Chemosphere 144 (2016) 107–112

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Toxicity of synthetic herbicides containing 2,4-D and MCPA moieties towards *Pseudomonas putida* mt-2 and its response at the level of membrane fatty acid composition

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HIGHLIGHTS

- Toxicity of herbicidal esterquats as potential herbicidal ionic liquids was tested using Pseudomonas putida mt-2.
- Precursors of herbicidal ionic liquids showed lower toxicity compared to commercialized synthetic herbicides.
- Herbicidal ionic liquids caused a toxicity depending isomerisation of cis to trans unsaturated fatty acids.

ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form
20 August 2015
Accepted 21 August 2015
Available online xxx

Keywords:
Herbicidal ionic liquids
Toxicity
EC 50
Pseudomonas putida
Cis–trans isomerisation
Fatty acid methyl esters (FAME)

ABSTRACT

One of the attempts to create more effective herbicidal compounds includes the use of ionic liquids. Herbicidal ionic liquids have more effective biological activity, they are less volatile, more thermally stable, and exhibit superior efficiency in comparison to typically employed herbicides, allowing the reduction of the herbicide dose applied per hectare. However, studies on the environmental toxicity of this group of compounds are very rarely available. Environmental toxicity is an important factor, showing the concentration of compounds that has negative effects on soil bacteria including those responsible for biodegradation processes. Therefore, potential toxicity of four herbicidal ionic liquids (HILs) precursors containing 2,4-D and MCPA moieties was tested with the well investigated model organism for toxicity and adaptation, *Pseudomonas putida* mt-2. Results were compared to those obtained for commercial 2,4-D and MCPA herbicides. Next to growth inhibition, given as EC_{50}, changes in the isomerisation of *cis* to *trans* unsaturated fatty acids were applied as proxy for cellular stress adaptation to toxic substances. The results revealed that all investigated precursors of HILs showed lower toxicity compared to commercialized synthetic herbicides 2,4-D and MCPA. The collected data on toxicity of HILs together with their physico-chemical properties might be useful for assessing the potential risk of the environmental pollution as well as guidelines for setting the legislation for their future use.

1. Introduction

Herbicides are widely used in agriculture to provide unimpeded grow of plants in monoculture. Derivatives of phenoxy acids such as (2,4-dichlorophenoxy)acetic acid (2,4-D) and (4-chloro-2-methylphenoxy)acetic acid (MCPA) are common synthetic herbicides. They have been produced since the 1940s and used in agriculture up to the present day. Commercial herbicidal formulations contain these active substances in several forms such as acids, sodium, and potassium salts, primary, secondary, and tertiary ammonium salts, and esters. Among them, esters belong to most active forms (Pernak et al., 2011).

However, attempts to create more efficient types of herbicides and minimize their negative influence on the environment are constantly made. One of the solutions includes the use of ionic liquids. New forms of phenoxyherbicides are herbicidal ionic liquids (HILs) defined as ionic compounds with melting temperature below 100 °C, where one of the ions possesses herbicidal activity. This kind of compounds, with the anion (MCPA) exhibited herbicidal activity, were first described by Pernak et al. (2011). In

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http://dx.doi.org/10.1016/j.chemosphere.2015.08.067
0045-6535/

subsequent papers, HILs contained other herbicide anions: 2,4-D (Pernak et al., 2012b; Praczyk et al., 2012), 2-(4-chloro-2-methylphenoxy)propionate (MCPP) (Pernak et al., 2012a), (3,6-dichloro-2-methoxy)benzoate (dicamba) (Cojocaru et al., 2013) were described. HILs with dual herbicidal function e.g. 2-chloroethyltrimethylammonium cation as a growth regulator with 2,4-D or MCPA (Pernak et al., 2013b) anions were already synthesized. HILs show more effective biological activity, they are less volatile, more thermally stable, and exhibit superior efficiency in comparison to typically employed herbicides, allowing for the reduction of the herbicide dose applied per hectare (Cojocaru et al., 2013). However, in spite of the 'environmental friendliness' ionic liquids (ILs) have been recognized as potentially hazardous and recalcitrant xenobiotics for both aquatic and terrestrial ecosystems (Jastorff et al., 2003). The need for risk assessment data has been a major driving force behind numerous recent research projects dedicated to evaluation of the environmental impact of ILs (Pham et al., 2010; Ranke et al., 2007). Most of the studies have been focused on the toxicity assessment towards terrestrial and aquatic organisms, whereas the number of reports dedicated to toxicity towards bacteria is limited. In the presented study, *Pseudomonas putida* as one of the best investigated model organisms for toxicity and adaptation was chosen (Heipieper et al., 1995). This bacterium has also the advantage that it contains of a very special urgent mechanism to adapt to environmental stress, the isomerisation of *cis* to *trans* unsaturated fatty acids (Heipieper et al., 1992). *Cis–trans* isomerisation of unsaturated fatty acids was proven to be independent of de novo fatty acid biosynthesis, allowing the bacteria to adapt rapidly to the presence of toxic organic compounds even under environmental conditions not supporting their growth (Heipieper et al., 1994, 2007).

Therefore, the concept of the present study was to investigate potential toxicity of HILs precursors containing 2,4-D or MCPA moieties. These compounds are quaternary ammonium salts with 2,4-D and MCPA in the cation as ester groups of the esterquats. The toxicity of four esterquats: [2,4-DDAEC$_{10}$][Br], [2,4-DDAEC$_6$][Br], [MCPADAEC$_{10}$][Br], [MCPADAEC$_6$][Br] was investigated towards the best known bacterial strain regarding stress adaptation, *P. putida* mt-2, in a direct comparison with the synthetic commercialized herbicides 2,4-D and MCPA as reference compounds.

2. Materials and methods

2.1. Chemical reagents

2.1.1. Fatty acids

Analytical standards of methylated fatty acids (C16:0, C16:1*trans*, C16:1*cis*, C18:0, C18:1*trans*, C18:1*cis*) were purchased from Sigma–Aldrich (Munich, Germany).

2.1.2. Phenoxyherbicides

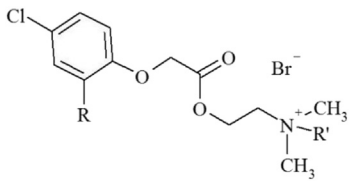
Commercial 2,4-D and MCPA were purchased from Sigma–Aldrich (Munich, Germany).

2.1.3. Precursors of herbicidal ionic liquids (HILs) – esterquats

Herbicidal esterquats were designed and synthetized in the Department of Chemical Technology in Poznan University of Technology. The synthesis and physico-chemical properties are provided in the Supplementary Material. Chemical structures are shown in Scheme 1 and Table 1.

2.2. Growth experiments with Pseudomonas putida mt-2

P. putida mt-2 (DSM 6125) was cultivated in a mineral medium as previously described by Hartmans et al. (1989) with 5 g/L Na_2-Succinate as carbon source. Cells were grown in 50 mL cultures in 250 mL flasks in a horizontally shaking water bath (160 U/min) at 30 °C.



Scheme 1. Chemical structure of synthetized esterquats.

Toxicant ([2,4-DDAEC$_6$][Br]; [2,4-DDAEC$_{10}$][Br]; [MCPADAEC$_6$][Br]; [MCPADAEC$_{10}$][Br]; 2,4-D; MCPA) at different concentrations was added to 50 mL of mineral medium in 250 mL flask shaking in water bath (30 °C, 160 U/min). The beginning of the growth experiment was established as the moment in which the turbidity (O.D.$_{560nm}$) of medium with toxicant was at a constant level. Depending on toxic compound it was after 1 h (2,4-D and MCPA) or 4–5 h (precursors of HILs). Parallel with that, an inoculum from an overnight culture (5 mL) was transferred to fresh medium. After 2 h of exponential growth, the culture was centrifuged and washed three times with phosphate buffer (50 mM, pH 7.0). The pellet of *P. putida* was suspended into medium with toxicant in flasks shaking in water bath (30 °C, 160 U/min). After that, the organisms continued to grow exponentially (with the small slope right after inserting pellet), but at reduced growth rates. One experiment lasted for approx. 5 h for 2,4-D and MCPA, and 7 h for precursors of HILs. Cell growth was measured by monitoring the turbidity (O.D.$_{560nm}$) of cell suspensions using a spectrophotometer. The growth rate μ of the cells was about 0.55 h^{-1}, which corresponds to a doubling time (t_D) of approx. 1 h 20 min.

2.3. Determination of growth inhibition

Growth inhibition caused by toxicants was calculated by comparing the differences in growth rates μ (h^{-1}) between intoxicated cultures with that of a control culture as described by Heipieper et al. (1995). The growth inhibition of different concentrations of toxicants is defined as the ratio of the growth rates μ (h^{-1}) of toxicant-treated and control cultures.

2.4. Lipid extraction and transesterification

The cells were harvested by centrifugation after 2 h in the presence of the toxic agents and washed with phosphate-buffer (50 mM, pH 7.0). After the next 1 h, lipids were extracted with chloroform/methanol/water as described by Bligh and Dyer (1959). Fatty acid methyl esters were prepared by a 15 min incubation at 95 °C in boron trifluoride/methanol using the method of Morrison and Smith (1964). Finally, the fatty acid methyl esters were extracted with hexane.

2.5. Determination of fatty acid composition

Fatty acid analysis was performed using gas chromatography (GC) (capillary column: CP-Sil 88; 50 m; temperature program from 160 °C to 220 °C; flame ionisation detector). The instrument used was a CP-9000 gas chromatograph (Chrompack-Packard). The fatty acids were identified with the aid of standards. The relative amounts of the fatty acids were determined from the peak areas of the methyl esters using a Chromatopac C-R6A integrator (Shimadsu, Kyoto, Japan). Replicate determinations indicated that the relative error [Relative error = (Standard deviation/mean) * 100%]