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Synthesis and characterization of polyethylene terephthalate (PET) precursors and potential degradation products: Toxicity study and application in discovery of novel PETases

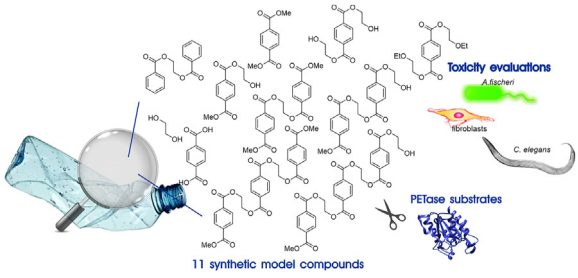
Milica Djapovic [a], Dusan Milivojevic [b], Tatjana Ilic-Tomic [b], Marija Lješević [c], Efstratios Nikolaivits [d], Evangelos Topakas [d], Veselin Maslak [a, **], Jasmina Nikodinovic-Runic [b, *]

[a] University of Belgrade, Faculty of Chemistry, Studentski Trg 16, P.O. Box 51, Belgrade, 11158, Serbia
[b] Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, 11000, Belgrade, Serbia
[c] University of Belgrade-Institute of Chemistry, Technology and Metallurgy, Njegoseva 12, 11000, Belgrade, Serbia
[d] Industrial Biotechnology & Biocatalysis Group, School of Chemical Engineering, National Technical University of Athens, Iroon Polytechniou 9, 15780, Athens, Greece

HIGHLIGHTS

- PET plastic precursors and possible degradation products toxicologically evaluated.
- Low toxicity observed in vitro on human fibroblasts.
- Three compounds harmful to nematode *Caenorhabditis elegans* only at high concentration.
- Six compounds classified as toxic and moderately toxic against *Allivibrio fischeri*.
- PET dimer and trimer are used as substrates for PET hydrolyzing enzyme.

GRAPHICAL ABSTRACT



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ABSTRACT

Polyethylene terephthalate (PET) is widely used material and as such became highly enriched in nature. It is generally considered inert and safe plastic, but due to the recent increased efforts to break-down PET using biotechnological approaches, we realized the scarcity of information about structural analysis of possible degradation products and their ecotoxicological assessment. Therefore, in this study, 11 compounds belonging to the group of PET precursors and possible degradation products have been comprehensively characterized. Seven of these compounds including 1-(2-hydroxyethyl)-4-methylterephthalate, ethylene glycol bis(methyl terephthalate), methyl bis(2-hydroxyethyl terephtahalate), 1,4-benzenedicarboxylic acid, 1,4-bis[2-[[4-(methoxycarbonyl)benzoyl]oxy]ethyl] ester and methyl tris(2-hydroxyethyl terephthalate) corresponding to mono-, 1.5-, di-, 2,5- and trimer of PET were synthetized and structurally characterized for the first time. *In-silico* druglikeness and physico-chemical properties of these compounds were predicted using variety of platforms. No antimicrobial properties were detected even at 1000 μg/mL. Ecotoxicological impact of the compounds against marine bacteria

* Corresponding author.
** Corresponding author.
E-mail addresses: vmaslak@chem.bg.ac.rs (V. Maslak), jasmina.nikodinovic@imgge.bg.ac.rs, jasmina.nikodinovic@gmail.com (J. Nikodinovic-Runic).

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Toxicity
PETase

Allivibrio fischeri proved that the 6 out of 11 tested PET-associated compounds may be classified as harmful to aquatic microorganisms, with PET trimer being one of the most toxic. In comparison, most of the compounds were not toxic on human lung fibroblasts (MRC-5) at 200 μg/mL with inhibiting concentration (IC50) values of 30 μg/mL and 50 μg/mL determined for PET dimer and trimer. Only three of these compounds including PET monomer were toxic to nematode *Caenorhabditis elegans* at high concentration of 500 μg/mL. In terms of the applicative potential, PET dimer can be used as suitable substrate for the screening, identification and characterization of novel PET-depolymerizing enzymes.

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1. Introduction

Since its discovery, its first synthesis, and its patenting in 1941, polyethylene terephthalate (PET) has become a widely used material in a number of industrial branches ranging from packaging, textile and fabrics, films, automotive, electronics and many more (Malik et al., 2017; Sargent et al., 2019). The global PET resin production reached 30.3 million tons in 2017 (www.statista.com). The high production rate is also reflected in the PET share in the post-consumer plastic waste (Ragaert et al., 2017). Although it can be perceived as an ideal plastic for recycling, due to its high melting temperature and the possibility to process it without the use of additives, coupled with possibility to easier separate and collect the main PET product, beverage bottles, only approximately 30% of PET is recycled (Ragaert et al., 2017; Wierckx et al., 2015). Nowadays in the focus of the research community are the innovative ways to use the post-consumer waste PET as a feedstock for new materials with higher values (Tiso et al., 2020; Zhou et al., 2019).

Due to its extensive use, PET is highly enriched in nature, but is generally considered inert and "safe" plastic (Zimmermann et al., 2019). It's toxicity is usually associated with the leach of antimony upon exposure to heat (Wittkowski et al., 2019). Nevertheless 'phthalates' have also been labeled as toxic and possible endocrine disruptors (Sathyanarayana, 2008; Sax, 2010). More recently, microplastics and bigger fragments of plastic are recognized as specific problem found worldwide in oceans and terrestrial environments (Gao et al., 2019; Zhang et al., 2020). Microplastics were recently found in the snow of once pristine Mount Everest (Napper et al., 2020), high mountain lakes of Alps (Pastorino et al., 2020), as well as in the deep-marine environments (Kane and Clare, 2019). PET debris is often eaten by fish and other marine creatures and as such enters the food chain (Webb et al., 2013). On the other side, plastic in agricultural soils poses a risk for drinking water supplies (Wanner, 2021). The first evidence of microplastic in human placenta was reported recently (Ragusa et al., 2021).

Microorganisms are often examined as a frontline against various pollution, including the plastic one (Amobonye et al., 2020). In 2016, bacterium *Ideonella sakaiensis* was reported to degrade amorphous PET when cultured in the presence of yeast extract as an additional carbon source (Yoshida et al., 2016). Subsequently, PETase and mono-(2-hydroxyethyl)TPA (MHET)ase enzymes, the ester-bond hydrolyzing enzymes of this strain have been reported and their activity was improved via protein engineering (Austin et al., 2018; Palm et al., 2019). It has been shown that PETase catalyzes the depolymerization of PET to bis(2-hydroxyethyl)-TPA (BHET), MHET, and terephthalic acid (TPA). MHETase converts MHET to TPA and ethylene glycol (EG) (Austin et al., 2018). Some known cutinases, including the ones from *Humilica insolens* (HiC), *Pseudomonas mendocina* (PmC), and *Fusarium solani* (FsC), were also shown to be catalytically active using low-crystallinity PET films as model substrates (Kawai et al., 2019; Ronkvist et al., 2009).

On the other side, only few species of bacteria and fungi have been described as capable of partially degrading PET to oligomers or even monomers (Kawai et al., 2019; Qiu et al., 2020; Ru et al., 2020). Noteworthy, all known PET hydrolases have been characterized using BHET as a model substrate of choice, and all have relatively low turnover rates, which makes their use for the efficient bioremediation almost impossible. Nevertheless, biodegradation and other biotechnological approaches offer a tremendous opportunity for waste treatment and valorization and are under intensive development. Novel PETases may be available from the untapped microbial diversity, therefore, a more suitable substrate allowing for the efficient screening methodology would be crucial.

Our goal was to synthetize the library of compounds that could be seen either as PET building blocks or as products of PET degradation (Fig. 1) and to assess their toxicity, as well as the potential to be used as substrates for PET hydrolyzing enzymes. We tested the toxicity of the compounds using the marine luminescent bacteria, *Allivibrio fischeri*, healthy human lung fibroblast cell line (MRC-5) and terrestrial nematode *Caenorhabditis elegans*.

2. Materials and methods

2.1. Compound synthesis and structural properties assessment

Model compounds (**5**–**11**; Fig. 1A) were synthesized according to established procedures for preparation of different esters. Two main approaches were explored: i) Schotten–Baumann reaction for acylation of alcohols with acyl halide in the presence of organic bases, and ii) esterification of acids with alcohols in the presence of DCC, known as Steglich esterification (Fig. S1). Synthetic protocols required optimizations in terms of ratio of reactants and other reaction conditions. All compounds were isolated as pure compounds and were well characterized using NMR and IR spectroscopy as well as mass spectrometry.

All chromatographic separations were performed on Silica 10–18, 60 Å, ICN Biomedicals. Standard techniques were used for the purification of reagents. ^{1}H and ^{13}C NMR spectra were recorded with Bruker Avance at 500 MHz (^{1}H at 500 MHz, ^{13}C at 125 MHz), and Varian/Agilent NMR 400 MHz (^{1}H at 400 MHz, ^{13}C at 100 MHz). Chemical shifts (δ) are expressed in ppm and coupling constant (J) in Hz. TMS was used as an internal standard. The following abbreviation were used for signal multiplicities (brs = broad singlet, s = singlet, t = triplet, q = quartet, dd = doublet of doublets, tt = triplet of triplets, m = multiplet). IR spectra (ATR) were recorded with a Perkin-Elmer-FT-IR 1725X spectrophotometer, ν values are given in cm^{-1}. Mass spectra were obtained on MS LTQ Orbitrap XL. Melting points were determined on the Electrothermal WRS1B apparatus and were reported uncorrected.

2.1.1. - (bis(2-Ethoxyethyl)terephthalate) (BEET)

A solution of terephthaloyl chloride **12** (10.16 g; 0.05 mol; 1 eq) in toluene (60 mL) was added dropwise to a solution of 2-ethoxyethanol **13** (12.10 mL; 0.125 mol; 2.5 eq) and pyridine

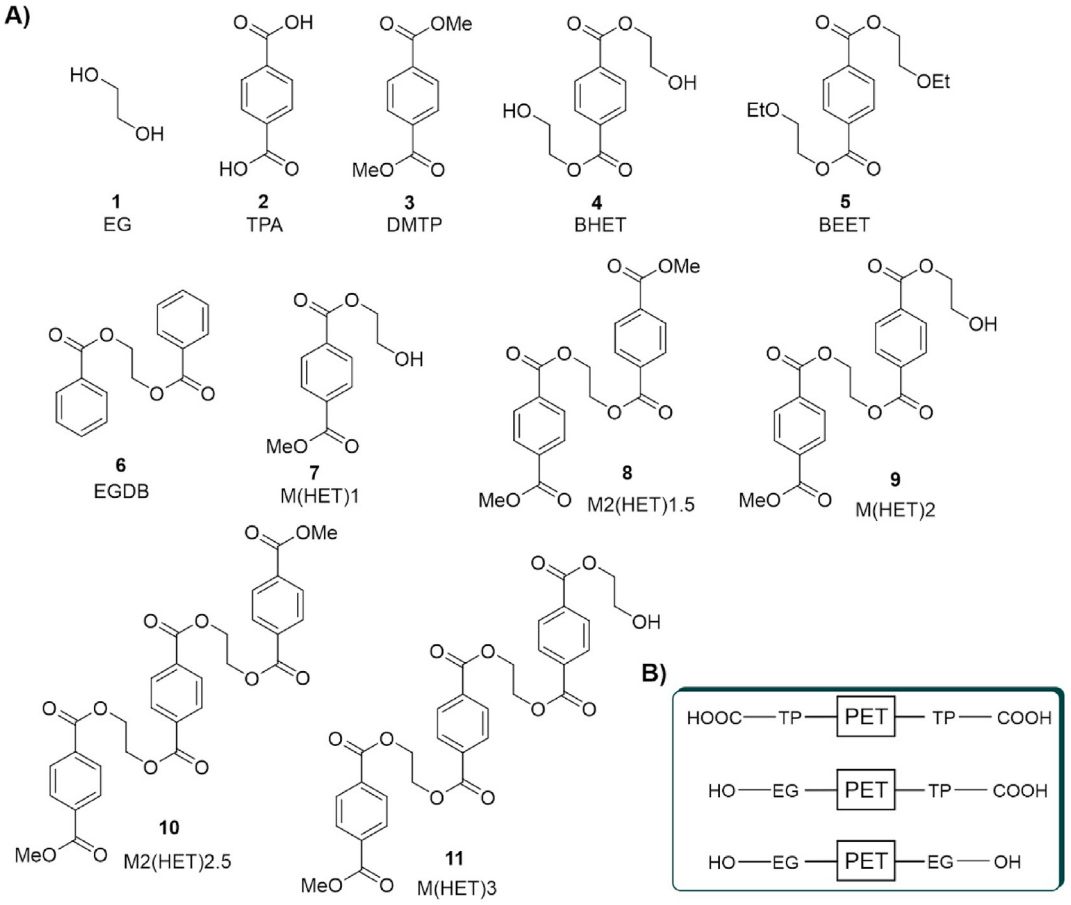

Fig. 1. Structures of PET precursors and possible degradation products: A) Commercially available compounds: EG = ethylene glycol, TPA = terephthalic acid, DMTP = dimethyl terephthalate, BHET = bis(2-hydroxyethyl) terephthalate. New or previously not structurally characterized compounds: BEET = bis(2-ethoxyethyl) terephthalate, EGDB = 1,2-Ethylene glycol dibenzoate, M(HET)1 = 1-(2-hydroxyethyl)-4-methylterephthalate, M2(HET)1.5 = ethylene glycol bis(methyl terephthalate), M(HET)2 = methyl bis(2-hydroxyethyl terephtahalate), M2(HET)2.5 = 1,4-Benzenedicarboxylic acid, 1,4-bis[2-[[4-(methoxycarbonyl)benzoyl]oxy]ethyl] ester and M(HET)3 = methyl tris(2-hydroxyethyl terephthalate). Compounds **9** and **11** are methyl esters of PET dimer and PET trimer, respectively. B) Schematic diagram of possible PET oligomers.

(10.10 mL; 0.125 mol; 2.5 eq) in toluene (40 mL) over 15 min. The reaction mixture was stirred at 60 °C for 5 h and the resulting pyridinium salt was separated by filtration. The filtrate was diluted with ethyl acetate (60 mL) and washed with 1 M HCl, saturated $NaHCO_3$ and brine. The organic solution was dried over anhydrous Na_2SO_4, concentrated in *vacuo* and purified by short-path distillation, to afford a diester **5** (Fig. 1) as colorless oil (11.17 g, 72%, bp 159–162 °C/0.2 mmHg) (Chase, 1963).

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.11 (s, 4H), 4.51–4.46 (m, 4H), 3.81–3.73 (m, 4H), 3.58 (q, J = 7.0 Hz, 4H), 1.23 (t, J = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δc 165.9, 134.1, 129.8, 68.4, 66.9, 64.8, 15.3. IR (ATR) ν_{max}: 2976, 2871, 1723, 1577, 1449, 1408, 1385, 1272, 1117, 732. HRMS (ESI): m/z $[M+Na]^+$ calculated for $C_{16}H_{22}O_6$: 333.1314; found: 333.1311.

6 - 1,2-Ethylene glycol dibenzoate (EGDB).

To a solution of ethylene glycol **1** (6.30 mL; 0.110 mol; 1 eq) and pyridine (17.8 mL; 0.220 mol; 2 eq) in toluene (120 mL) benzoyl chloride (33.1 mL; 0.275 mol; 2.5 eq) was added dropwise over 15 min. The reaction mixture was heated at 120 °C for 4 h and the resulting pyridinium salt was separated by filtration. The filtrate was diluted with ethyl acetate (80 mL) and washed with 1 M HCl, saturated $NaHCO_3$ and brine, successively. The organic solution was dried over anhydrous Na_2SO_4. The drying agent was filtered off and the filtrate was concentrated in *vacuo*. The crude product was recrystallized from ethanol, and diester **6** was obtained as white solid (27.60 g; 93%, mp 71 °C) (Ishido et al., 1977; Ren et al., 2011).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 8.06 (dd, J = 8.2, 1.0 Hz, 4H), 7.57 (tt, J = 7.0, 1.2 Hz, 2H), 7.44 (t, J = 7.7 Hz, 4H), 4.67 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δc 166.5, 133.3, 130.0, 129.8, 128.6, 62.9. IR (ATR) ν_{max}: 3145, 3068, 2977, 2911, 1713, 1601, 1481, 1453, 1338, 1258, 1119, 704.

7 - 1-(2-Hydroxyethyl)-4-methylterephthalate (M(HET)1; PET monomer).

Monomethyl terephthalate (15): KOH (1.46 g; 26 mmol; 1 eq) was dissolved in methanol (52.2 mL) with stirring at 80 °C and solution of dimethyl terephthalate **3** (5.00 g; 26 mmol; 1 eq) and 57.4 mL of toluene was added. The white suspension was heated at 120 °C for 5 h. The mixture was filtered, and solid salt was washed with dichloromethane. The unreacted dimethyl terephthalate was removed in this way. The solid salt was dissolved in water (70 mL), filtered to clarity and acidified with concentrated HCl. The precipitate of monomethyl terephthalate was filtered, washed with water and dried to yield a product as a white solid (3.65 g; 78%, mp 214 °C) (Konosonoks et al., 2005; Li et al., 2015).

^{1}H NMR (400 MHz, DMSO): δ_H 8.05 (s, 4H), 3.87 (s, 3H), 3.63 (brs, COOH). ^{13}C NMR (100 MHz, DMSO): δc 166.7, 165.8, 134.9, 133.3, 129.7, 129.5, 52.6. IR (ATR) ν_{max}: 3428, 3014, 2959, 1721, 1505, 1438, 1410, 1340, 1259, 1127, 1019, 957.

1-(2-Benzyloxyethyl)-4-methylterephthalate (16): A solution of dicyclohexylcarbodiimide (DCC) (0.68 g; 3.32 mmol; 1.2 eq) and dichloromethane (5 mL) was added dropwise to a cold (0 °C) suspension of monomethyl terephthalate **15** (0.50 g; 2.77 mmol; 1 eq),

2-(benzyloxy)ethanol **18** (0.46 g; 3.01 mmol; 1.1 eq), 4-dimethylaminopyridine (DMAP) (68 mg; 0.56 mmol; 0.2 eq) and dichloromethane (20 mL). The reaction was carried out at room temperature for 4 h. The byproduct dicyclohexylurea (DCU) was removed by filtration and precipitate was washed three times with ethyl acetate. The filtrate was concentrated and the residue was purified by dry flash chromatography (SiO_2; eluent: petroleum ether/ethyl acetate = 9:1), to afford 0.72 g (82%) of benzyl ether **16**, as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 8.11 (s, 4H), 7.38–7.27 (m, 5H), 4.61 (s, 2H), 4.55–4.50 (m, 2H), 3.95 (s, 3H), 3.84–3.80 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ_c 166.4, 165.9, 138.0, 134.1, 134.0, 129.8, 129.6, 128.6, 127.9, 127.8, 73.3, 67.9, 64.7, 52.6. IR (ATR) ν_{max}: 3385, 3061, 3031, 2953, 2861, 1723, 1557, 1500, 1438, 1409, 1380, 1274, 1104, 1102, 878, 732, 701.

1-(2-Hydroxyethyl)-4-methyl terephthalate (7): A mixure of benzyl ether **16** (0.72 g; 2.3 mmol), ethyl acetate (40 mL) and 10% palladium on charcoal (35 mg) was exposed to hydrogenolysis at 45 psi for 3 h in Parr apparatus at room temperature. The mixture was filtered through a plug of celite and the solvent was removed in *vacuo*. PET monomer **7** was obtained as a white solid after recrystallization from methanol (0.47 g, 91%, mp 87 °C) (Kudrna, 1964; Yasukawa et al., 2016).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 8.14–8.08 (m, 4H), 4.52–4.47 (m, 2H), 4.00–3.96 (m, 2H), 3.95 (s, 3H), 1.95 (brs, OH). ^{13}C NMR (100 MHz, $CDCl_3$): δc 166.2, 166.0, 134.1, 133.6, 129.6, 129.5, 67.0, 61.2, 52.4. HRMS *m/z* $[M+Na]^+$ calculated for $C_{11}H_{12}O_5$: 247.0582; found: 247.0586.

8 - Ethylene glycol bis(methyl terephthalate) (M2(HET)1.5).

A solution of methyl 4-(chlorocarbonyl)benzoate **(17)** (16.68 g; 0.084 mol; 2 eq) and dichloromethane (80 mL) was added over 30 min into the solution of ethylene glycol (2.35 mL; 0.042 mol; 1 eq), pyridine (10.2 mL; 0.126 mol; 3 eq) and dichloromethane (100 mL) at 0 °C. The reaction mixture was stirred for 12 h at room temperature. The resulting pyridinium salt was filtered, and filtrate was washed successively with saturated aqueous $CuSO_4$, water, saturated $NaHCO_3$ and brine, then dried over Na_2SO_4. The organic solution was concentrated and residue was purified by dry flash chromatography (SiO_2; eluent: dichloromethane/petroleum ether/ethyl acetate = 60:40:5), to afford of diester **8** as a white solid (10.25 g, 63%, mp 163 °C) (Fuentes et al., 2015).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 8.10 (brs, 8H), 4.70 (s, 4H), 3.94 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δc 166.3, 165.7, 134.3, 133.6, 129.82, 129.77, 63.1, 52.6. IR (ATR) ν_{max}: 3423, 3059, 2965, 1723, 1505, 1443, 1410, 1378, 1292, 1127, 1102, 977, 725. HRMS (ESI): *m/z* $[M+Na]^+$ calculated for $C_{20}H_{18}O_8$: 409.0899; found: 409.0893.

9 - Methyl bis(2-hydroxyethyl terephthalate) (M(HET)2, PET dimer).

bis(2-Benzyloxyethyl) terephthalate (19): A solution of terephthaloyl chloride **12** (1.06 g; 5.2 mmol; 1 eq) and toluene (10 mL) was added dropwise to a solution of 2-(benzyloxy)ethanol **18** (1.98 g; 13.0 mmol; 2.5 eq), pyridine (1.1 mL; 13.0 mol; 2.5 eq) and toluene (10 mL) during 15 min. The reaction mixture was stirred at 60 °C for 5 h and the resulting pyridinium salt was separated by filtration. The filtrate was diluted with ethyl acetate (15 mL) and washed with 1 M HCl, saturated $NaHCO_3$ and brine, successively. The extract was dried over anhydrous Na_2SO_4, concentrated in *vacuo*. The crude product was purified by dry flash chromatography (SiO_2; eluent: toluene/ethyl acetate = 8:2). The product **19** was obtained as colorless oil, which solidified after several days (2.04 g, 89%, mp 47 °C).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 8.10 (s, 4H), 7.36–7.23 (m, 10H), 4.60 (s, 4H), 4.54–4.48 (m, 4H), 3.83–3.78 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δc 165.7, 137.8, 133.9, 129.6, 128.4, 127.8, 127.7, 73.15, 67.8, 64.5. IR (ATR) ν_{max}: 3062, 3030, 2951, 2863, 1722, 1580, 1499, 1453, 1409, 1378, 1360, 1272, 1099, 1022, 731, 700.

4-((2-(Benzyloxy)ethoxy)carbonyl)benzoic acid (20): Potassium hydroxide (0.26 g; 4.7 mmol; 1 eq) was dissolved in 2-(benzyloxy)ethanol (0.71 g; 4.7 mmol; 1 eq) with stirring and gentle heating. Then, solution of *bis*(2-benzyloxyethyl) terephthalate **19** (2.04 g; 4.7 mmol; 1 eq) and toluene (45.0 mL) was added, and reaction mixture was heated at 120 °C for 3.5 h. After cooling to room temperature, the reaction mixture was filtered. The solid ionic salt was washed with ethyl acetate. Solid product was then dissolved in water (40 mL), acidified with HCl (conc.) to pH = 2, whereby the product separated as a colloidal precipitate. After 12 h, the mixture was filtered, and solid product was washed with water and dried. 4-((2-(Benzyloxy) ethoxy)carbonyl)benzoic acid **20** was obtained as white solid (0.58 g; 42%, mp 185 °C).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 8.20–8.13 (m, 4H), 7.39–7.27 (m, 5H), 4.63 (s, 2H), 4.57–4.52 (m, 2H), 3.86–3.81 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δc 170.8, 165.8, 137.9, 134.8, 133.2, 130.3, 129.9, 128.6, 128.0, 127.9, 73.3, 67.9, 64.7. IR (ATR) ν_{max}: 3361, 3061, 3031, 3008, 2960, 2892, 2855, 1718, 1577, 1499, 1470, 1446, 1389, 1366, 1268, 1136, 1268, 1136, 1093, 1012, 987, 870, 788, 727, 690.

2-((4-((2-(Benzyloxy)ethoxy)carbonyl)benzoyl)oxy)ethyl methyl terephthalate (21): A catalytic amount of DMAP (26.6 mg; 0.22 mmol; 0.2 eq) was added into the solution of PET monomer **7** (270.0 mg; 1.20 mmol; 1.1 eq), acid **20** (328.8 mg; 1.09 mmol; 1 eq) and dichloromethane (12 mL). The mixture was cooled in an ice bath, and a solution of DCC (269.8 mg; 1.31 mmol; 1.2 eq) in dichloromethane (3 mL) was added dropwise. The obtained reaction mixture was stirred at 0 °C for 5 min, and then at room temperature for 20 h. The byproduct, dicyclohexylurea (DCU), was removed by filtration and washed with ethyl acetate three times. The combined organic solution was concentrated on rotavapor, and residue was purified by dry flash chromatography (SiO_2; dichloromethane/petroleum ether/ethyl acetate = 60:40:5). Protected PET dimer **21** was obtained as a white solid (353.5 mg, 64%, mp 79 °C).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 8.12 and 8.11 (two overlaping singlets in ratio 4:4, 8H), 7.35–7.27 (m, 5H), 4.71 (s, 4H), 4.61 (s, 2H), 4.52–4.49 (m, 2H), 3.94 (s, 3H), 3.84–3.79 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δc 166.3, 165.8, 165.7, 138.0, 134.34, 134.28, 133.7, 133.6, 129.9, 129.82, 129.79, 129.77, 128.6, 128.0, 127.8, 73.3, 67.9, 64.7, 63.1, 52.6. IR (ATR) ν_{max}: 2933, 2857, 1725, 1649, 1503, 1451, 1409, 1373, 1278, 1102, 1021, 876, 732, 701.

Methyl bis(2-hydroxyethyl terephthalate (9): The catalytic amount of 10% palladium on charcoal (16 mg) was added into the solution of benzyl ether **21** (320.4 mg; 0.63 mmol) and 1,4-dioxane (8 mL). The hydrogenolysis of protected dimer was performed under hydrogen atmosphere, with rubber balloon filled with hydrogen, at room temperature for 4 h. The reaction mixture was filtered through a plug of celite, solvent was removed in *vacuo* and the crude product was purified by dry flash chromatography. The unreacted protected dimer was eluted using dichloromethane/ethyl acetate = 9:1, and then the deprotected dimer was eluted with ethyl acetate. PET dimer **9** was obtained as a white solid (230.1 mg, 87%; mp 128 °C).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 8.11and 8.10 (two overlapping singlets in ratio 4:4, 8H), 4.70 (s, 4H), 4.51–4.47 (m, 2H), 4.00–3.96 (m, 2H), 3.94 (s, 3H), 1.94 (brs, OH). ^{13}C NMR (100 MHz, $CDCl_3$): δc 166.3, 166.1, 165.7, 165.6, 134.3, 134.0, 133.8, 133.6, 129.9, 129.83, 129.80, 129.7, 67.2, 63.2, 63.1, 52.6. IR (ATR) ν_{max}: 3390, 3013, 2962, 1718, 1581, 1506, 1439, 1412, 1388, 1344, 1276, 1130, 1069, 1021, 894, 871, 727. HRMS *m/z* $[M+Na]^+$ calculated for $C_{21}H_{20}O_9$: 439.1005; found: 439.0992.

10–1,4-Benzenedicarboxylic acid, 1,4-bis[2-[[4-(methoxycarbonyl)benzoyl]oxy]ethyl] ester (M2(HET)2.5).

A solution of methyl 4-(chlorocarbonyl)benzoate **17** (595.8 mg; 3.0 mmol; 2 eq) and dichloromethane (5 mL) was added into the

solution of bis(2-hydroxyethyl)terephthalate **4** (381.4 mg, 1.5 mmol, 1 eq), pyridine (364 μl; 4.5 mmol; 3 eq) and dichloromethane (10 mL) at 0 °C over 15 min. The reaction mixture was stirred at room temperature for 12 h. Resulting pyridinium salt was separated by filtration. The filtrate was washed with saturated aqueous $CuSO_4$, water, saturated $NaHCO_3$ and brine successively, and then dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure, and the residue was purified by dry flash chromatography (SiO_2; eluent: dichloromethane/toluene/ethyl acetate = 60:40:3). Product **10** was obtained as a white solid (537.6 mg, 62%, mp 199 °C) (Brooke et al., 2002).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 8.11 and 8.10 (two overlapping singlets in ratio 4:8, 12H), 4.70 (s, 8H), 3.94 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δc 166.3, 165.7, 165.6, 134.3, 133.9, 133.6, 129.9, 129.81, 129.77, 63.2, 63.1, 52.6. IR (ATR) ν_{max}: 3429, 3013, 2960, 1724, 1505, 1470, 1437, 1410, 1340, 1266, 1128, 1021, 838, 870, 723. HRMS: m/z $[M+Na]^+$ calculated for $C_{30}H_{26}O_{12}$: 601.1322; found: 601.1314.

11 - Methyl tris(2-hydroxyethyl terephthalate) (M(HET)3, PET trimer).

Protected PET trimer (22): A solution of DCC (198.0 mg; 0.96 mmol; 1.2 eq) and dichloromethane (2 mL) was added dropwise into the solution of PET dimer **9** (366.5 mg; 0.88 mmol; 1.1 eq), acid **20** (240.1 mg; 0.80 mmol; 1 eq), DMAP (48.9 mg; 0.4 mmol; 0.5 eq) and dichloromethane (8 mL) at 0 °C. Then reaction mixture was stirred at room temperature for 24 h. DCU, as a byproduct, was removed by filtration. The precipitate was washed three times with ethyl acetate. The combined organic filtrate was collected and concentrated on rotavapor. The residue was purified by dry flash chromatography (SiO_2; dichloromethane/petroleum ether/ethyl acetate = 60:40:3). The product **22** was obtained as a white solid (268.4 mg, 48%, mp 134–136 °C).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 8.115, 8.101, 8.098 (three overlapping singlets in ratio 4:4:4, 12H), 7.36–7.27 (m, 5H), 4.704, 4.698 (two overlapping singlets in ratio 4:4, 8H), 4.60 (s, 2H), 4.53–4.49 (m, 2H), 3.94 (s, 3H), 3.83–3.79 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 166.3, 165.8, 165.70, 165.68, 165.6, 137.9, 134.35, 134.33, 133.9, 133.65, 133.59, 129.9, 129.8, 129.78, 129.76, 128.6, 127.9, 127.8, 73.3, 67.9, 64.1, 63.19, 63.13, 52.6. IR (ATR) ν_{max}: 3030, 2927, 2854, 1719, 1627, 1577, 1503, 1449, 1410, 1377, 1342, 1275, 1128, 1020, 876, 729.

PET trimer (11): The catalytic amount of 10% palladium on charcoal (10 mg) was added into the solution of protected PET trimer **22** (204.2 mg; 0.29 mmol) and 1,4-dioxane (6 mL). The hydrogenolysis of protected PET trimer was performed under hydrogen atmosphere, with rubber balloon filled with hydrogen, at room temperature for 6 h. The reaction mixture was filtered through a plug of celite, solvent was removed in *vacuo* and the crude product was purified by dry flash chromatography. The unreacted protected trimer was eluted using dichloromethane/ethyl acetate = 9:1, and then product of deprotection was eluted with dichloromethane/ethyl acetate = 1:1. PET trimer **11** was obtained as a white solid (141.2 mg, 80%, mp 173 °C).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 8.113, 8.100 and 8.098 (three overlapping singlets in ratio 4:4:4, 12H), 4.71 and 4.70 (two overlapping singlets in ratio 4:4, 8H), 4.51–4.47 (m, 2H), 4.00–3.96 (m, 2H), 3.94 (s, 3H), 1.60 (s, OH). ^{13}C NMR (100 MHz, $CDCl_3$): 166.4, 166.1, 165.7, 165.63, 165.61, 134.3, 134.1, 133.88, 133.87, 133.79, 133.6, 129.89, 129.88, 129.84, 129.81, 129.77, 67.2, 63.19, 63.16, 63.1, 61.4, 52.6. IR (ATR) ν_{max}: 3425, 3014, 2964, 1717, 1581, 1506, 1469, 1439, 1411, 1389, 1343, 1271, 1129, 1070, 1021, 971, 870, 725. HRMS (ESI): m/z $[M+Na]^+$ calculated for $C_{31}H_{28}O_{13}$: 631.1428; found: 631.1433.

Structural properties, logP, pKa, logD, solubility and geometry were predicted using software available from https://chemicalize.com/. Physico-chemical properties of PET molecules used for drug likeness determination were predicted using software available from https://www.molinspiration.com/.

2.2. Biological evaluations

2.2.1. Bacterial MIC assay

To compare planktonic antimicrobial activity, *Escherichia coli* (NCTC 9001) and *Staphylococcus aureus* (ATCC 25923) were used as test microorganisms in the microdilution assay in 96-well format recommended by the National Committee for Clinical Laboratory Standards (M07-A8). The highest concentrations of compounds tested were 1000 μg/mL (which corresponded to 16.1 mM concentration of compound **1** and 1.64 mM concentration of compound **11**) and the inoculum was 5×10^5 cfu/mL.

2.2.2. Aliivibrio fischeri toxicity test

The inhibitory effect on the light emission of *A. fischeri* (also called *Vibrio fischeri*) was determined according to the ISO 11348 standard, using freeze-dried bacteria. Toxicity analysis was performed with BioFix® Lumi-10 (Macherey-Nagel GmbH & Co. KG, Duren, Germany). Freeze-dried bacteria (*A. fischeri* NRRL B-11177, Macherey-Nagel GmbH & Co. KG, Duren, Germany) were reconstituted with the provided solution before the test. Bacteria were incubated at 15 °C with 1 mL of 2% (w/v) NaCl with different concentrations of PET compounds (**1**–**11**). Stock solutions of PET compounds (50 mg/mL in DMSO) were diluted in 2% (w/v) NaCl up to 500 μg/mL. All samples except **1** (EG), **4** (BHET) and **5** (BEET) formed a suspension when mixed with NaCl, hence, all of the 500 μg/mL sample solutions were centrifuged for 5 min at 10,000 rpm. The supernatants were transferred to a 1.5 mL tube and used for the preparation of serial dilutions. Serial dilutions of all tested samples were prepared by diluting each starting concentration by 50% (500–15.625 μg/mL). The bioluminescence was monitored after 15 min of incubation with the test solution.

2.2.3. Cytotoxicity assay

Cytotoxicity, as a measure of anti-proliferative effect on healthy human lung fibroblast MRC-5 cell line (obtained from ATCC) was determined using standard colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Hansen et al., 1989). Each tested compound was added to the cells at a concentration ranging from 25 to 250 μg mL^{-1} and the treatment lasted for 48 h. The MTT assay was performed two times in four replicates and the extent of MTT reduction was measured spectrophotometrically at 540 nm using a Tecan Infinite 200 Pro multiplate reader (Tecan Group Ltd., Männedorf, Switzerland). Cytotoxicity was expressed as the concentration of the compound inhibiting cell growth by 50% (IC50) in comparison to control (DMSO-treated cells).

2.2.4. Caenorhabditis elegans survival assay

C. elegans is a genetically tractable multicellular organism with metabolically active digestive, reproductive, endocrine, sensory and neuromuscular systems that has been widely used in toxicity screens (Hunt, 2017; Wittkowski et al., 2019).

Caenorhabditis elegans N2 (*glp-4*; *sek-1*) was propagated under standard conditions, synchronized by hypochlorite bleaching, and cultured on nematode growth medium using *E. coli* OP50 as a food source, as described previously (Stiernagle, 2006). The *C. elegans* survival assay was carried out as described previously with some modifications (Brackman et al., 2011; Scoffone et al., 2016). In brief, synchronized worms (L4 stage) were suspended in a medium containing 95% M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, and 1 mL of 1 M $MgSO_4 \times 7H_2O$ in 1 L of water), 5% LB broth (Oxoid, UK), and 10 μg of cholesterol (Sigma-Aldrich) per mL.

Experiment was carried out in 96-well flat bottomed microtiter plates (Sarstedt, Germany) in final volume of 100 μL per well. Aliquotes (25 μL) of this suspension of nematodes (25–35 nematodes) were transferred to the wells of a 96-well microtiter plate where 50 μL of medium were previously added. Next, 25 μL solvent control (DMSO) or 25 μL of a concentrated compound solution was added to the test wells. Final concentrations of the compounds were 500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL made out from stock solutions (50 mg/mL in DMSO) of each compound. Subsequently the plates were incubated at 25 °C for 2 days. The fraction of dead worms was determined after 48 h by counting the number of dead worms and the total number of worms in each well, using a stereomicroscope (SMZ143-N2GG, Motic, Germany). The compounds were tested at least three times in each assay and each assay was repeated at least two times ($n \geq 6$). As negative control experiment, nematodes were exposed to medium containing 1% (v/v) DMSO.

2.3. Application of PET dimer and trimer (9 and 11) in PETase activity assessment

2.3.1. PET-hydrolase gene synthesis and expression

Synthetic gene of *Humicola insolens* cutinase (HiC) was constructed based on its deposited amino acid sequence (PDB ID: 4OYY) and was codon optimized for expression in *Escherichia coli*, while cloned in pET26b(+) vectors (GenScript Biotech BV, the Netherlands). Recombinant expression vector was transferred to *E. coli* BL21 (NewEngland Biolabs, Ipswich, Massachusetts, USA) and enzyme was expressed based on a methodology described (Dimarogona et al., 2015). Briefly, overnight bacterial cultures (1%, v/v) were used to inoculate 500 mL LB medium supplemented with 100 μg mL^{-1} ampicillin, and culture was incubated at 37 °C to mid-exponential phase (OD_{600} of approximately 0.6). Then cultures were cooled down to 16 °C during 1 h, when 0.2 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to the medium and was left to incubate overnight. After that time *E. coli* cells were harvested by centrifugation at 4000×*g* for 15 min at 4 °C and resuspended in 50 mM Tris-HCl pH 8, 300 mM NaCl buffer. Cell suspension was disrupted by sonication during four 1-min cycles (8 s pulses and 5 s pause) at 40% amplitude using a 20 kHz high intensity (400 W) ultrasonic processor (VC 400, Sonic & Materials, Newtown, CT, USA). Cell debris was removed by centrifugation at 20,000×*g*, 30 min, 4 °C twice and loaded onto an immobilized metal-ion (Co^{2+}) affinity chromatography (IMAC), as previously described (Nikolaivits et al., 2016). The purity of isolated enzyme was checked on SDS-PAGE electrophoresis (12.5% (w/v) polyacrylamide) and protein concentration was determined by measuring the absorbance at 280 nm, based on the calculated molar extinction coefficient.

2.3.2. Enzymatic hydrolysis reactions and analysis of hydrolysis products

Reactions containing 1 mg/mL of each substrate (BHET, **8** (M2(HET)1.5), **9** (M(HET)2) and **10** (M2(HET)2.5)) in 0.1 M sodium phosphate buffer pH 8 were initiated by adding 0.5 μM of HiC and left to incubate at either 30 °C or 60 °C for 24 h. After that time 0.1% of 6 M HCl was added in each reaction and centrifuged at 5000×*g* at 10 °C. Supernatant was collected and analyzed on a SHIMADZULC-20AD HPLC equipped with a SIL-20A autosampler. The column used was a C-18 reverse-phase NUCLEOSIL®100-5 (Macherey-Nagel, Germany) and the mobile phase was 20% acetonitrile, 20% 10 mM sulfuric acid in ultrapure water at a flow rate of 0.8 mL/min. Detection of terephthalic acid (TPA) and its derivatives took place with a photodiode array detector Varian ProStar at 241 nm. Quantification of TPA, MHET and BHET was performed by constructing calibration curves with standard concentrations in the range of 0.01–1 mM.

2.4. Statistical analysis

Descriptive statistics (mean and standard deviation, SD) were used to describe the level of inhibition in different toxicity assays. Differences in effective concentration between the treatment in comparison to the untreated control were assessed using the standard non-parametric test (ANOVA), and results were considered statistically significant at $p < 0.01$. Statistical analysis was performed with STATISTICA 13.3 software.

3. Results and discussion

3.1. Synthesis and characterization

It is of a great importance to obtain pure oligomers of PET in order to study their biological properties and to assess them as platform molecules. They could be obtained either via partial hydrolysis of PET (top-down approach) or via synthesis step by step (bottom up approach). Preparation of oligomers by partial hydrolysis of PET is less controllable process and usually followed by prolonged and tedious chromatographic purification of the hydrolysis products. Synthesis of PET oligo esters, starting from ethylene glycol (**1**) and terephthalic acid (**2**), as well as their differently functionalized derivatives were developed over the last 60 years. Pioneering synthetic work in this field was carried out by Zahn et al. (Pénisson and Zahn, 1970; Zahn and Gleitsman, 1963), followed by Haslin et al. (1980) and more recently by Brooke et al. (2002). Possible PET oligomers can have free carboxylic group and hydroxyl group or both of them on the ends of the chain (Fig. 1B). In addition, these groups could be functionalized as ester and ether. It is well known that molecules with polar groups, such as –OH and –COOH, usually have better solubility in water and polar solvents and these properties could be tuned by chemical modification. With this in mind, we determined a set of model compounds **1**–**11** for this study (Fig. 1A).

Model compounds **5**, **6**, **8** and **10** were obtained by Schotten–Baumann reaction, starting from acid chlorides and alcohols. This is typical procedure for preparation of esters, involving the addition of acid chloride into the solution of alcohol and the organic base. However, the yield of these esters was highly dependent on the reaction conditions and required substantial optimization to achieve yields of more than 70%. Diesters **5** and **6** were obtained by addition of acid chloride into the solution of appropriate alcohol/diol and pyridine in toluene at 0 °C (Fig. S1). After usual work up products were isolated in good yields (72% and 71%). Compound **10** was identified as main side product of the synthesis of compound **8** and both were obtained in more than 60% yields in separate reactions (Fig. S1).

PET monomer **7**, dimer **9** and trimer **11** were prepared by coupling reaction between carboxylic acid and alcohols in the presence of dicyclohexyl carbodiimide (DCC), well known as Steglich procedure (Neises and Steglich, 1978). This mild procedure was applied in reaction with sensitive substrates such as alcohols **18**, **7** and **9**, which in reaction with appropriate acids gave benzyl protected monomer **16**, dimer **21** and trimer **22** in 82%, 64% and 48% yields, respectively (Fig. S1). Deprotection of these compounds was performed by hydrogenolysis and PET models **7**, **9** and **11** were obtained in excellent yields (80–91%). All NMR spectra showing purity of the compounds are depicted in Fig. S2-S14.

Molecular descriptors that characterize electronic and structural properties of compounds have provided useful predictive information on the toxicity of compounds (Bakire et al., 2018).

Calculated structural properties, including logP, pKa, logD, solubility and geometry were determined for all 11 compounds with a view of providing information that is important for biological properties determination (Table 1 and Table 2). In addition, predicted physico-chemical properties of PET molecules used for drug likeness determination were also estimated (Table 3).

The acid dissociation constant (pKa), the 1-octanol–water partition coefficients (Kow) and logP as the 10-base logarithmic measure of the Kow and solubility were estimated for all compounds being useful for the environmental risk assessment of chemicals (Table 2). For example 1-octanol–water systems display similarities to the partition of compounds between water and the biological membranes of microorganisms or cells, due to the amphiphilic nature of 1-octanol, similar to a generalized lipid phase in terms of their dielectric properties (Turner and Williamson, 2005). Correlations between the toxicity results and Kow values have been successfully obtained for diverse classes of compounds (Czerwinski et al., 2006), Szwej et al. (2015). Given that compounds having ionizable groups exist in solution as a mixture of different ionic forms, the ionization of those groups, depends on the pH of the environment, therefore LogD (the distribution constant) is the more appropriate descriptor for lipophilicity of ionizable compounds because it accounts for the pH dependence of a molecule in aqueous solution (Table 2). Solubility as an ability for a given substance to dissolve in a solvent and logS as the 10-based logarithm of the solubility measured in mol/L was also predicted for each of the compounds using H_2O as a solvent (Table 2). Polarity and polarizability presented in Table 1 are also useful descriptors for the prediction of chemical reactivity and bioactivity of given compounds (Tandon et al., 2020).

Compounds **1**, **2** and **4** have polar groups on the ends, while compounds **3**, **5**, **6**, **8** and **10** have ether or ester non-polar groups on the ends. PET oligomers **7**, **9** and **11** have free OH group on the end and methyl ester on the other side of the chain. Overall, polar compounds among PET-associated library are **1**, **2** and **4**, while majority of monoesters (**7**, **9** and **11**) and diesters (**3**, **5**, **6**, **8** and **10**) are nonpolar (Tables 1 and 2). Compounds **8**, **9** and **10**, corresponding to 1.5-, di- and 2.5-mer of PET, can accept 4,5 and 6 hydrogen bonds (Table 1). With logP of higher than 4, predicted for **10** (M2(HET)2.5) and **11** (M(HET)3) these compounds can be considered hydrophobic (Table 2).

In the search for new potential drug candidates establishing druglikeness is very important step. Druglikeness allows the assessment of the pharmacokinetic profile of the tested molecules based on the prediction of their absorption and distribution. An effective methodology for estimation of potential solubility and permeability of potential new drug based on the molecular weight, octanol/water partition coefficient, number of H-bond donors and number of H-bond acceptors is known as “Rule of five” (Lipinski, 2004). Briefly, poor absorption or permeation are more likely to occur when the molecule has molecular weight more than 500, logP over 5, and contains more than 5 H-bond donors or 10 (2×5) H-bond acceptors. The critical limit for acceptable drug-likeness is that no more than one violation of the rule exists in molecule. We have subjected all PET related compounds to this *in silico* assessment and notably, only **10** (M2(HET)2.5) and **11** (M(HET)3) have three violations each (Table 3). The rest of investigated PET compounds obey the “Rule of five” and meet all criteria for good solubility and permeability.

For investigated compounds with miLogP below 4 (all except **8** (M(HET)1), **10** (M2(HET)2.5)) and **11** (M(HET)3)) can be suggested that they have favorable physicochemical profiles for oral bioavailability (Veber et al., 2002). In addition, sufficient oral bioavailability is expected for molecules with 10 rotatable bonds or fewer (Veber et al., 2002), which is fulfilled in 6 different PET compounds with **10** (M2(HET)2.5) and **11** (M(HET)3) having 18 and 20 ratable bonds, respectively (Table 3).

Topological polar surface area (TPSA) can be defined as a sum of the surface areas occupied by the oxygen and nitrogen atoms and the hydrogen atoms attached to them and represent the hydrogen bonding capacity of the molecules, as such represents good predictor of the drug transport properties, drug absorption. Molecules with TPSA <140 $Å^2$ have good intestinal absorption, while those with TPSA <60 $Å^2$ show good blood–brain barrier penetration (Prasanna and Doerksen, 2009). Only compounds **10** (M2(HET)2.5) and **11** (M(HET)3) do not fall into this category, and **1** (EG), **3** (DMTP) and **6** (EGDB) have the potential to cross the blood–brain barrier (Table 3). Having concluded that most of the compounds have good solubility and good potential to cause effect of living cells we have assessed them across different systems to evaluate their toxicity.

3.2. Toxicity analysis

Aquatic toxicity is required in the assessment of the toxicity of organic chemicals to marine and freshwater organisms, therefore *A. fischeri* (also called *Vibrio fischeri*) model system was employed in the assessment of PET model compounds (Fig. 2). *A. fischeri* is a bioluminescent, Gram-negative marine bacterium that can be found free living and in a mutualistic association with certain squids and fishes and has been successfully employed as model organism in toxicity evaluations of number of chemicals (Di Nica

Table 1
Predicted[a] structural properties of PET model compounds **1**–**11**.

PET compound	1	2	3	4	5	6	7	8	9	10	11
Atom count	10	18	24	32	44	34	28	46	50	68	72
Non-hydrogen atom count	4	12	14	18	22	20	16	28	30	42	44
Asymmetric atom count	0	0	0	0	0	0	0	0	0	0	0
Rotatable bond count	1	2	4	8	12	7	6	11	13	18	20
Ring count	0	1	1	1	1	2	1	2	2	3	3
Aromatic ring count	0	1	1	1	1	2	1	2	2	3	3
Hetero ring count	0	0	0	0	0	0	0	0	0	0	0
Fsp3[b]	1	0	0.2	0.33	0.5	0.12	0.27	0.2	0.24	0.2	0.23
Hydrogen bond donor count	2	2	0	2	0	0	1	0	1	0	1
Hydrogen bond acceptor count	2	4	2	4	4	2	3	4	5	6	7
Formal charge	0	0	0	0	0	0	0	0	0	0	0
Topological polar surface area ($Å^2$)	40.46	74.6	52.6	93.06	71.06	52.6	72.83	105.2	125.43	157.8	178.03
Polarizability ($Å^3$)	5.72	15.08	19.21	24.2	31.65	28.76	21.7	37.7	40.2	56.2	58.69
Molar refractivity (cm^3/mol)	14.55	40.57	50.11	62.69	81.69	74.2	56.4	98.25	104.54	146.39	152.68

[a] predicted values obtained using https://chemicalize.com/.
[b] Fsp3 = sp3 carbon atom count/total carbon atom count.

Table 2
Predicted[a] pKA, LogP and solubility of PET compounds **1**–**11**.

PETcompound											
	1	2	3	4	5	6	7	8	9	10	11
pKa	14.83	3.32	nd[b]	14.79	nd	Nd	15.1	nd	15.1	nd	15.1
LogP	−1.209	1.288	1.98	0.6	2.60	3.782	1.29	3.789	3.099	5.598	4.907
pH	**logD**										
1.7	−1.21	1.28	1.98	0.6	2.60	3.78	1.29	3.79	3.10	5.60	4.91
4.6	−1.21	−0.32	1.98	0.6	2.60	3.78	1.29	3.79	3.10	5.60	4.91
6.5	−1.21	−3.67	1.98	0.6	2.60	3.78	1.29	3.79	3.10	5.60	4.91
7.4	−1.21	−4.91	1.98	0.6	2.60	3.78	1.29	3.79	3.10	5.60	4.91
8	−1.21	−5.40	1.98	0.6	2.60	3.78	1.29	3.79	3.10	5.60	4.91
pH	**Solubility [logS]**										
1.7	1.14	−1.6	−2.03	−1.71	−2.77	−4.06	−1.88	−4.48	−4.24	−6.35	−6.01
4.6	1.14	0	−2.03	−1.71	−2.77	−4.06	−1.88	−4.48	−4.24	−6.35	−6.01
6.5	1.14	0.39	−2.03	−1.71	−2.77	−4.06	−1.88	−4.48	−4.24	−6.35	−6.01
7.4	1.14	0.39	−2.03	−1.71	−2.77	−4.06	−1.88	−4.48	−4.24	−6.35	−6.01
8	1.14	0.39	−2.03	−1.71	−2.77	−4.06	−1.88	−4.48	−4.24	−6.35	−6.01
Intrinsic solubility	1.14	−1.61	−2.03	−1.71	−2.77	−4.06	−1.88	−4.48	−4.23	−6.35	−6.02
Solubility category	High	High	High	High	High	Moderate	High	Moderate	Moderate	Low	Low

[a] Predicted values obtained using https://chemicalize.com/.
[b] nd = not determined using the prediction software.

Table 3
Predicted[a] physico-chemical properties of PET model molecules **1**–**11** used for drug likeness determination.

PET compound	1	2	3	4	5	6	7	8	9	10	11
miLogP	−0.95	1.76	2.28	0.83	2.62	3.71	1.55	4.06	3.33	5.83	5.11
TPSA	40.46	74.6	52.61	93.07	71.08	52.61	72.84	105.22	125.45	157.83	178.06
natoms	4	12	14	18	22	20	16	28	30	42	44
Mw	62.07	166.13	194.19	254.24	310.35	270.28	224.21	386.36	416.38	578.53	608.55
nON	2	4	4	6	6	4	5	8	9	12	13
nOHNH	2	2	0	2	0	0	1	0	1	0	1
nviolations	0	0	0	0	0	0	0	0	0	**3**	**3**
nrotb	1	2	4	8	12	7	6	11	13	18	20
volume	62.27	138.05	173.1	223.22	291.88	244.99	198.16	334.05	359.11	495	520.06

LogP – Octanol-water partition coefficient.
TPSA – Molecular Polar Surface Area.
natoms – Number of atoms in molecule.
Mw – Molecular weight.
nON – Number of nitrogen and oxygen atoms in molecule.
nOHNH – Number of amino and hydroxyl groups.
nviolations – Number of violated Lipinski rules.
nrotb – Number of rotable bonds.
volume – Volume of molecule.
[a] predicted values obtained using www.molinspiration.com.

et al., 2017). The inhibition of bioluminescence in *A. fischeri* is considered more sensitive for nonspecific toxicity than some other available bioanalytical approaches (Neale et al., 2012).

Compound **6** (EGDB) showed the highest toxicity to *A. fischeri* with EC_{50} value lower than 7.81 μg/mL (Table S1). In addition to **6**, compound **11** (M(HET)3) with EC_{50} lower than 10 μg/mL could also be considered as toxic (Passino and Smith, 1987; Ventura et al., 2016), while compounds **2** (TPA) and **5** (BEET) with EC_{50} between 10 and 100 μg/mL (27.1 and 43.0, respectively) were deemed as moderately toxic. The rest of the compounds could be considered harmless (EC50 values higher than 125 mg/L). Compounds **1**,**4**, **7**, **8**, **9**, **10** have showed very low inhibition of *A. fischeri* bioluminescence, and the highest inhibition was around 30% for the compounds **4**, **7** and **10** (Fig. 2). Since all observed effects were lower than 50%, EC50 could not be calculated. With the exception of compound **9**, there was no concentration-inhibition dependence observed, and thus EC20 and EC10 values could not be calculated either (Table S1).

Observed results for EG (**1**) are in line with the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) registration dossier for EG and higher glycols that are considered not toxic and not harmful to aquatic organisms. The NOEC (no observed effect concentration) on fish, daphnia and algae for EG is above 100 mg/L (European Chemicals Agency, dossier 15,973). The EC_{50} for the algae *Pseudokirchneriella subcapitata* is 36.64 g/L (Aruoja et al., 2014). According to Macario et al. (Macário et al., 2018), the EC_{50} value after 15 min of *A. vibrio* exposure to ethylene glycol was 87.75 g/L. In a different report, the EC_{50} value after 15 min was 621 ppm, corresponding to approximately 0.62 g/L (Docherty and Kulpa, 2005). TPA (**2**) also has no adverse effect in both short and long term ecotoxicity tests on aquatic organisms. The lowest concentration of test substance which results in a 50 percent reduction in growth rate (ErC50) in the tests with fish, *Daphnia* and algae was for algae (ErC50: >19.0 mg TPA/L) (European Chemicals Agency, dossier 15,563). Ventura et al. (2016) showed that there was no toxic effect on *V. fisheri* (the EC_{50} could not be determined). This differs from our results, since we have obtained the EC50 value of 27.1 mg/L for TPA. In their study, Ventura et al. stated that TPA is non-toxic and that EC50 could not be defined due to very low solubility in water, and there is no report of the concentrations used for the test. In our study, the stock solutions of compounds were prepared in DMSO, which could account for the

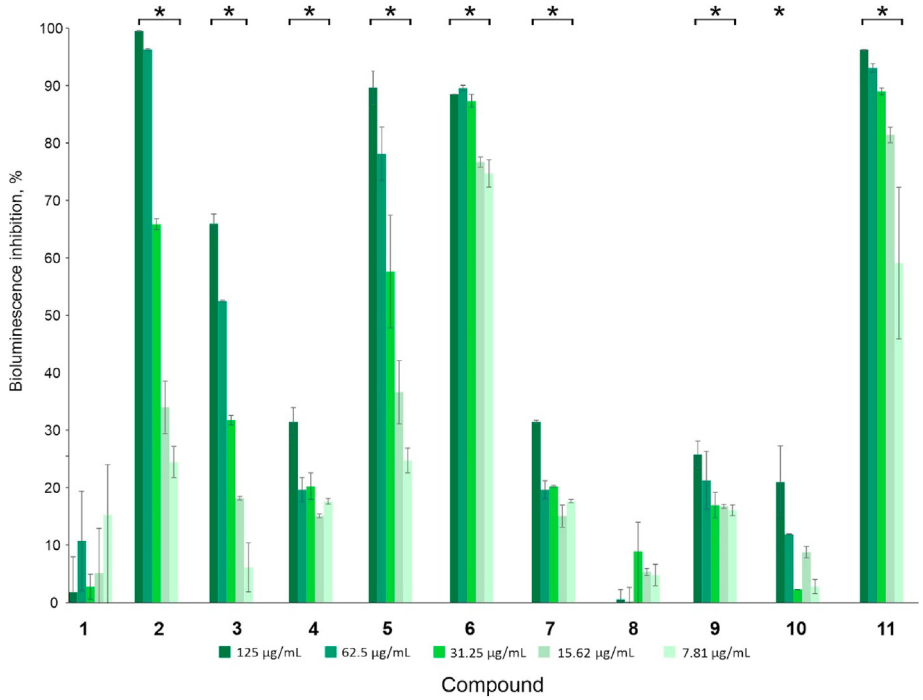

Fig. 2. Inhibition of *A. fischeri* bioluminescence upon exposure to PET compounds (average ± the SD and comparison to the untreated control, $p < 0.01$).

observed difference. TPA levels in the environment have been estimated by the US Environment Protection Agency and range from 0.038 mg/L in marine water to 50 mg/L in a sewage treatment plants (data available from https://www.epa.gov/risk/regional-screening-levels-rsls). These values are also available for compounds **1** (0.004 μg/L of tap water and 0.013 mg/kg of residential soil) and **3** (0.019 mg/L of tap water and 0.08 mg/kg of residential soil) and not for **4** (BHET).

The effect of compounds on pathogenic bacteria, healthy human lung fibroblast MRC-5 cell line and nematode *C. elegans* was assessed in order to gain deeper understanding and more specific effects (Fig. 3). None of the compounds were affecting the growth of common human pathogens *E. coli* and *S. aureus* even at 1000 μg/mL (results not shown). This result may indicate that they do not possess any antimicrobial properties, however they can possibly be internalized and bio-augmented by bacteria throughout the food chain, which is a risk to be taken into consideration. Most of the compounds were not toxic to cells at 200 μg/mL (Fig. 3A), however for **9** (M(HET)2), **11** (M(HET)3) and **7** (M(HET)1) IC_{50} values of 30, 50 and 100 μg/mL, respectively were calculated. These three being monoesters with free hydroxyl group (Fig. 1). Compound **11** (PET trimer), although violating Lipinski 'Rule of Five' and as such not predicted to have biological activity, exhibited inhibition of cell proliferation, possibly due to the formation of suspension upon application onto cells. **10** (M2(HET)2.5) was causing about 20–30% cell death irrespective of the amount applied (Fig. 3A). When the compounds were assessed using nematode model system, **7** (M(HET)1) and **3** (DMTP) were the most toxic, in the concentration of 500 μg/mL, while most of the compounds were not toxic in 250 μg/mL (Fig. 2B). Noteworthy is the fact that concentrations of test compounds of 500 μg/mL are considered quite high in *C. elegans* model.

TPA (**2**) although on the Hazardous Substance List due to the fact that can cause irritation of nose, throat and lungs upon breathing, but widely used even as an additive to poultry feeds (Slinger et al., 1962), did not show any adverse effects on cells and *C. elegans*, but showed acute toxicity on *A. fischeri* (Figs. 2 and 3). BHET (**4**) is also produced and used on the large scale and no toxicity or environmental hazards data are available (Qiu et al., 2020). In this study, **4** was not harmful to *A. fischeri* and *C. elegans*, while it caused 30% decrease in MRC-5 cell proliferation at 200 μg/mL (Figs. 2 and 3). PET bottled water that contained BHET didn't induce any cytotoxic, genotoxic or endocrine disruptive effect (Bach et al., 2013; Mao et al., 2005). BHET is the common model compound used in the screen for enzymatic depolymerization of PET and it is also major intermediate in biotechnological upcycling of PET (Allen, 2019; Palm et al., 2019; Tiso et al., 2020).

In summary, PET trimer (**11**) was toxic on *A. fischeri* and MRC-5 cells, PET monomer (**7**) was toxic on cells and nematode, while PET dimer (**9**) was toxic on cells and not on other two systems. Dimethyl terephthalate (**3**) was toxic on nematode and *A. fischeri*. Overall, not any clear tendency in respect to the chemical features of the compounds studied could be inferred about their toxicity levels in three systems employed within this study. Possibly the effect of the presence of free hydroxyl groups contributed to toxicity of **11**, **9** and **7** on different systems. Also, the most hydrophobic compound **11**, was the most toxic in two studied systems. It has been previously shown that increasing hydrophobicity of furans and derivatives decreased toxicity on *A. fischeri* (Ventura et al., 2016). However, the results depicted in Figs. 2 and 3 show that the hydrophobic/hydrophilic nature of these compounds is not enough to explain all the results, and that other structural descriptors may play the important role.

3.3. Enzymatic hydrolysis of PET model compounds

Degradability of polymeric materials is a function of their structures, the presence of degradative microbial population and the environmental conditions that encourage microbial growth. Our goal in this study was to test whether these novel synthesized PET oligomers could be hydrolyzed by a proven PET depolymerase and act as a substrate for screening and characterization of such

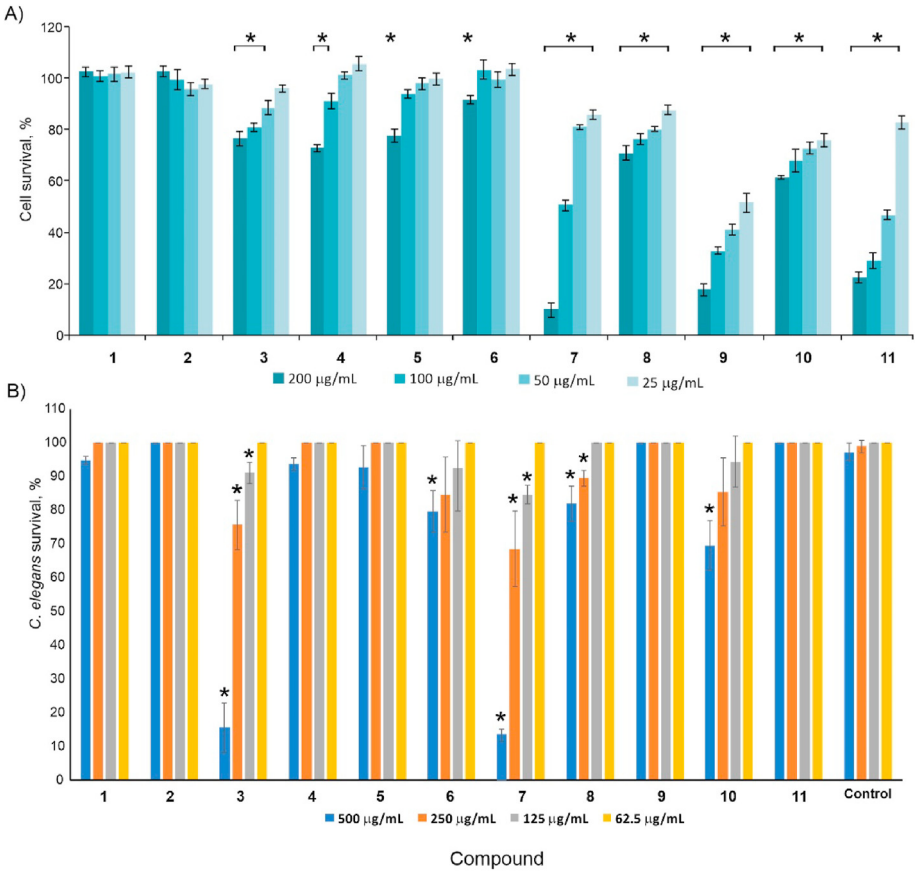

Fig. 3. Effect of PET model compounds on A) healthy human fibroblast MRC-5 cell line survival rate in comparison to DMSO treated control that was set to 100%, and B) percent survival of *C. elegans* (average ± the SD). The results are expressed as the percent survival after 48 h of treatment and compared to the untreated control ($p < 0.01$).

enzymes. HiC has proven to be an efficient depolymerase having the ability to hydrolyze various types of polyesters (Gigli et al., 2019; Su et al., 2020; Weinberger et al., 2017). However, the majority of studies performed so far utilize enzymes of bacterial origin for the degradation of PET (Kawai et al., 2019). HiC was used for the first time in 2009 probably as a crude preparation provided by Novozymes for the degradation of low crystallinity (lcPET – 7% crystallinity) and biaxially oriented (boPET – 35% crystallinity) PET films (Ronkvist et al., 2009). Even though HiC resulted in 97% weight loss of lcPET after 96 h at 70 °C, the degradation of boPET was a lot lower. However, the authors reported that the hydrolysis product was almost exclusively TPA. On the other hand, Carniel et al. studied the synergism between HiC (Novozym©51,032 crude enzymatic preparation) and lipase CALB aiming to increase TPA yield and overall hydrolysis of pretreated PET-bottle films (Carniel et al., 2017). The combination of these enzymes increased PET to TPA yield 7.7 times leading to mole fraction of TPA up to 0.88 after 14 days of reaction at 50 °C. Additionally, HiC has been used for treatment of chemically treated PET fibers, increasing the TPA yield from 85% to 97% (after 24 h at 50 °C) by hydrolyzing residual oligomers (Quartinello et al., 2017).

In this work, we utilized the purified recombinant form of HiC in order to limit interference from other enzymes or medium components. Reactions were performed at pH 8 at either 30 or 60 °C. The pH was selected considering that HiC could act well at pH 8, but also that most PET-hydrolyzing enzymes have an optimum pH at 8–9 (Nikolaivits et al., 2018). The two temperatures were chosen in order to cover the cases of both thermophilic and mesophilic enzymes.

HiC was firstly tested on BHET at a concentration of 1 mg/mL. At 60 °C BHET was autohydrolyzed completely after 24 h to MHET and TPA. Nonetheless, at 30 °C only a small amount was hydrolyzed at the control reactions (approx. 0.6 mM MHET), when the enzyme completely hydrolyzed BHET producing almost completely MHET and a small amount of TPA (0.06 mM). Even so, BHET is a water-soluble compound that can be hydrolyzed by various esterases/lipases that do not necessarily hydrolyze insoluble hydrophobic PET (Barth et al., 2016; Ion et al., 2020; Palm et al., 2019). Consequently, we tested the activity of HiC on the insoluble compounds **8** (M2(HET)1.5), **9** (M(HET)2) and **10** (M2(HET)2.5), which constitute of 1.5, 2 and 2.5 PET monomers, respectively. Overall, five products were detected with the applied method: **2** (TPA), MHET, **4** (BHET), monomethyl TPA (MTPA) and **7** (M(HET)1), which is the methylated MHET (Fig. 4, Table S2; Fig. S15).

Compound **10** (M2(HET)2.5) proved to be a very resilient substrate to autohydrolysis and enzymatic hydrolysis, with only traces of the products detected after 24 h (Table S2). However, comparing the different reaction temperatures we can see that at 60 °C only TPA and methyl TPA were detected, while at 30 °C mostly MHET and MTPA with fewer amounts of BHET and M(HET)1. We suspect that, since the enzymatic mechanism in both temperatures is the same, TPA at 60 °C was derived from the autohydrolysis of enzymatically produced MHET. Similarly, the absence of M(HET)1 might be a result of its autohydrolysis to methyl TPA. Considering that HiC has a very low activity on MHET (according to the test reaction with BHET), the enzyme probably preferentially cleaves **10** (M2(HET)2.5) at the first main-chain ester bond from the sides of the molecule, releasing two MTPA units and BHET, which is further converted to

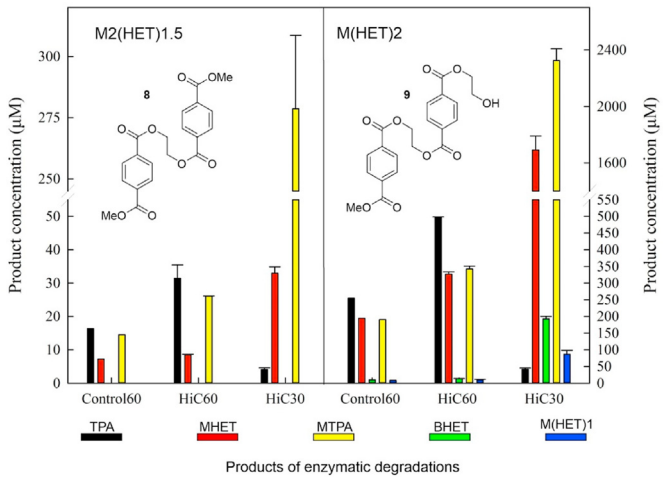

Fig. 4. Products of enzymatic reactions identified after incubation of HiC with 1 mg/mL of each M2(HET)1.5 and M(HET)2 for 24 h at 30 °C or 60 °C.

MHET. Hence, compound **10** (M2(HET)2.5) can be utilized as a substrate for screening purposes for the discovery or protein engineering of highly PET-active enzymes, especially thermophilic ones, even in a high throughput manner in a 96-well microtiter plate format. Recently, this approach was applied for the PET derived nanoparticles (Pfaff et al., 2021).

Using compounds **8** and **9**, 1.5- and dimer respectively, we noticed that the latter results in 10 times higher product concentration in all cases (Fig. 4). For both compounds, there was also a high amount of products detected at 60 °C in control reactions (absence of enzyme) compared to the control reactions at 30 °C, where no products were detected. High temperature in combination with slightly alkaline pH led to autohydrolysis of these substrates. In both cases, the concentration of products detected for the enzymatic reaction were 2-fold higher compared to the control reactions. At 30 °C autohydrolysis for both starting compounds was zero, so we could more easily observe the enzyme's mode of action. For **8** (M2(HET)1.5) the main product was methylated TPA (MTPA) (279 μM). Even though one would suspect that equal amounts of M(HET)1 would be released, what we noticed were the small amounts of MHET (33 μM) and TPA (4 μM). These could derive from the cleavage of the resulting M(HET)1 either at the methyl or the ethylene glycol ester bonds.

The reaction that led to the highest product release was the one with the compound **9** (M(HET)2), representing the PET dimer. In this case, MTPA was the main product (2.3 mM) followed by MHET (1.7 mM) and BHET (0.2 mM). Clearly HiC cleaves at the first main-chain ester bond from the MTPA side releasing MTPA and BHET, which is further converted to MHET and a small amount in TPA (42 μM). The small amount of liberated M(HET)1 detected (87 μM) might show a secondary first cleavage point at the center of the molecule. Studies of the hydrolysis of compound **9** (M(HET)2) with a another polyesterase have shown a different mode of action for the two enzymes (Nikolaivits et al., 2020). This fact could imply that PET dimer can be used as a substrate for various polyesterases and potentially correlate their hydrolysis mechanism (based on product release) with their ability to degrade PET polymer.

The first and most used PET model substrate is bis (benzoyloxyethyl) terephthalate (3PET), resembling **10** (M2(HET)2.5), and has been successfully utilized for the discovery of microbes and enzymes that had the ability to modify the surface of PET fabrics (Fischer-Colbrie et al., 2004; Heumann et al., 2006). 3PET has also been used as a sole carbon source for screening microbes in liquid cultures or agar plates, but also as an inducer for polyester-hydrolyzing enzymes (Liebminger et al., 2007; Ribitsch et al., 2011). Recently, ethylene glycol bis(*p*-methylbenzoate), resembling **8** (M2(HET)1.5), has been utilized as substrate for the development of a turbidimetric assay for high-throughput screening of PET oligomer-hydrolyzing enzymes (Belisário-Ferrari et al., 2019). These model compounds contain benzoic acid moiety and due to that are more amenable to microbial and enzymatic attack, in contrast to compounds **7**–**11**, which were designed to encompass only TPA and EG fragments, resembling more closely to realistic PET fragments (Fig. 1), leading to the discovery of more efficient PET depolymerases.

4. Conclusion

Microplastics have moved into virtually every crevice on Earth, with PET being in the top of widely utilized materials. Recent efforts to biotechnologically degrade waste PET and valorize building blocks are evident (Tan et al., 2019; Tiso et al., 2020). In a view of their extensive occurrence either from natural degradation of PET waste accumulated in nature or from the (bio)industrial degradation, library of 11 compounds, building blocks or hydrolysis products of PET, have been synthesized and thoroughly characterized in terms of structure and their toxicity. The structural and toxicity data for most of these important molecules was not existing in the available literature.

Different levels of toxicity are found among these closely related compounds, across panel of tests, which may indicate different mechanisms of toxicity with the main driving force for the observed toxicity compounds' lipophilicity, and thus their penetration into the cell. Some of the compounds exhibited a considerable level of toxicity to *A. fischeri* indicating their environmental hazard. Overall PET-trimer (M(HET)3), methyl tris(2-hydroxyethyl terephthalate), may have the highest toxicity, indicating the longer the oligomers released the greater the risk. Our understanding of polymer degradation has been advanced in recent years, but the lack of information available on the participating microorganisms and particularly enzymatic mechanisms involved is evident. The obtained results suggest that compounds reported in this study, especially PET dimer, may be a more suitable substrate for the assessment of novel PETases and, as such, should be adopted as an alternative to BHET.

CRediT author contribution statement

Milica Djapovic: Synthesis methodology, Investigation, Data curation, Writing - original draft. Dusan Milivojevic: Toxicity assessment, Data curation, Writing - editing. Tatjana Ilic-Tomic: Investigation, Cell toxicity methodology, Writing - original draft. Marija Lješević: Investigation, eco-toxicity assessment, Writing - original draft. Efstratios Nikolaivits: Investigation, Enzymatic degradation and HPLC methodology, Writing - review and editing. Evangelos Topakas: Investigation, Resources, Writing – editing. Veselin Maslak: Conceptualization, Resources, Writing - review and editing. Jasmina Nikodinovic-Runic: Conceptualization, Funding acquisition, Writing - review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.130005.

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