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Preliminary experiments into colonization of microorganisms from activated sludge on different types of plastics

Preliminarni poskusi kolonizacije različnih tipov plastike z mikroorganizmi iz aktivnega blata

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Abstract: The presence of plastics in the environment is currently one of the most pressing global environmental problems. Microorganisms start to form biofilms on plastic surfaces when they first come in contact with the biosphere; however, these interactions and processes are little understood, especially in freshwaters. This study aimed to better understand the colonization process of microorganisms from activated sludge on plastic materials exhibiting different surface characteristics. We inoculated synthetic fabric (PET), water bottles (PET), and plastic bags for packing vegetables and fruits (HDPE) with microorganisms from activated sludge. Mixtures of plastics and activated sludge, as well as the control, were incubated at 22-24°C in Bushnell Haas (BH) liquid medium and shaken at 120 rpm for two months. The mixtures were sub-sampled weekly and seeded into fresh BH medium with test plastic materials to avoid feeding microorganisms on dead biomass. The colonization was followed by measuring optical density (OD_{600}) of liquid medium, by measurements of isotopic composition of carbon ($\delta^{13}C$) in untreated and treated plastic materials and, with inspecting the plastics surface with scanning electron microscopy (SEM). Overall, the study confirmed differences between colonizing microorganisms on different plastic material when comparing SEM micrographs of materials from the flasks inoculated with activated sludge. The texture of the HDPE bag changed during the experiment in both, control and inoculated flasks, but it is not clear whether the observed changes were due to abiotic or biotic factors. We concluded that microorganisms from activated sludge are capable of colonizing both PET and HDPE materials, and biofilm formation is most probably influenced by the chemical composition of plastics and their surface characteristics.

Keywords: biofilm, plastics, SEM, isotopic composition of carbon, co-cultivation, UV sterilization

Izvleček: Prisotnost plastike v okolju postaja eden izmed največjih globalnih problemov. Prvi stik plastike z biosfero je običajno s kolonizirajočimi mikroorganizmi, ki tvorijo biofilm, vendar je ta interakcija dokaj neznana, še posebej v celinskih vodah. Cilj študije je bil bolje razumeti proces mikrobne kolonizacije različnih plastičnih materialov z različnimi površinskimi lastnosti. Uporabili smo sintetična vlakna blaga (PET), plastenke za vodo (PET) in plastične vrečke za pakiranje zelenjave in sadja (HDPE) ter jih zmešali z okoljskim vzorcem aktivnega blata. Erlenmajerica z mešanico različnih plastik, inokulirana z vzorcem aktivnega blata v Bushnell Haas (BH) tekočem gojišču, ter negativna kontrola (mešanica plastik v sterilnem BH gojišču) so bile 2 meseca inkubirane na 22-24°C in stresane s 120 rpm. Vzorce smo tedensko prestavljali v sveža BH gojišča s testnimi plastičnimi materiali, da smo izključili rast na odmrli biomasi. Proces smo spremljali z merjenjem OD_{600} v tekočem mediju, z meritvami izotopske sestave ogljika ($\delta^{13}C$) v plastiki, in z opazovanjem površine plastike z vrstičnim elektronskim mikroskopom (SEM). S študijo smo potrdili različno rast mikroorganizmov na različnih materialih. V primeru HDPE vrečke se je spremenila tekstura tako v sterilnem kontrolnem gojišču kot v gojišču z mikroorganizmi iz aktivnega blata, vendar ni jasno, če zaradi abiotskih ali biotskih faktorjev. Zaključili smo, da so bakterije iz aktivnega blata zmožne kolonizacije plastike, ki je v vsakdanji uporabi, in da je pestrost in struktura biofilma odvisna od kemične sestave in površinskih lastnosti plastičnih materialov.

Ključne besede: biofilm, plastika, SEM, izotopska sestava ogljika, ko-kultivacija, UV sterilizacija

Introduction

Continuous increase in the production of synthetic or semi-synthetic organic compounds (plastics) and its wide use in every aspect of human life has led to increasing occurrence of plastic waste in freshwater environments (Li et al. 2018). During its time in the environment, larger plastic debris undergoes fragmentation due to weathering processes generating secondary microplastics (MP) (i.e. particles <5 mm). MP can also be manufactured as such and used as resin pellets to produce larger items or used directly (primary MP), as used in cosmetic products (Wagner and Lambert 2018). In concordance with global production rates most commonly found polymers in the environment are high- and low-density polyethylene (HD/LD-PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC). They occur mostly as fragments (rounded, angular), pellets (cylinders, disks, spherules), filaments (fibres), and granules (Wagner et al. 2014).

The presence of MP in the environment, as a new type of emerging contaminant, has become an issue of great concern and has drawn the attention of public and government authorities (Li et al. 2018). The main concerns are that MP particles can be easily ingested throughout the food chain; MP is a vector for toxic contaminants including metals and persistent, bioaccumulative and toxic compounds such as pharmaceuticals and endocrine-disrupting compounds; and MP can act as a vector for waterborne (human) pathogens influencing the hygienic water quality (Wagner et al. 2014, Eckert et al. 2018). There are many sources of MP for freshwater systems, with the largest portion from wastewater treatment plants (WWTP) (Li et al. 2018). When entering the environment, the plastic is exposed to physical factors (temperature, UV light, abrasion, etc.) and adsorbs organic and inorganic substances due to its high adsorptive properties (Rummel et al. 2017). After that, further colonization by bacteria, algae, fungi, and protozoa occur, which results in biofilm formation (McCormick et al. 2014, Rummel et al. 2017, Jemec Kokalj et al. 2019, Parrish and Fahrenfeld 2019).

The interaction between microorganisms and plastics occurring in freshwaters is not well known, even though these interactions and potential for biodegradation is a highly relevant topic in the field of environmental remediation (Wu et al. 2016). MP-associated microbial assemblages in forms of biofilms are likely to influence the distribution, impacts and fate of these pollutants, but most research has focused on marine environments (Harrison et al. 2018). Several researchers pointed out, that plastic particles are rapidly colonized once submerged in marine waters (Lobelle and Cunliffe 2011, Dang and Lovell 2016, Jacquin et al. 2019). In streams, biofilms are primary sites for carbon and nutrient transformations; thus they are also essential for pollutant biodegradation (Battin et al. 2016, Harrison et al. 2018). A recent study of Parrish and Fahrenfeld (2019) demonstrated that biofilm community structures varied as a function of source water and that PS spheres had different microbial community structures from PE microparticles indicating that the characteristics of plastics that is being colonized affects the biofilm structure.

Biofilm formation is composed of four distinct phases: (i) adsorption of dissolved organic molecules, (ii) attachment of bacterial cells, (iii) attachment of unicellular eukaryotes, and (iv) attachment of larvae and spores (Dobretsov 2010). Bacterial adhesion is divided into two stages, primary (docking) and secondary (locking) bacterial adhesion (Dunne 2002). Generally, plastics are first covered by inorganic and organic matter, referred to as "conditioning film" and shortly after by bacteria (mainly *Gammaproteobacteria* and *Alphaproteobacteria*) (Oberbeckmann et al. 2015). Microorganisms attach more firmly to hydrophobic materials, which is opposite to hydrophilic materials such as glass (Dobretsov 2010). The factors that affect the composition of microbial communities attached to artificial substrates are not well known (Caruso 2020), but they certainly gain advantages through surface colonization and biofilm formation, the most critical being better access to resources (Dang and Lovell 2016). Certain bacterial groups belonging to the phyla Proteobacteria, Bacteriodetes, Firmicutes, and Cyanobacteria are associated with plastic as colonizers more than others, suggesting an ecological niche for some taxonomic groups and indicating metabolic adaptation (Roager and Sonnenschein 2019). However, more information is needed to better understand how bacteria are associating with different type of plastics and under what conditions. A study conducted by Khatoon et al. (2014) used activated sludge as seed in their experiments. Similarly, they used SEM to characterize biofilm and surface morphology, but of polypropylene (PP) balls. A study by Huang and Cui (2012) also used activated sludge but investigated poly(lactic acid) (PLA), poly(butylene succinate) (PBS) and poly(caprolactone) (PCL). Using SEM, they found that biodegradation of PCL is best, PLA follows, and lastly PBS. Although some papers study HDPE degradation using SEM, the bacterial seeds come mostly from plastic waste dumpsites' soil or plastic samples and to lesser extent from activated sludge (Kowalczyk et al. 2016, Awasthi et al. 2017).

This study aimed to better understand the colonization process of microorganisms from activated sludge on plastics differing in chemical (PET and HDPE) and surface characteristics (textile fabric, thin plastic bags, thick plastic bottles). The colonization process and biofilm formation were observed on PET and HDPE materials, which are commonly present in treated wastewaters (Lv et al. 2019) and also occur as pollutants in freshwater environments (Koelmans et al. 2019) and, to our knowledge, have never been studied in a similar experiment. We hypothesized that the microorganisms from the activated sludge would be able to colonize and utilize carbon from experimental plastics in order to survive, that different biofilms would form on the PET textile, PET bottle, and HDPE bag, and that the thin plastic bag would reveal the greatest structural surface changes due to its thinness. Since scanning electron microscopy (SEM) enables visualization of bacterial colonization and biofilm architecture, including extracellular polymeric substance (EPS) deposits, we used it to investigate the colonization process by microorganisms from activated sludge, which are investigated less often than microorganisms from landfill and dump sites (Matjašič et al. in prep.). Moreover, we tested whether the measurements of the isotopic composition of carbon ($\delta^{13}C$), as proposed by Lucas et al. (2008), can provide reliable information on microbial degradation of experimental plastics. This preliminary study

also identified methodological improvements necessary for further experiments where we will try to better understand the survival and colonization processes on plastic materials by microorganisms from different polluted sites. Our final, long term aim is to isolate environmental microorganisms capable of efficient biodegradation.

Material and methods

Preparation of plastic materials

We used three types of plastics in this study. The store-bought new materials consisted of synthetic fabric (textile for clothes), plastic water bottles (bottle), and plastic bags used for packing vegetables and fruits in grocery stores (bag). The determination of the chemical composition of the materials was carried out using Fourier-transform infrared spectroscopy (FTIR) (Perkin Elmer Spectrum Two) in ATR mode. FTIR identifies the functional groups present in organic or inorganic compounds and characterizes covalent bonding information by measuring their absorption of infrared radiation over a range of wavelengths (Smith 2011). By FTIR, we can identify the analysed polymer, but cannot see the detailed chemical composition, including additives. The textile and water bottle were identified as polyethylene terephthalate (PET) and the bag as high-density polyethylene (HDPE), all with 98% similarity (Fig. 1).

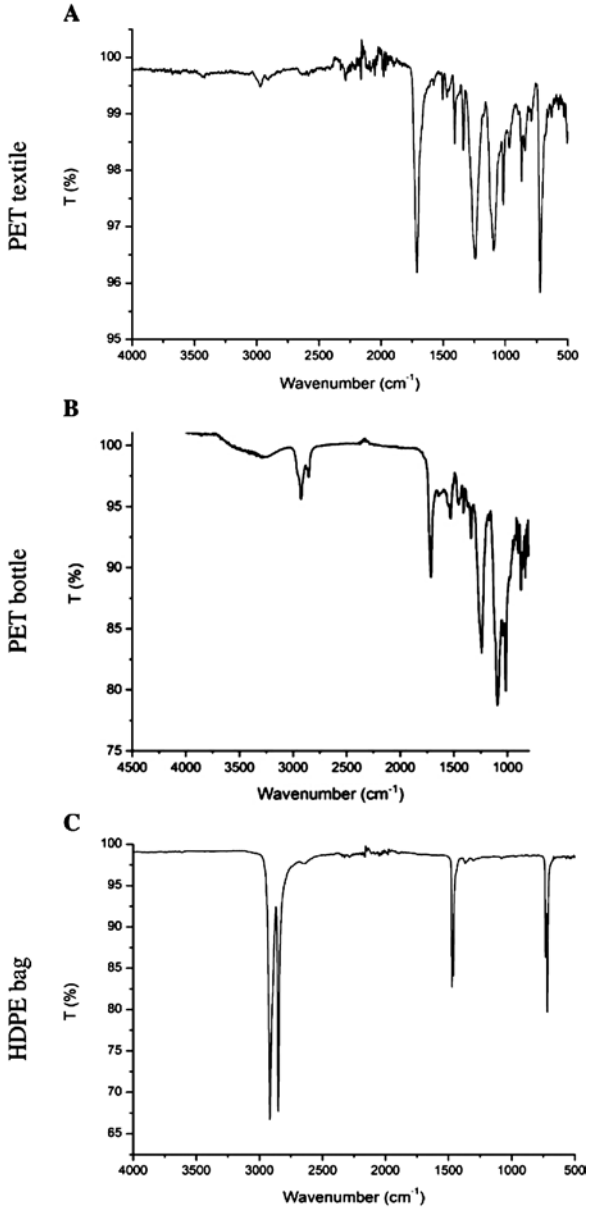

Figure 1: FTIR spectra of PET textile (A), PET bottle (B) and HDPE bag (C).

Slika 1: FTIR spekter PET blaga (A), PET plastenke (B) in HDPE vrečke (C).

Usually, in colonization studies, sterilization with 70% of ethanol is applied (Arkatkar et al. 2009, Arkatkar et al. 2010, Francis et al. 2011, Mohanrasu et al. 2018) but, because treatment with ethanol was indicated not to be sporicidal (McDonnell and Russell 1999, Yoo 2018), we decided to expose plastic materials to UV light. The materials were first exposed to UV-A light (365 nm) for seven days (lamp Osram Ultra-Vitalux, 300 W) to partly mimic the exposure of materials to natural light present in the environment. Next, the plastics were cut into 2 x 2 cm squares and exposed to UV light (ozone free UV-C ($\lambda = 253.7$ nm), with a UV radiation level of 15 mW/cm^2/sec; (UVC/T-M-AR Cleaner Box, BioSan) for 30 minutes, turning the materials half-way through to sterilize them.

Co-cultivation experiment

Erlenmeyer flasks (100 mL) were sterilized (dry sterilization, 5h, 180 °C) twice within a two-day window. To each flask, 27 mL Bushnell Haas (BH; 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 1.0 g NH_4NO_3, 0.2 g $MgSO_4$, 0.05 g $FeCl_3$, 0.02 g $CaCl_2$, in 1000 mL of deionized water) liquid medium (Bushnell and Haas 1941) was added, together with two pieces (2 x 2 cm) of each type of plastic material. We used BH medium because it was designed as a medium for bacteria that degrade hydrocarbons (Bushnell and Haas 1941), and recent studies dealing with microbial plastic degradation used it as a suitable medium without added carbon (Mohan et al. 2016, Auta et al. 2017, Auta et al. 2018, Mohanrasu et al. 2018). We inoculated one flask with microorganisms from an activated sludge sample, and one flask was used as a negative control. We collected the environmental sample of activated sludge on June 17th, 2019, at the wastewater treatment plant (WWTP) Domžale-Kamnik, located near Ljubljana, Slovenia. The volume of inocula was 3 mL. The flasks were incubated for two months at room temperature (22-24 °C) with continuous shaking (120 rpm, HS 501 digital, IKA Labortechnik). The samples were shaken because the medium and the plastic were settling, and because we wanted to maximize the available surface for bacteria. Shaking also increased the probability of bacteria encountering the plastic surface used in the experiments. During incubation, we carried out weekly subculturing to exclude growth on dead biomass, so that bacteria could use plastics as the sole carbon source. The first inoculation was conducted on June 18th, 2019. Then, regular weekly subculturing was carried out three times by transferring 3 mL of mixture from the inoculated flask and the negative control into flasks with fresh BH medium containing plastics. One and a half month after the last subculturing, OD_{600} of liquid media was measured (Lambda UV/Vis spectrophotometer, PerkinElmer, Waltham, MA, USA) and samples for the SEM and isotopic analysis were taken.

Characterization of biofilms on plastics

We took pieces of plastics from the final subculturing where plastic was exposed to microorganisms for month and a half (treated plastics) and corresponding negative controls (control plastics) for further investigation of colonization by SEM. The biofilm growth was characterized by comparing the SEM micrographs of plastics from the mixture with activated sludge with those of negative control and plastics, both not exposed (untreated) and exposed to UV-C (UV exposed), in order to obtain information on their initial texture. The potential biofilms on plastics were fixed with 2.5 % glutaraldehyde and 0.4 % paraformaldehyde in a 0.1 M Phosphate Buffer, pH 7.4, for 2 hours and subsequently subjected to desiccation in sorted series of ethanol concentrations (10%, 20%, 30%, 40%, 50%, 75%, 85%, 95%, 100%). The samples were coated using an ion-beam precision etching coating system (PECS 682, Gatan Inc. USA) with 5 nm thick conductive Au-Pd layer and observed at various magnifications under scanning electron microscope (SEM, JSM 7600F, JEOL, Japan) at SEM accelerating voltage of 10 or 5 kV. The whole surface of colonized plastic materials was systematically examined by using 5000 x magnification, and, in cases of indication on biofilm formation or microbial occurrence the selected area was inspected under 6000, 10 000 and 15 000 x magnifications. Highly indicative micrographs were extracted for this study. Due to space limitation, the presented micrographs (Fig. 2 – Fig. 6) are the most representative images.

Isotopic analysis

Following the co-cultivation experiment, plastics were pre-prepared for isotope analysis. Materials were taken from the final subculturing, same as for OD_{600}, submerged in deionized water separately, and rinsed two more times with fresh deionized water to remove the potential biofilm (treated material). Similarly treated were plastics from control flasks (control material) and material that was not incubated in the flask (untreated material). The isotopic composition of carbon in plastics was determined using a Europa 20-20 continuous flow IRMS ANCA-SL preparation module. Approximately 0.5 mg of plastics (textile - PET, bottle - PET, bag - HDPE) were weighed in a tin capsule for carbon analysis. The isotopic composition of carbon was determined after combustion of the capsules in a hot furnace (temperature 1000°C). Generated products were reduced in a Cu tube (600°C), where excess O_2 was absorbed. H_2O was trapped on a drying column composed of $MgClO_4$. Gases were separated on a chromatographic column and ionized. IAEA CH-3 (-24.724±0.041), CH-7 (-32.151±0.050), CH-6 (-10.449±0.033) reference materials were used to relate the analytical results to the VPDB standards. The sample reproducibility for carbon was ±0.2‰. The isotopic composition of carbon ($\delta^{13}C_{sample}$) is expressed in ‰ and was reported in the δ notation:

$$\delta^{13}C_{sample}\ (‰) = (({}^{13}C/{}^{12}C_{sample})-({}^{13}C/{}^{12}C_{RM}))/({}^{13}C/{}^{12}C_{RM}) * 1000\ [‰]$$

with VPDB – Vienna Pee Dee Belemnite as the reference material (RM).

Results and discussion

Visual analysis of the surface of untreated and treated plastics

The surface of the three types of untreated plastics, as seen by SEM micrographs, was smooth and without any visible surface overgrowth (Fig. 2). Treatment of HDPE bag with UV-C resulted in a seemingly smoother surface (Fig. 2D).

During the two months incubation in sterilized liquid BH (negative controls), plastic pieces of PET textile (Fig. 3) and PET bottles (Fig. 4) did not show substantial textural changes. However, individual rod-shaped cells or coccoid microorganisms occurred on the plastics, mostly on PET bottles, indicating microbial contamination. On the contrary, changes in surface texture were obvious for HDPE bags in control flasks (Fig. 5), which at the end of experiments showed rougher texture with cracks. PET bottles, PET textile and HDPE bag materials from flask inoculated with activated sludge, differed in both density of microorganisms and biofilm development. The highest microbial diversity and the most developed biofilm was observed on material from the PET bottle, and the least or almost none was found on PET textile. Colonization on PET bottles included the formation of deposits of extracellular polymeric substance (EPS) and the presence of different types of cells (coccoid, rods, spirals, corkscrews) in the form of single cells, pairs, chains or clusters (Fig. 6). The low colonization rate on textiles may be due to impregnation with antimicrobial additives, physical characteristics of the material, or other factors, such as not suitable growth temperature. FTIR analysis did not indicate substantial differences in PET structure and composition between the PET bottle and PET textile. However, some antimicrobial additives could be present in the PET textile that were not detected by FTIR. Antimicrobial additives are widely used in the production of polyester (PET) and polyamide (PA) fibres in order to avoid pathogenic microorganism infection, control microbe infestation, limit the deterioration of textiles, control the spread of disease, reduce the formation of odour by microbial metabolism, and protect the textile products from staining, discolouration, and quality deterioration (Al-Balakocy and Shalaby 2017). The plastics of PET bottles as substrate lead to the highest perceived variety of cell morphology and the most structured and mature biofilm (Fig. 6). Despite this high growth, no obvious textural changes in the plastics itself could be

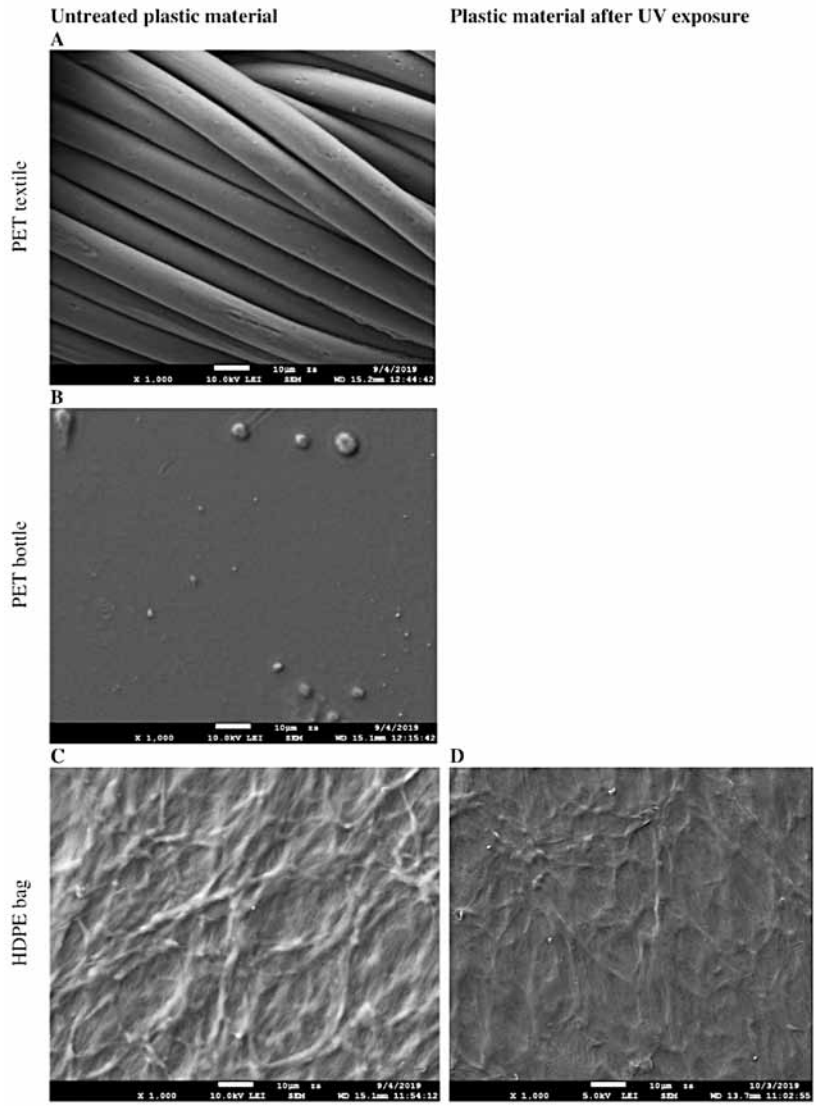

Figure 2: SEM micrographs of untreated PET textile (A), PET bottle (B) and HDPE bag (C) at 1000 x magnification and of HDPE bag (D) exposed for 7 days to UV light at 1000 x magnification.

Slika 2: SEM mikrograf neobdelanega PET blaga (A), PET plastenke (B), HDPE vrečke (C) in HDPE vrečke (D), 7 dni izpostavljene UV svetlobi na 1000 x povečavi.

observed. The HDPE bag, a material successfully sterilized with UV-C, supported the growth of microorganisms from activated sludge (Fig. 5). As with the PET bottles, the microorganisms seem to form a biofilm with cells embedded in the matrix. Contrary to PET plastics, the HDPE texture changed during the experiment; however, these changes were observed with and without activated sludge. Therefore, it is not clear whether the changes are due to abiotic or biotic factors.

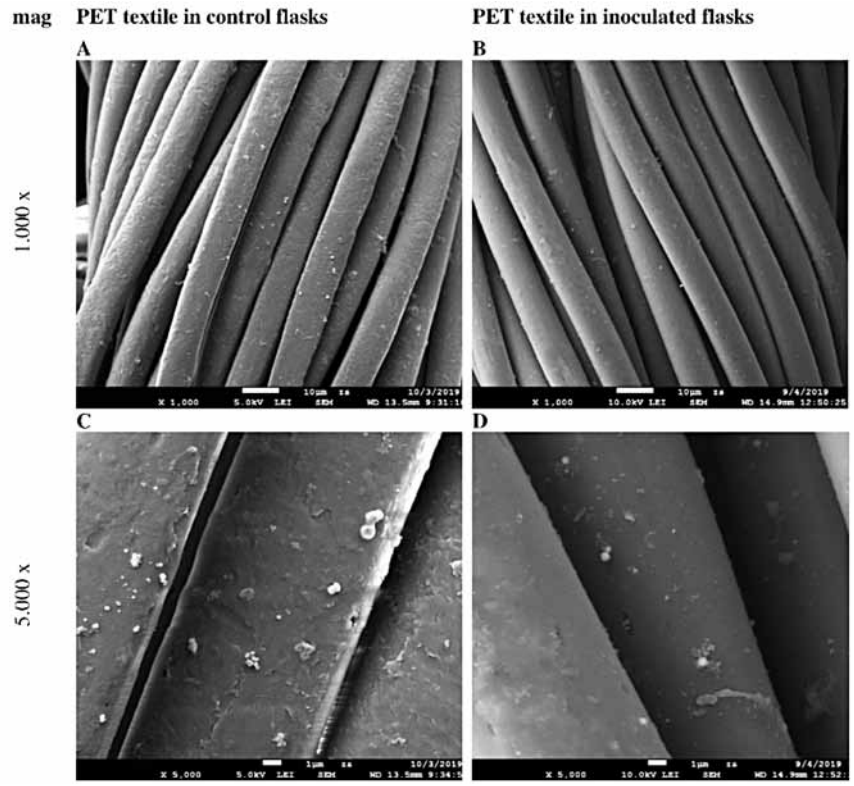

Figure 3: SEM micrographs of PET textile incubated for two months in sterilized media (A, C), and PET textile incubated in media with added microorganisms from activated sludge (B, D). Magnification (mag) showed left.

Slika 3: SEM mikrograf PET blaga, inkubiranega 2 meseca v steriliziranem gojišču (A, C) in PET blaga, inkubiranega v gojišču z dodanimi mikroorganizmi iz aktivnega blata (B, D). Povečava (mag) prikazana levo.

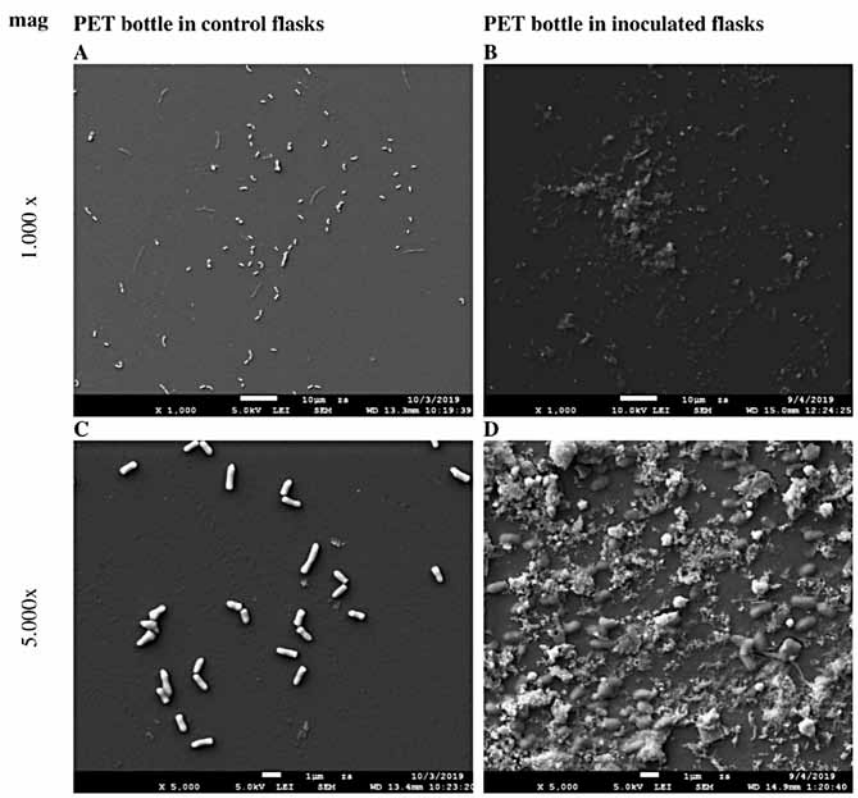

Figure 4: SEM micrographs of PET bottles incubated for two months in sterilized media (A, C), and PET bottles incubated in media with added microorganisms from activated sludge (B, D). Magnification (mag) showed left.

Slika 4: SEM mikrograf PET plastenk, inkubiranih 2 meseca v steriliziranem gojišču (A, C) in PET plastenk, inkubiranih v gojišču z dodanimi mikroorganizmi iz aktivnega blata (B, D). Povečava (mag) prikazana levo.

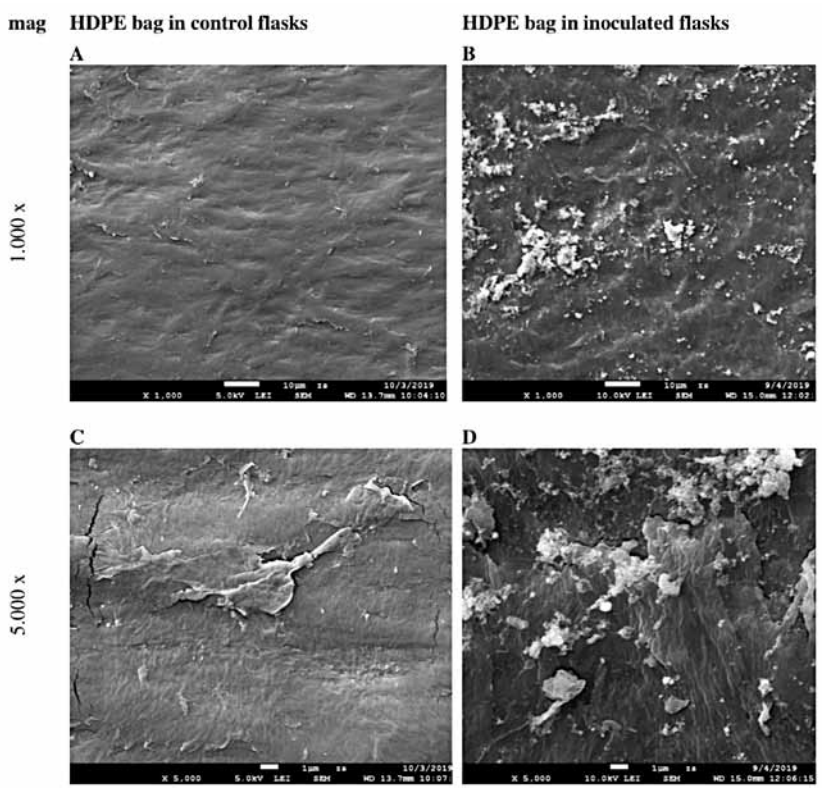

Figure 5: SEM micrographs of HDPE bag incubated for two months in sterilized media (A, C), and HDPE bag incubated in media with added microorganisms from activated sludge (B, D). Magnification (mag) showed left.

Slika 5: SEM mikrograf HDPE vrečk, inkubiranih 2 meseca v steriliziranem gojišču (A, C) in HDPE vrečk, inkubiranih v gojišču z dodanimi mikroorganizmi iz aktivnega blata (B, D). Povečava (mag) prikazana levo.

Overall, this study confirmed the capabilities of plastics to support growth of microorganisms and formation of biofilms from samples of activated sludge. An ecotoxicity study of primary MP by Jemec Kokalj et al. (2019) demonstrated that primary MP from cosmetic products became coated by organic/inorganic material and possibly microorganisms when incubated for three weeks in different environmental samples (spring water, river water, landfill leachate, WWTP effluent), as visualized by light microscopy. They observed the most pronounced overgrowth on MP incubated in the leachate collected from a landfill collection basin and the least in those from WWTP effluent (Jemec Kokalj et al. 2019). Since they did not use samples from activated sludge, a direct comparison with our study is not possible. Nevertheless, our observation of biofilm formation on plastics after exposure to activated sludge is in general agreement with their results. A study by Khatoon et al. (2014) revealed biofilm succession associated with degradative effects on plastic (PP) and contaminants in the sludge. Their surface analysis of plastics by SEM revealed the emergence of profound bacterial growth on the surface of PP beads. Biofilm development started after the third week of incubation. Six-week-old biofilm showed maximum growth and long chains of bacilli, which were succeeded by bacilli of larger sizes, followed after nine weeks by the predominance of mostly rod-shaped bacteria embedded in thick EPS. During biofilm formation they identified

13 microbial strains (*Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes, Salmonella typhimurium, Proteus vulgaris, Alcaligenes faecalis, Shigella dysenteriae, Klebsiella pneumonia, Bacillus subtis, Staphylococcus aureus, Micrococcus luteus, Streptococcus lactis,* and *Cornyebacterium xerosis*) by biochemical characterization. Since a global study by Wu et al. (2019) demonstrated that activated sludge over the globe has a small, core bacterial community (28 operational taxonomic units), we expect that strains from our samples, at least to some extent, coincide with the strains identified by Khatoon et al. (2014).

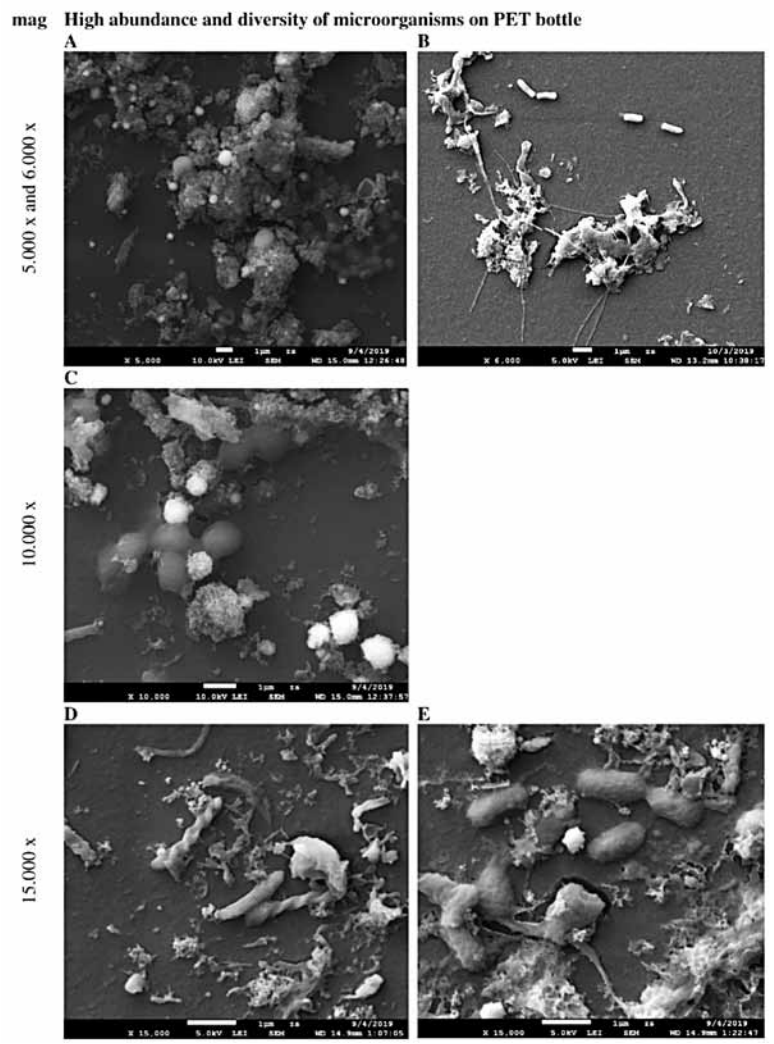

Figure 6: SEM micrographs indicating high abundance and diversity of microorganisms growing on PET bottles. The magnification was 5.000 x (A), 6.000 x (B), 10.000 x (C), 15.000 x (D, E). Magnification (mag) showed left.

Slika 6: SEM mikrografi nakazujejo visoko abundanco in diverziteto mikroorganizmov, rastočih na PET plastenkah. Povečava je 5.000 x (A), 6.000 x (B), 10.000 x (C), 15.000 x (D, E). Povečava (mag) prikazana levo.

Microbial diversity, based on observation of their cell morphology, was different on different types of plastics. We found the highest diversity of cell morphological diversity on PET water bottle and the lowest on PET textile fibres. PET water bottles are more closely regulated for any toxic compounds because they are used for storing drinking water consumed by humans, which may explain the highest cell morphological diversity found in them. Textile fibres, however, may be treated with different antimicrobial chemicals widely used in textile production (Al-Balakocy and Shalaby 2017). Parrish and Fahrenfeld (2019) found differences in biofilm composition between polystyrene (PS) and polyethylene (PE) microparticles that were exposed to environmental samples for only 48 hours. In their study, Deltaproteobacteria and Acidomicrobia (two classes common to wastewater), and Saprospirae of Bacteroidetes prevailed in wastewater PS and PE biofilms, and they observed elevated Gammaproteobacteria in PE microparticle biofilm and Betaproteobacteria in the PS biofilm in river water. They concluded that differences were due to the morphology/surface texture rather than polymer composition. They did not observe differences in biofilm due to particle size (particles smaller or larger than 250 µm), and they proved that biofilm microbial communities differed from communities in the surrounding water. Ogonowski et al. (2018) exposed ambient Baltic bacterioplankton to plastic substrates commonly found in marine environments (PE, PP, and PS) as well as native (cellulose) and inert (glass beads) particles for two weeks under controlled conditions. They found significant differences between plastics and non-plastic substrates in their community composition and diversity. Through operational taxonomic units (OTUs) data, the PE and PP communities were quite similar, whereas that on PS was more distinct. They determined that the observed differences were most probably due to surface hydrophobicity of the materials. Similarly, significantly different communities were observed on low density polyethylene (LDPE), polyethylene terephthalate (PET), and polypropylene (PP) materials from marine environments using denaturing gradient gel electrophoresis (DDGE) profiles (Oberbeckmann et al. 2014).

Microorganisms growth in liquid BH media

After one and a half months, measurements of optical density (OD_{600}) did not indicate microbial growth in the liquid medium. The OD_{600} of BH media was low (0.0603) and similar to the one in the control flask (0.0678). Optical density (OD) measurements of microbial growth are one of the most common techniques used in microbiology, including investigations of growth under different nutritional or stress environments, where the OD value obtained is assumed to be proportional to the cell number (Stevenson et al. 2016). For example, for *E. coli* cell cultures, OD_{600} of 1 corresponds to 8×10^8 cells mL^{-1}. However, to be accurate, the OD method needs thorough calibration and depends on the type of instrument, size and shape of the cells, and changes in the refraction of medium or cells (Stevenson et al. 2016). Our results indicate no microbial growth in liquid media in comparison with the growth on plastics itself as seen by SEM. We speculate that the microbial growth was not present in liquid BH medium containing only minerals, because of nutrient limitations. The food was present only in the form of carbon bonded within synthetic polymers (i.e. particles of PET textile and bottle and HDPE bag) localizing at the bottom of the 250 mL flasks. In contrast, when comparing surrounding media and biofilm, the study of Eckert et al. (2018) demonstrated that after 15 days, the bacterial community composition was not different between biofilm and free-living communities in the 750 mL vessels with different concentrations of PS microparticles (sizes 4 mm x 4 mm x 0.1 mm). Nevertheless, their experiment aimed to demonstrate that microplastics can be a vector for microorganisms from the WWTPs, not a source of carbon. Consequently, they used liquid media with a carbon source in the form of chitin. Moreover, the plastic particles added to the experiment were continually floating in the water column enabling uniform distribution of microorganisms within the experimental vessels.

Isotopic analysis

Values for $\delta^{13}C$ in untreated and treated materials, incubated in liquid BH medium in this study are presented in Table 1. The comparison

of the $\delta^{13}C$ values in treated and untreated materials, indicated that the $\delta^{13}C$ value increased (enriched with heavy carbon isotope $\delta^{13}C$) in both the control and inoculated flasks (Table 1). Berto et al. (2017) preliminary study reported on $\delta^{13}C$ values for various plastic materials, including PET bottles for drinking water and HDPE bags. They characterized plastic polymers using EA/IRMS where mean $\delta^{13}C$ for PET was -27.84 ± 1.71 ‰ as opposed to this study where $\delta^{13}C$ for untreated PET was -29.0 ±0.2 ‰. Mean $\delta^{13}C$ value for HDPE bag was -33.97 ± 0.70 ‰ in the study of Berto et al. (2017) and we measured -30.2 ± 0.1 ‰ for untreated HDPE in this study. Plastics degraded in the marine environment showed an increase of $\delta^{13}C$ values (Berto et al. 2017), which was also the case in our study. The shift of $\delta^{13}C$ could be related to physical/chemical or biological degradation although it was not possible to evaluate the degradation rate. The results indicated that this method has the potential to be used in biodegradation studies but careful experimental design is needed.

Critical evaluation of methodology

The chosen sterilization approach for plastics, namely UV-C, was found not to be totally efficient since microorganisms were detected on the plastics from control flasks. The UV-C sterilization was more suitable for thinner plastic bags of HDPE and less for PET textile and thicker plastic of PET water bottles. The morphology of microorganisms observed on the two materials from control flasks was different. Coccoid microorganisms were present in PET textile (Fig. 3), and rod-shaped cells were dominant in PET bottles (Fig. 4). This indicates that the contamination is not a consequence of an experimental error but instead is linked to the materials themselves. Most likely, the contamination is due to the limited penetration of UV-C through these materials. The results indicate that a combination of sterilization approaches may be required and will be used in further studies. The observations are in line with a previous study of Meechan and Wilson (2006) which shows that while UV-C is germicidal and virucidal, it does not penetrate well and will only disinfect the outer

Table 1: Isotopic composition of carbon ($\delta^{13}C$) measured in plastic materials prior to the experiment (untreated material) and these incubated in control flask and flask inoculated with activated sludge (treated material). The average values and SD of the replicate measurements on the same sample are shown. Sign ε (enrichment factor) represents the difference to respective untreated material.

Tabela 1: Izotopska sestava ogljika ($\delta^{13}C$) merjena v plastičnih materialih pred poskusom (netretiran material) in po poskusu v kontrolni Erlenmajerici in Erlenmajerici z dodanim inoculum iz aktivnega blata (tertian material). Prikazane so srednje vrednosti in SD zaporednih meritev istega vzorca. Znak ε (obogatitveni faktor) predstavlja razliko do neobdelanega materiala.

	Untreated material $\delta^{13}C$	**Treated material $\delta^{13}C$**	
		Control flask	Flask with activated
	[‰ ± s‰]	[‰ ± s‰]	sludge [‰ ± s‰]
Textile (PET)	-28.9 ± 0.1	-28.2 ± 0.1	-28.1 ± 0.1
		ε = 0.7	ε = 0.8
Bottle (PET)	-29.0 ± 0.2	-28.6 ± 0.1	-28.6 ± 0.2
		ε = 0.4	ε = 0.4
Bag (HDPE)	-30.2 ± 0.1	-29.7 ± 0.1	-29.8 ± 0.2
		ε = 0.5	ε = 0.4

surface of a material. Plastics of PET bottles were also not efficiently sterilized with UV-C before to the experiment and showed contamination (Fig. 4). However, the final abundance of microbial growth in control was far below that observed on plastics exposed to activated sludge.

Conclusions

This study represents one of the rare insights into the differences in interactions between PET textile, PET bottle, and HDPE bag and microorganisms outside of the marine environments, namely with microorganisms from activated sludge. Through a combination of SEM and stable isotopic analyses, we demonstrated that the chemical composition of plastics and its surface characteristics (morphology, texture) play a significant role in biofilm development regarding both its diversity and complexity, presumably because of easier adhesion. The value of $\delta^{13}C$ slightly increased in all tested materials compared to the source material suggesting certain level of degradation. The study results show that microorganisms are capable of colonizing plastics in environments without other carbon sources. Among the PET textile, PET bottles, and HDPE bags, the last two promoted the most abundant growth and seemingly more structured and mature biofilm as evidenced by SEM micrographs. Further studies with improved experimental design, including metagenomic approaches, which may be useful in identifying the microorganisms present in activated sludge that are more successful in the colonization of plastics. As potential bio-degraders, these microorganisms are of interest to isolate in pure cultures as well.

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Povzetek

Posledica povečanja produkcije sintetičnih ali pol-sintetičnih organskih spojin (plastike) in njihove vse bolj razširjene uporabe je tudi povečano pojavljanje plastike v celinskih vodah. V okolju najdemo različne vrste plastičnih materialov (npr. polietilen visoke oz. nizke gostote (HD/LD-PE), ali polietilen tereftalat (PET)), ki se pojavljajo v različnih oblikah (npr. fragmenti, peleti, filamenti). Prvi stik plastike z biosfero so običajno kolonizacijski mikroorganizmi, vendar je ta interakcija v celinskih vodah slabo raziskana. V študiji smo poskušali bolje razumeti, kako poteka kolonizacijski proces mikroorganizmov iz aktivnega blata na različnih plastičnih materialih, prepoznali smo tudi nekatere metodološke izboljšave, ki jih bomo upoštevali v nadaljnjih eksperimentih.

V preliminarni raziskavi smo uporabili plastične materiale, ki so se razlikovali tako v kemični sestavi (PET in HDPE) kot tudi v površinskih lastnostih (blago, debelejše plastenke in tanjše plastične vrečke). Materiale smo najprej teden dni izpostavili UV-A, s čimer smo simulirali sončno svetlobo, nato smo ga pred vnosom v gojišče sterilizirali z UV-C. V erlenmajerice z BH gojiščem smo zraven različnih kosov plastike (2 x 2 cm) dodali še mikrobe iz vzorca aktivnega blata iz centralne čistilne naprave (CČN) Domžale-Kamnik, eno smo pustili ne-inokulirano kot negativno kontrolo. Erlenmajerice smo 2 meseca stresali na sobni temperaturi (22-24°C) in 120 rpm ter jih redno prestavljali v sveže gojišče s testnimi plastičnimi materiali, da bi bakterijam preprečili rabo ogljika, sproščenega iz odmrlih bakterij. Ob koncu eksperimenta smo v tekočih gojiščih izmerili OD_{600} ter izvedli analizo izotopske sestave ogljika ($\delta^{13}C$) in vizualno analizo z uporabo vrstičnega elektronskega mikroskopa (SEM) vseh treh plastičnih materialov.

Ugotovili smo, da so mikroorganizmi iz aktivnega blata sposobni kolonizirati plastiko brez dodatnih virov ogljika in da kemijska sestava plastike najverjetneje vpliva na razvoj biofilma. Biofilm, ki se je tvoril na PET plastenkah in HDPE vrečkah, je bil glede na SEM mikrografe na videz bolj strukturiran in zrel, na kar najverjetneje vpliva kemična sestava plastike, aditivi in njene površinske lastnosti. Ugotovili smo tudi, da sterilizacija plastike z UV ni zadostna. Vrednost $\delta^{13}C$ je bila

višja v materialih izpostavljenih mikroorganizmom v primerjavi z ne-tretiranim materialom, kar najverjetneje nakazuje degradacijo. V nadaljnjih eksperimentih bomo uporabili več različnih okoljskih virov mikroorganizmov, izboljšan način sterilizacije in metagenomske pristope, s končnim ciljem izolirati seve, ki so sposobni razgrajevati težko razgradljive plastične materiale.

References

Al-Balakocy, N., Shalaby, S. E., 2017. Imparting antimicrobial properties to polyester and polyamide fibers-state of the art. Journal of the Textile Association, 78, 179-201.

Arkatkar, A., Arutchelvi, J., Bhaduri, S., Uppara, P. V., Doble, M. 2009. Degradation of unpretreated and thermally pretreated polypropylene by soil consortia. International Biodeterioration and Biodegradation, 63(1), 106-111.

Arkatkar, A., Juwarkar, A. A., Bhaduri, S., Uppara, P. V., Doble, M., 2010. Growth of *Pseudomonas* and *Bacillus* biofilms on pretreated polypropylene surface. International Biodeterioration and Biodegradation, 64(6), 530-536. doi: https://doi.org/10.1016/j.ibiod.2010.06.002

Auta, H. S., Emenike, C. U., Fauziah, S. H., 2017. Screening of *Bacillus* strains isolated from mangrove ecosystems in Peninsular Malaysia for microplastic degradation. Environmental Pollution, 231, 1552-1559. doi: 10.1016/j.envpol.2017.09.043.

Auta, H. S., Emenike, C. U., Jayanthi, B., Fauziah, S. H., 2018. Growth kinetics and biodeterioration of polypropylene microplastics by *Bacillus* sp. and *Rhodococcus* sp. isolated from mangrove sediment. Marine pollution bulletin, 127, 15-21. doi: https://doi.org/10.1016/j.marpolbul.2017.11.036

Awasthi, S., Srivastava, P., Singh, P., Tiwary, D., Mishra, P. K., 2017. Biodegradation of thermally treated high-density polyethylene (HDPE) by *Klebsiella pneumoniae* CH001. 3 Biotech, 7(5), 332. doi: 10.1007/s13205-017-0959-3

Battin, T. J., Besemer, K., Bengtsson, M. M., Romani, A. M., Packmann, A. I., 2016. The ecology and biogeochemistry of stream biofilms. Nature Reviews Microbiology, 14(4), 251-263. doi: 10.1038/nrmicro.2016.15

Berto, D., Rampazzo, F., Gion, C., Noventa, S., Ronchi, F., Traldi, U., Giorgi, G., Cicero, A. M., Giovanardi, O., 2017. Preliminary study to characterize plastic polymers using elemental analyser/isotope ratio mass spectrometry (EA/IRMS). Chemosphere, 176, 47-56. doi: https://doi.org/10.1016/j.chemosphere.2017.02.090

Bushnell, L. D., Haas, H. F., 1941. The utilization of certain hydrocarbons by microorganisms. Journal of Bacteriology, 41(5), 653-673.

Caruso, G., 2020. Microbial Colonization in Marine Environments: Overview of Current Knowledge and Emerging Research Topics. Journal of Marine Science and Engineering, 8(2), 78.

Dang, H., Lovell, C. R., 2016. Microbial Surface Colonization and Biofilm Development in Marine Environments. Microbiology and Molecular Biology Reviews, 80(1), 91-138. doi: 10.1128/mmbr.00037-15

Dobretsov, S., 2010. Marine Biofilms. In: S., Dürr and J. C., Thomason (Eds.), Biofouling (pp. 123-136).

Dunne, W. M., 2002. Bacterial Adhesion: Seen Any Good Biofilms Lately? Clinical Microbiology Reviews, 15(2), 155-166. doi: 10.1128/cmr.15.2.155-166.2002

Eckert, E. M., Di Cesare, A., Kettner, M. T., Arias-Andres, M., Fontaneto, D., Grossart, H.-P., Corno, G., 2018. Microplastics increase impact of treated wastewater on freshwater microbial community. Environmental Pollution, 234, 495-502. doi: https://doi.org/10.1016/j.envpol.2017.11.070

Francis, V., Subin, S. R., Bhat, S. G., Thachil, E. T., 2011. Characterization of Linear Low-Density Polyethylene/Poly(vinyl alcohol) Blends and Their Biodegradability by *Vibrio* sp. Isolated from Marine Benthic Environment. Journal of Applied Polymer Science, 124, 257-265. doi: 10.1002/app.34155

Harrison, J. P., Hoellein, T. J., Sapp, M., Tagg, A. S., Ju-Nam, Y., Ojeda, J. J., 2018. Microplastic-Associated Biofilms: A Comparison of Freshwater and Marine Environments. In M. Wagner and S. Lambert (Eds.), Freshwater Microplastics : Emerging Environmental Contaminants? (pp. 181-201). Cham: Springer International Publishing.

Huang, J. T., Cui, C. N., 2012. Study on Biodegradable Behavior of Polyesters in the Soil of FuJian Local. Advanced Materials Research, 472-475, 1881-1884. doi: doi.org/10.4028/www.scientific.net/amr.472-475.1881

Jacquin, J., Cheng, J., Odobel, C., Pandin, C., Conan, P., Pujo-Pay, M., Barbe, V., Meistertzheim, A.-L., and Ghiglione, J.-F., 2019) Microbial Ecotoxicology of Marine Plastic Debris: A Review on Colonization and Biodegradation by the "Plastisphere". Frontiers in Microbiology, 10(865). doi: 10.3389/fmicb.2019.00865

Jemec Kokalj, A., Kuehnel, D., Puntar, B., Žgajnar Gotvajn, A., Kalčikova, G., 2019. An exploratory ecotoxicity study of primary microplastics versus aged in natural waters and wastewaters. Environmental Pollution, 254, 112980. doi: https://doi.org/10.1016/j.envpol.2019.112980

Khatoon, N., Naz, I., Ali, M. I., Ali, N., Jamal, A., Hameed, A., Ahmed, S., 2014. Bacterial succession and degradative changes by biofilm on plastic medium for wastewater treatment. Journal of Basic Microbiology, 54(7), 739-749. doi: 10.1002/jobm.201300162

Kowalczyk, A., Chyc, M., Ryszka, P., Latowski, D., 2016. *Achromobacter xylosoxidans* as a new microorganism strain colonizing high-density polyethylene as a key step to its biodegradation. Environmental Science and Pollution Research, 23(11), 11349-11356. doi: 10.1007/s11356-016-6563-y

Li, J., Liu, H., Paul Chen, J., 2018. Microplastics in freshwater systems: A review on occurrence, environmental effects, and methods for microplastics detection. Water Research, 137, 362-374. doi: https://doi.org/10.1016/j.watres.2017.12.056

Lobelle, D., Cunliffe, M., 2011. Early microbial biofilm formation on marine plastic debris. Marine Pollution Bulletin, 62(1), 197-200. doi: https://doi.org/10.1016/j.marpolbul.2010.10.013

Lv, X. Dong, Q., Zuo, Z. ,Liu, Y. Huang, X., Wu, W.-M., 2019. Microplastics in a municipal wastewater treatment plant: Fate, dynamic distribution, removal efficiencies, and control strategies. Journal of Cleaner Production, 225, 579-586. doi: https://doi.org/10.1016/j.jclepro.2019.03.321

McCormick, A., Hoellein, T. J., Mason, S. A., Schluep, J., Kelly, J. J., 2014. Microplastic is an Abundant and Distinct Microbial Habitat in an Urban River. Environmental Science and Technology, 48(20), 11863-11871. doi: 10.1021/es503610r

McDonnell, G., Russell, A. D., 1999. Antiseptics and Disinfectants: Activity, Action, and Resistance. Clinical Microbiology Reviews, 12(1), 147-179. doi: 10.1128/cmr.12.1.147

Meechan, P. J., Wilson, C., 2006. Use of Ultraviolet Lights in Biological Safety Cabinets: A Contrarian View. Applied Biosafety, 11(4), 222-227.

Mohan, A. J., Sekhar, V. C., Bhaskar, T., Nampoothiri, K. M., 2016. Microbial assisted High Impact Polystyrene (HIPS) degradation. Bioresource Technology, 213, 204-207. doi: https://doi.org/10.1016/j.biortech.2016.03.021

Mohanrasu, K., Premnath, N., Siva Prakash, G., Sudhakar, M., Boobalan, T., Arun, A., 2018. Exploring multi potential uses of marine bacteria; an integrated approach for PHB production, PAHs and polyethylene biodegradation. Journal of Photochemistry and Photobiology, 185, 55-65. doi: 10.1016/j.jphotobiol.2018.05.014

Oberbeckmann, S., Löder, M. G. J., Labrenz, M., 2015. Marine microplastic-associated biofilms – a review. Environmental Chemistry, 12(5), 551-562. doi: https://doi.org/10.1071/EN15069

Oberbeckmann, S., Loeder, M. G. J., Gerdts, G., Osborn, A. M., 2014. Spatial and seasonal variation in diversity and structure of microbial biofilms on marine plastics in Northern European waters. FEMS Microbiology Ecology, 90(2), 478-492. doi: 10.1111/1574-6941.12409

Ogonowski, M., Motiei, A., Ininbergs, K., Hell, E., Gerdes, Z., Udekwu, K. I., Bacsik, Z., Gorokhova, E., 2018. Evidence for selective bacterial community structuring on microplastics. Environmental Microbiology, 20(8), 2796-2808. doi: 10.1111/1462-2920.14120

Parrish, K., Fahrenfeld, N. L., 2019. Microplastic biofilm in fresh- and wastewater as a function of microparticle type and size class. Environmental Science: Water Research and Technology, 5(3), 495-505. doi: 10.1039/C8EW00712H

Roager, L., Sonnenschein, E. C., 2019. Bacterial Candidates for Colonization and Degradation of Marine Plastic Debris. Environmental Science and Technology, 53(20), 11636-11643. doi: 10.1021/acs.est.9b02212

Rummel, C. D., Jahnke, A., Gorokhova, E., Kühnel, D., Schmitt-Jansen, M., 2017. Impacts of Biofilm Formation on the Fate and Potential Effects of Microplastic in the Aquatic Environment. Environmental Science and Technology Letters, 4(7), 258-267. doi: 10.1021/acs.estlett.7b00164

Smith, B. C., 2011. Fundamentals of Fourier transform infrared spectroscopy: CRC press.

Stevenson, K., McVey, A. F., Clark, I. B. N., Swain, P. S., Pilizota, T., 2016. General calibration of microbial growth in microplate readers. Scientific Reports, 6, 38828. doi: 10.1038/srep38828

Wagner, M., Lambert, S., 2018. Freshwater Microplastics Springer International Publishing.

Wagner, M., Scherer, C., Alvarez-Muñoz, D., Brennholt, N., Bourrain, X., Buchinger, S., Fries, E., Grosbois, C., Klasmeier, J., Marti, T., Rodriguez-Mozaz, S., Urbatzka, R., Vethaak, A. D., Winther-Nielsen, M., Reifferscheid, G., 2014. Microplastics in freshwater ecosystems: what we know and what we need to know. Environmental Sciences Europe, 26(1), 12. doi: 10.1186/s12302-014-0012-7

Wu, L. Ning, D., Zhang, B. Li, Y., Zhang, P., Shan, X., Zhang, Q., Brown, M., Li, Z., Van Nostrand, J. D., Ling, F., Xiao, N., Zhang, Y., Vierheilig, J., Wells, G. F., Yang, Y., Deng, Y., Tu, Q., Wang, A., Acevedo, D., Agullo-Barcelo, M., Alvarez, P. J. J., Alvarez-Cohen, L., Andersen, G. L., de Araujo, J. C., Boehnke, K., Bond, P., Bott, C. B., Bovio, P., Brewster, R. K., Bux, F., Cabezas, A., Cabrol, L., Chen, S., Criddle, C. S., Deng, Y., Etchebehere, C., Ford, A., Frigon, D., Gómez, J. S., Griffin, J. S., Gu, A. Z., Habagil, M., Hale, L., Hardeman, S. D., Harmon, M., Horn, H., Hu, Z., Jauffur, S., Johnson, D. R., Keller, J., Keucken, A., Kumari, S., Leal, C. D., Lebrun, L. A., Lee, J., Lee, M., Lee, Z. M. P., Li, Y., Li, Z., Li, M., Li, X., Ling, F., Liu, Y., Luthy, R. G., Mendonça-Hagler, L. C., de Menezes, F. G. R., Meyers, A. J., Mohebbi, A., Nielsen, P. H., Ning, D., Oehmen, A., Palmer, A., Parameswaran, P., Park, J., Patsch, D., Reginatto, V., de los Reyes, F. L., Rittmann, B. E., Robles, A. N., Rossetti, S., Shan, X., Sidhu, J., Sloan, W. T., Smith, K., de Sousa, O. V., Stahl, D. A., Stephens, K., Tian, R., Tiedje, J. M., Tooker, N. B., Tu, Q., Van Nostrand, J. D., De los Cobos Vasconcelos, D., Vierheilig, J., Wagner, M., Wakelin, S., Wang, A., Wang, B., Weaver, J. E., Wells, G. F., West, S., Wilmes, P., Woo, S.-G., Wu, L., Wu, J.-H., Wu, L., Xi, C., Xiao, N., Xu, M., Yan, T., Yang, Y., Yang, M., Young, M., Yue, H., Zhang, B., Zhang, P., Zhang, Q., Zhang, Y., Zhang, T., Zhang, Q., Zhang, W., Zhang, Y., Zhou, H., Zhou, J., Wen, X., Curtis, T. P., He, Q., He, Z., Brown, M., Zhang, T., He, Z., Keller, J., Nielsen, P. H., Alvarez, P. J. J., Criddle, C. S., Wagner, M., Tiedje, J. M., He, Q., Curtis, T. P., Stahl, D. A., Alvarez-Cohen, L., Rittmann, B. E., Wen, X., Zhou, J., Global Water Microbiome, C., 2019. Global diversity and biogeography of bacterial communities in wastewater treatment plants. Nature Microbiology, 4(7), 1183-1195. doi: 10.1038/s41564-019-0426-5

Yoo, J.-H., 2018. Review of Disinfection and Sterilization – Back to the Basics. Infection and Chemotherapy, 50(2), 101-109. doi: doi.org/10.3947/ic.2018.50.2.101