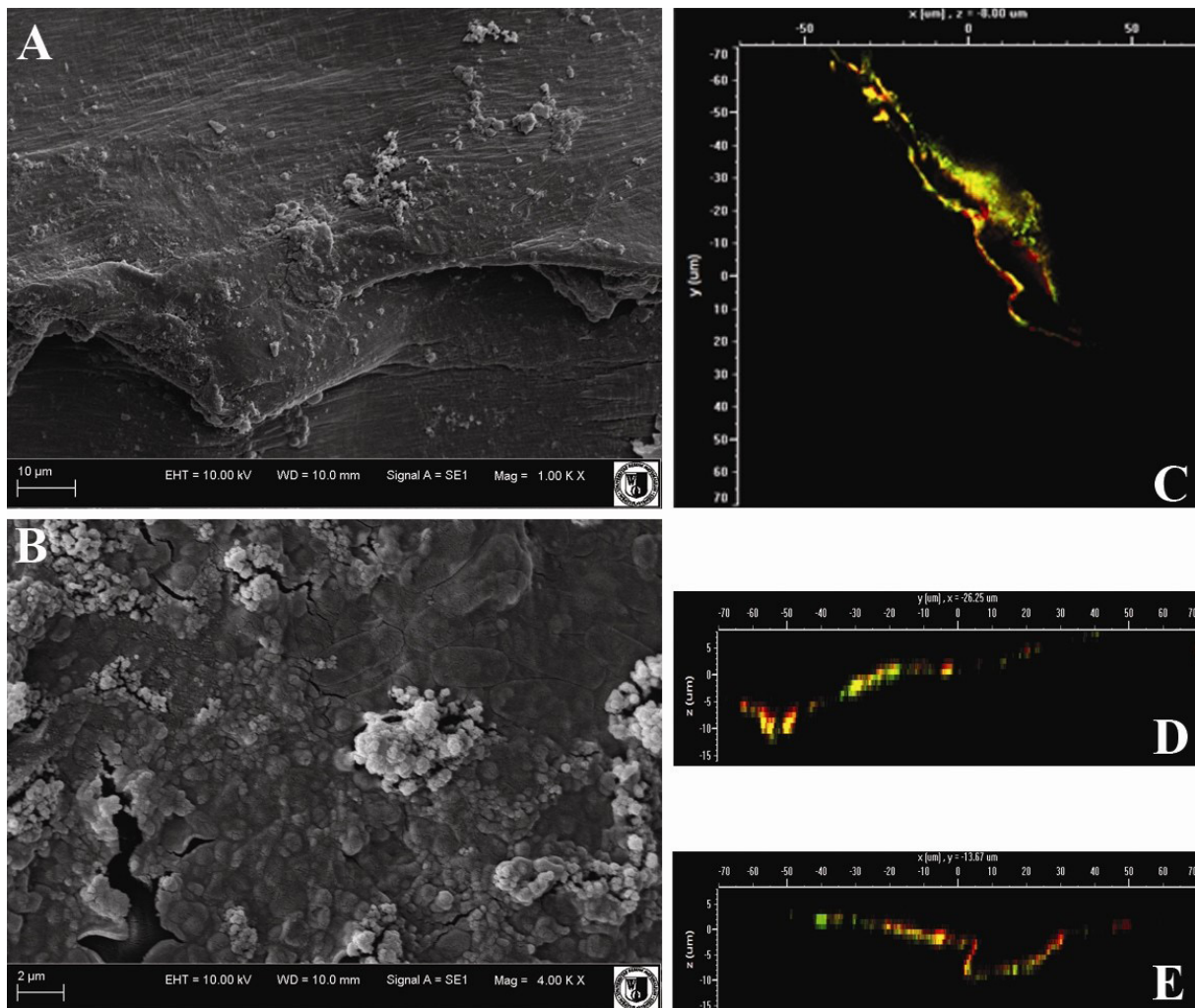
of microorganisms to degrade sorbic acid, which is used as an antibacterial coating. In his work, Farber noted that this chemical compound greatly facilitated the bacterial adhesion to the surface. Paradoxically, this antibacterial substances, facilitates the adhesion of microorganisms on the hydrophobic surface of polyvinyl chloride. This process is associated with production of adaptive enzymes and decomposing this compound (Farber *et al.,* 1995).

It was confirmed that a metabolic profile of a biofilms formed on various materials indicates specific metabolic activity which may be inversely proportional to an increase of biofilm mass (Simoes *et al.,* 2007). Polypropylene is a material which is highly colonized by microorganisms (Traczewska and Sitarska, 2012). Bellon also confirmed the fibrous structure of polypropylene promoted bacterial adhesion (Bellon *et al.,* 2001).

Henne noted that the varied species structure of a biofilm largely depended on the type of surface (Henne *et al.,* 2012).

Yu confirmed significant differences in the structure of the biofilm, depending on the material on which it is formed. He observed that the materials such as polyethylene, polybutylene, were well colonized by microorganisms. Yu also noted that the bacteria can release different compounds from plastic pipes (Yu *et al.,* 2010). In this study, ultra-high molecular weight polyethylene was used, which was characterized with high resistance to abrasion.

According to Moskovitz, decomposition of L-methionine sulfoxide suggested the presence of methionine sulfoxide reductase, which is an enzyme counteracting environment stress and catalysed decomposition sulfoxide to methionine (Moskovitz *et al.,* 1997).

The interactions of the biofilm microorganisms are not fully understood. Further study of interspecies communication (quorum sensing) and metabolic pathways of various microorganisms in biofilm may play an important role in both adhesion and biofilm maturation processes (Donlan, 2002).

CONCLUSIONS

1. It was found that on each of the three materials, microorganisms catabolized unique compounds which were related to the bacteria adaptation to the biofilm formation on different surface.

2. The greatest diversity of active metabolic pathways characterized biofilm grown on polypropylene.

3. Metabolic profile of a biofilm from different materials can be used as a method of monitoring the physiological state of microorganism. Information about potential metabolic biofilm may be used to determine the viability of the bacterial consortium in extreme environmental conditions.

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