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ORIGINAL PAPER

Bio-oxidation of tripropylene glycol under aerobic conditions

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Abstract Aerobic biodegradation of tripropylene glycol (PG3) was investigated under the conditions of the OECD screening test 301E and the Continuous Flow Activated Sludge Simulation test (CFAS). A modified two-chamber facility with a denitrification stage was used for the CFAS test. Primary PG3 biodegradation was measured by the HPLC with fluorimetric detection and analyte derivatisation. Metabolites were identified by LC-MS with electrospray ionisation and GC-MS with electron impact ionisation, as well as semiquantitatively determined by the LC-MS technique. PG3 was found to be inherently biodegradable and it exhibits a strong poisonous effect on activated sludge after exceeding the threshold concentration (10 mg l^{-1}). Metabolite accumulation onto the activated sludge is probably responsible for this poisonous effect. Probable biotransformation products of tripropylene glycol under the aerobic conditions include metabolites with a single terminal aldehyde or a ketone group and metabolites with two terminal aldehyde or ketone groups. Their concentration rises at the end of the OECD screening test.

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Abbreviations

PG3	Tripropylene glycol
CFAS	Continuous flow activated sludge simulation test
PPG	Poly(propylene glycol)
NIC	1-Naphthyl isocyanate
OECD	Organization for Economic Co-operation and Development

Introduction

Propylene glycol, and di- and tripropylene glycol, the first three members of a homologous series of poly(propylene glycol)s (PPG), are completely water and ethanol soluble, as well as miscible with most organic solvents. Tripropylene glycol (PG3) is widely used as an initiator for urethane polyol synthesis from epoxides and as a polyol in some polyurethane foam systems (O'Sickey 2002). PG3 is an excellent solvent in applications where high solvent power is needed. Its water solubility, solvent power, low volatility, and high boiling point makes it a material of choice for textile soaps, lubricants and many similar products. PG3 has extensive applications—emulsifiers, emulsion stabilizers or viscosity modifiers. Due to inherent

antimicrobial activity against a wide range of bacterial and fungal species, PG3 is used as a preservative. Commercially available PG3 is a mixture of isomers (Fluka-Riedel-de-Haën 2005/2006).

PG3 can be released into the environment directly, or as a product of the biodegradation process of compounds containing the oxypropylene chain (PPG copolymers or alkyl derivatives). PG3, when released into water, is not readily biodegraded. Only 1–2% of PG3 is biodegraded under the conditions of the OECD 301 C test performed at concentration of 100 mg l^{-1} according to BOD measurements, as well as 0% according to TOC analysis and 0–3% from GC analysis (OECD SIDS Report 1994). PG3 is not toxic. For example its toxicity for Daphnia magna described as EC_{50} is above 1 g l^{-1} (OECD SIDS Report 1994).

Moreover, little information concerning biodegradation of PPGs is published (Balson and Felix 1995; Rychlowska et al. 2003; Tachibana et al. 2003; Zgola-Grzeskowiak et al. 2006). According to Rychlowska et al. (2003) biodegradation of PPGs is slow and leads to unknown products. PPGs are thought to be non-toxic for humans. Studies of PG3 proved its low toxicity as its acute toxicity for rats is 3 g kg^{-1} (OECD SIDS Report 1994). However, the toxicity of PPGs biodegradation products is unknown. Therefore, biodegradation studies of these compounds in the aquatic environment are highly significant.

Numerous tests are used for measuring the biodegradation of organic compounds, reflecting different situations of the process (Pagga 1997). Static screening tests performed under aerobic batch conditions are used for the determination of the "ready biodegradability". The following tests belong to this group (Pagga 1997): DOC Die away test (OECD 301A or ISO 7827), CO_2 evolution test (OECD 301B or ISO 9439), Closed bottle test (OECD 301D or ISO 10708), Modified OECD screening test (OECD 301E or ISO 7827), Respirometric test (OECD 301F or ISO 9408) and CO_2 head space test (ISO 14593). Substances, which pass these tests, are classified as readily biodegradable. The tested compound is the sole source of organic carbon. Apart from the tested substance, the tested mixture contains an inorganic test medium and an unadapted inoculum. Generally, ultimate biodegradability is determined by measuring summary parameters, such as DOC (alternatively COD), BOD or CO_2 evolution. The primary biodegradation determination of tested compounds requires the application of more sophisticated analytical methods. Even more advanced analytical methods are required for identification and determination of metabolites of tested compounds.

Apart from readily biodegradable and persistent substances, inherently biodegradable substances may be distinguished as an intermediate class. These substances are not able to pass tests for "ready biodegradability" but do pass tests for "inherent biodegradability", which have enhanced biodegrading power. The semi-continuous activated sludge test (OECD 302A or ISO 9887) and Zahn-Wellens test (OECD 302B or ISO 9888) belong to this group (Pagga 1997).

The continuous flow activated sludge simulation test (OECD 303 or ISO 11733) simulates conditions of a biological sewage treatment plant (Pagga 1997). A tested substance is continuously introduced into the test facility together with artificial sewage. The tested compound is treated only during a hydraulic retention time i.e. several hours. Apart from a simple single-chamber facility working under aerobic conditions, a two-chamber facility with an additional denitrifying chamber is used (Gronenberg and Schöberl 1994). The two-chamber facility contains an aeration chamber (aerobic conditions) and a denitrifying chamber (anoxic conditions) and better corresponds to modern sewage treatment plant conditions.

The aim of this work was the investigation of tripropylene glycol primary biodegradation under aerobic conditions, as well as the identification of PG3 metabolites. Both static and continuous flow tests were used. The general conditions of the OECD 301E test were applied for the static test, however, with specific PG3 determination during the test and significantly reduced mineral nutrients concentration. The continuous flow activated sludge simulation test was performed in the two-chamber facility with a denitrifying chamber (Gronenberg and Schöberl 1994). This test better corresponds to the conditions of real biological sewage treatment plants than the single chamber facility recommended by the OECD 303 test. The HPLC method with fluorimetric detection and analyte derivatisation was used for quantitative determination of PG3. The LC-MS with electrospray ionisation and GC-MS with electron

impact ionisation were used for the identification and LC-MS was used for semiquantitative determination of PG3 metabolites.

Experimental

Reagents and chemicals

Tripropylene glycol (PG3) was from Fluka (Switzerland). Chloroform, sodium chloride and sodium hydrogen carbonate of analytical grade were obtained from POCh (Poland). Acetonitrile was from J.T. Baker (The Netherlands). 1-Naphthyl isocyanate (NIC) from Aldrich (USA) was used for derivatisation. HPLC-grade methanol from J.T. Baker was used for HPLC measurements. The HPLC-grade water was prepared by reverse osmosis in a Demiwa system from Watek (Czech Republic), followed by double distillation from a quartz apparatus. Only freshly distilled water was used. All the reagents used for preparation of synthetic sewage were from POCh.

Biodegradation tests

The continuous flow activated sludge simulation test

Apparatus A continuous flow activated sludge (CFAS) plant with a denitrifying chamber (Gronenberg and Schöberl 1994) was used. It consisted of a denitrifying chamber connected to an aeration vessel, as well as to a clarifier. The total volume of the denitrifying chamber and aeration vessel was 7.8 l (2.8 and 5.0 l, respectively). Synthetic sewage was supplied from a receiver to the denitrifying chamber at a rate of 0.5 l h^{-1}. Settled sludge was periodically returned from the clarifier to the denitrifying chamber. Concentration of dissolved oxygen was maintained at between 1.5 mg l^{-1} and 2.5 mg l^{-1} in the aeration vessel and below 0.03 mg l^{-1} in the denitrifying chamber. Activated sludge used in the experiments was brought from the sewage treatment plant in Szamotuly, located near Poznan, Poland, which treats typical municipal sewage.

Synthetic sewage The synthetic sewage was prepared according to Schöberl (1991). The following were dissolved in 1 l water: peptone, 80 mg; beef extract, 55 mg; urea, 15 mg; NaCl, 3.5 mg; $CaCl_2 \cdot 2H_2O$, 2 mg; $MgSO_4 \cdot 7H_2O$, 1 mg; K_2HPO_4, 14 mg and $NaHCO_3$, 98 mg. The synthetic sewage was freshly prepared each day from solids using distilled water.

Procedure The plant was started by filling the aeration vessel with activated sludge with a suspended solids concentration of 3.2 g l^{-1}. The concentration of activated sludge (suspended solids) was measured gravimetrically. Synthetic sewage (without PG3) was applied. The synthetic sewage containing PG3 (10 mg l^{-1}) was supplied after 1 week's delay. Concentration of dissolved oxygen, suspended solids, as well as pH and temperature were controlled during the experiment. Temperature in the aeration vessel was kept within the range of 20 ± 0.5°C. pH measured in the aeration vessel varied between 7.4 and 7.8.

Samples of treated sewage were collected on selected days of the experiment and the day prior to the supply of the synthetic sewage containing the PG3 under test. The latter samples served for the determination of 'blanks'. The samples were extracted, derivatised and analysed by HPLC.

The OECD screening test

In the OECD screening test activated sludge was used as an *inoculum*. The sludge was placed in the CFAS plant (Schöberl 1991) for a 1-week cleaning process. During this time, synthetic sewage was delivered to the CFAS plant to clean the sludge. After the purification process was finished, the activated sludge concentration in water from the CFAS plant was measured gravimetrically. Briefly, 100 ml of activated sludge solution was filtrated and the filtrate was dried and weighed.

The OECD screening test was performed in bottles filled with 200 ml of a medium consisting of water spiked with PG3, an aliquot of activated sludge suspension and a solution of mineral salts. Final amounts of the constituents in 1 l of the solution was as follows: PG3, 10 or 5 mg; activated sludge, 0.2 g; KH_2PO_4, 8.5 mg; K_2HPO_4, 21.5 mg; $Na_2HPO_4 \cdot 2H_2O$, 33.5 mg; NH_4Cl, 20 mg; $MgSO_4 \cdot 7H_2O$, 22.5 mg; $CaCl_2$, 27.5 mg; $FeCl_3 \cdot 6H_2O$, 250 μg; $MnSO_4 \cdot H_2O$, 40 μg; H_3BO_3, 57.5 μg; $ZnSO_4 \cdot 7H_2O$, 42.5 μg; $(NH_4)_6Mo_7O_{24}$, 35 μg.

The samples were left in open bottles protected against dust and light at room temperature. All the bottles were shaken on a rotary shaker to provide oxygen. The samples were analysed on selected days of the biodegradation test.

Sample preparation

Sample preparation was performed as described in a previous paper (Zgola-Grzeskowiak et al. 2006). Briefly, 15 g of sodium chloride and 0.1 g of sodium hydrogen carbonate were added to a 50 ml water sample containing tripropylene glycol. The sample was extracted with three portions of chloroform. The extracts were combined and filled up to 25 ml. An aliquot of the combined extracts was evaporated to dryness and reconstituted to 200 µl of acetonitrile. 6 µl of NIC was added to a sample and the solution was sonificated for 20 min at 35°C. The reaction was quenched by the addition of 10 µl of methanol with a subsequent 10 min sonification at 35°C. The sample was evaporated and reconstituted to 1 ml of a mixture of methanol:water (6:4).

Chromatography

A chromatographic system from Dionex (USA), delivered by Polygen (Poland), consisting of a P580 A LPG gradient pump, ASI-100 autosampler, STH 585 oven and a RF 2000 fluorescence detector were used. 25 µl samples were injected into a 250 mm × 4.6 mm I.D. analytical column packed with 5 µm Hypersil BDS C18 from ThermoQuest (USA), delivered by Polygen with a guard column packed with 3 µm Hypersil BDS C18 from the same supplier. The column was flushed in a water:methanol gradient at a flow-rate of 1.5 ml min^{-1} at 35°C. The mobile phase consisted of solvent A (water) and solvent B (methanol) in the following gradient: 0 min. 60% B; 10 min. 100% B. Signal responses were measured by fluorescence detection at wavelengths set at 225 nm for excitation and 362 nm for emission.

Mass spectrometry

LC-MS

The mass spectra and LC-MS analyses were performed using a Waters/Micromass (UK) ZQ mass spectrometer. The electrospray (ESI) source potentials were: capillary 3 kV, lens 0.5 kV, extractor 4 V and cone voltage 30 V. The source temperature was 120°C and desolvation temperature, 300°C. Nitrogen was used as the nebulising and desolvation gas at flow rates of 100 and 300 l h^{-1}, respectively.

The instrument was coupled to a Waters model 2690 HPLC pump (USA). Using an autosampler, the sample solutions were injected on the Nova Pak C18 RP column (150 mm × 3.9 mm I.D., Waters). A gradient of acetonitrile and 5% formic acid in a water:methanol 9:1 v/v mixture was applied. The linear gradient started from 0% of acetonitrile reaching 95% of acetonitrile after 15 min and the latter concentration was maintained for 5 min.

GC-MS

GC-MS analysis was obtained on an AMD-402 two-sector mass spectrometer (AMD Intectra, Germany) of B/E geometry with an acceleration voltage of 8 kV, an electron energy of 70 eV and an ion source temperature of 200°C. The analyzed mixture was introduced into the mass spectrometer using a Hewlett-Packard Model 5890II gas chromatograph. The instrument was equipped with a DB-1 fused-silica capillary column (J&W Scientific, 25 m × 0.2 mm i.d.). The carrier gas was helium at a flow rate 1 ml/min. Column temperature was programmed from 80°C (held for 2 min) at a rate of 10°C/min to 300°C.

Results and discussion

The OECD screening test 301E for PG3 was performed. The tested PG3 was the sole source of organic carbon and was introduced only at the beginning of the test. Diluted activated sludge was the *inoculum*. Recommended PG3 concentration equal to 10 mg l^{-1} was applied. The results are shown in Fig. 1A. No significant PG3 biodegradation was observed during the 20 days of the test. Additional two replications of the test gave similar results. Then the test was repeated with a lower PG3 concentration (5 mg l^{-1}) and a longer test time. The results shown in Fig. 1B exhibit satisfactory biodegradation progress. Thus, PG3 is biodegraded, however, exceeding a certain PG3 concentration threshold leads to biodegradation failure.

The biodegradation of PG3 under the conditions of the continuous flow activated sludge experiment was tested over 27 days. Tested PG3 (10 mg l^{-1}) was continuously supplied together with synthetic sewage, containing both organic and inorganic nutrients. Within 7.5 h of the retention time, the introduced PG3 was completely replaced by a newly introduced tested solution. Apart from the PG3 concentration, concentration of activated sludge was measured. The results for PG3 concentration are shown in Fig. 2A and for activated sludge concentration, in Fig. 2B. Relatively high PG3 biodegradation was observed at the very beginning of the test. However, on the third day and the following days, the biodegradation dropped to approximately 70%, and after 2 weeks biodegradation, it became dramatically worse and only 20% of PG3 was being biodegraded by the end of the experiment.

This deterioration of the PG3 degradation process was connected with a rapid reduction in the concentration of activated sludge at the beginning of the second week of the test (Fig. 2B). After 1 week of growth, the concentration of activated sludge decreased rapidly. As a result, the rate of the biodegradation process decreased a few days later.

Poisoning of activated sludge by PG3 metabolites is a probable reason for the observed effects. The poisonous metabolite is probably accumulated on the surface or in the microorganisms of the activated sludge. In the authors' opinion, this is why the effect of reduction of activated sludge concentration was observed only after 8 days delay, when a certain level of the poison concentration was exceeded. However, the reduction in the activated sludge concentration did not involve an immediate drop in the degree of PG3 biodegradation. The next several days were needed to observe the biodegradation failure. Thus, the activated sludge concentration drop is not the direct reason for the PG3 biodegradation failure. It is worth stressing that the tested solution is fully

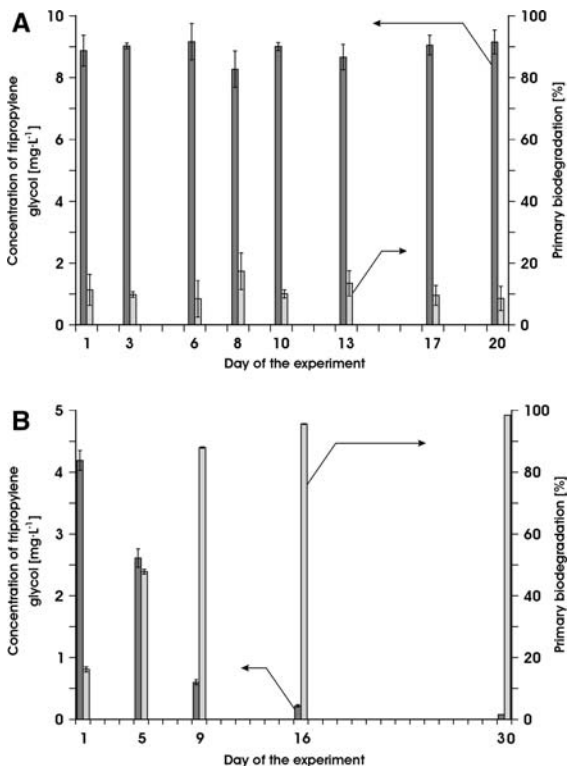

Fig. 1 OECD screening test of tripropylene glycol: residual concentration and primary biodegradation of tripropylene glycol. The initial concentration of tripropylene glycol (mg l^{-1}): (**A**) 10, (**B**) 5. Inoculum: activated sludge

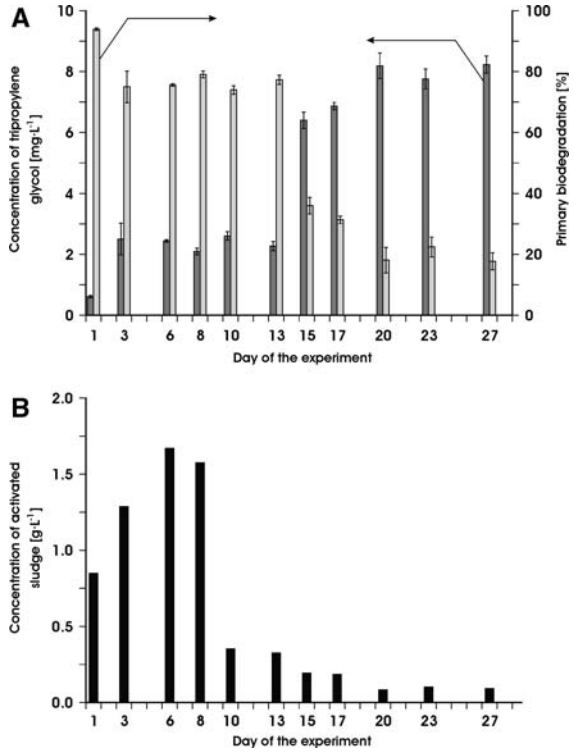

Fig. 2 Continuous flow activated sludge simulation test of tripropylene glycol. (**A**) Effluent concentration and primary biodegradation of tripropylene glycol. (**B**) Concentration of activated sludge. Influent concentration of tripropylene glycol (mg l^{-1}): 10

exchanged within 7.5 h, i.e. during hydraulic retention time in the continuous flow activated sludge test. Thus, only suspended solids of activated sludge are able to preconcentrate the poisonous PG3 metabolite. Most probably, the poisonous effect of the metabolites of PG3 does not affect bacteria strains adapted to PG3 bio-oxidation, but does affect the other members of the activated sludge consortia. This firstly caused a very dramatic drop in suspended solids concentration (see Fig. 2B) while PG3 bio-oxidation remained high. However, very low concentration of suspended solid causes PG3 oxidizing bacteria to be washed out from the apparatus. This is why the bio-oxidation of PG3 fails with several days delay as compared to the suspended solids concentration failure. All these data show that PG3 can be classified rather as an inherently biodegradable substance than as a readily biodegradable one.

In order to identify PG3 metabolites, mass spectra of chloroform extracts of samples from the final period of the OECD screening test (5 mg l^{-1}) were measured. Spectra of two main metabolites were found. The first metabolite contains two hydrogen atoms less than molecule PG3. The second metabolite contains four hydrogen atoms less than molecule PG3. Two possible processes may lead to the loss of a pair of hydrogen atoms: oxidation of terminal hydroxy groups to aldehyde and ketone groups and also dehydrogenation of the PG3 chain leading to double carbon–carbon bonds. More probable is the first process belonging to typical alcohol behaviour. The mass spectrum corresponding to day 26 of the test (see Fig. 3) shows clear signals corresponding to m/z equal to 191 and 213. The first probably belongs to the protonated metabolite of PG3 containing a single terminal aldehyde or ketone group (see chemical formulae in the figure). The signal at 213 probably corresponds to the complex ion of the same metabolite with sodium. Formation of aldehyde or ketone group is a result of the bio-oxidation of the primary or secondary alcohol group, respectively. The investigated PG3 is a mixture of different isomers containing both primary as well as secondary alcohol groups.

The spectrum corresponding to day 33 of the test is given in Fig. 4. Apart from the signals at 191 and 213, the spectrum shows clear signals at m/z equal to 189 and 211, probably corresponding to ions of the metabolite with two terminal aldehyde or ketone

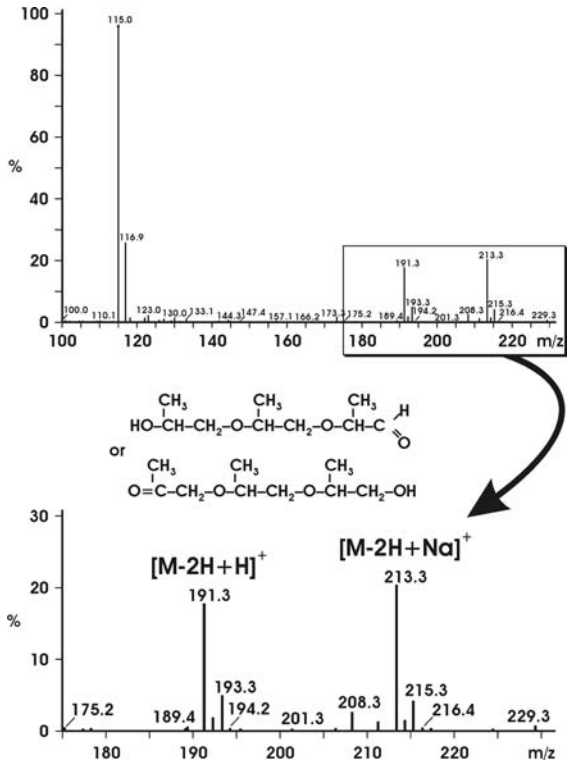

Fig. 3 The mass spectrum of chloroform extract corresponding to day 26 of the OECD screening test. The initial concentration of tripropylene glycol (mg l^{-1}): 5

groups (see chemical formula in the figure). As before, the signal at 189 corresponds to the protonated metabolite and the signal at 211 corresponds to the complex ion of the same metabolite with sodium. Both spectra show traces of the signal at m/z equal to 229. This probably corresponds to the complex ion of carboxytaled PG3 with sodium. In contrast to the ketone group, the aldehyde group can be oxidized to the carboxyl group. The very weak signal of this metabolite may be evidence both that the ketone groups outnumbered the aldehyde groups or that the carboxyl groups are speedily biodegraded.

Presence of the ketone or aldehyde group among PG3 metabolites was also confirmed with GC-MS with electron impact ionisation spectra of a chloroform extract of a sample from the day 29 of the experiment. Three peaks from PG3 and its metabolites were present on the GC chromatogram (see Fig. 5). Spectra of these peaks are also shown in Fig. 5. Appropriate chemical structures with their fragmentation patterns corresponding to metabolite

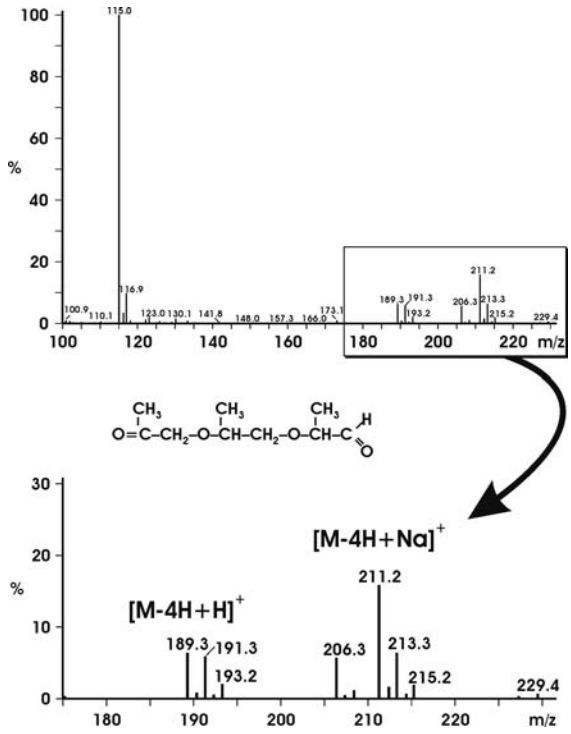

Fig. 4 The mass spectrum of chloroform extract corresponding to the sample from day 33 of the OECD screening test. The initial concentration of tripropylene glycol (mg l^{-1}): 5

with the ketone group are added to each peak spectrum. The first peak on the GC chromatogram belongs to PG3 (A). The fragment having M/z of 59 forms the main peak of the spectrum. It corresponds to $CH_2CH_2CH_2OH$ or $CH_2CH(OH)CH_3$ i.e. the end fragment of primary or secondary alcohol. Apart from this fragment, two other much smaller peaks having M/z of 45 and 103 are also apparent. The first probably corresponds to $CH(OH)CH_3$, while the second probably corresponds to $CH(CH_3)OCH_2CH_2CH_2OH$ or $CH(CH_3)OCH_2CH(OH)CH_3$. The second peak on GC chromatogram belongs to PG3 metabolites without 2 hydrogen atoms (B). These compounds contain both alcohol as well as ketone (or aldehyde) groups. Thus, fragments characteristic to both alcohol and ketone (or aldehyde) are present as both groups are present in double dehydrogenated PG3. The fragments coming from ketone (or aldehyde) group can be ascribed to M/z of 57 which probably corresponds to the CH_2CH_2CHO or $CH_2(CO)CH_3$ fragment, M/z of 43 which corresponds to the $COCH_3$ fragment, M/z of 101 which corresponds to the $CH(CH_3)OCH_2CH_2CHO$ or $CH(CH_3)OCH_2(CO)CH_3$ fragment and 115 which corresponds to the $CH_2CH(CH_3)OCH_2CH_2CHO$ or $CH_2CH(CH_3)OCH_2(CO)CH_3$. The third peak on GC chromatogram belongs to PG3 metabolites without 4 hydrogen atoms (C). The fragment having M/z of 57 forms the main peak of the spectrum. It probably corresponds to CH_2CH_2CHO or $CH_2(CO)CH_3$ i.e. aldehyde or ketone. Apart from this fragment, several other much smaller peaks having M/z of 43, 101 and 115 are also apparent. These were identified above during peak B discussion as fragments from ketone (or aldehyde) group. Some fragments typical to alcohol are also present in the third spectrum as peaks B and C are not completely resolved.

Metabolites containing aldehyde or ketone terminal groups were also identified during PPG having M.W. of 425 and 725 biodegradation under aerobic conditions (Zgola-Grzeskowiak et al. 2007). Thus the oxidation of terminal groups seems to be the initial stage of bio-oxidation of the oxypropylene chain.

The LC-MS technique provided semiquantitative information concerning PG3 metabolite concentration. Single ion chromatograms of the sample from day 33 of the test corresponding to m/z equal to 189, 191 and 193, as well as to 211, 213 and 215 are shown in Fig. 6. The first series corresponds to the protonated PG3 and its metabolites and the second series corresponds to ions of PG3 and its metabolites with sodium. On the basis of single ion chromatograms, the changes in PG3 metabolite analytical signals during the final stage of the OECD screening test were investigated. The results are shown in Fig. 7. Growing analytical signals of both metabolites at the period between days 26 and 29 of the test are observed. The analytical signal of the metabolite which probably contains a single terminal aldehyde or ketone group is much higher than that which probably contains two terminal aldehyde or ketone groups and forms a maximum at the very end of the test. The lack of standards hinders the quantitative metabolite determination.

Conclusions

Tripropylene glycol is inherently biodegradable and exhibits a strong poisonous effect towards activated sludge after exceeding threshold concentration (10 mg l^{-1}). Metabolite accumulation onto the

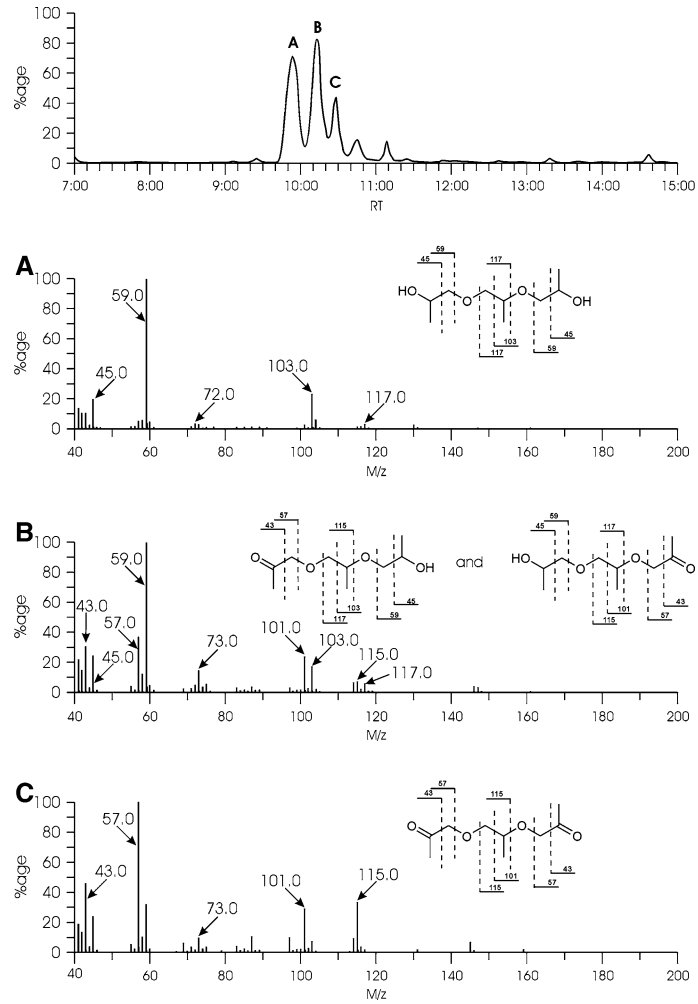

Fig. 5 The GC-MS chromatogram of chloroform extract corresponding to the sample from day 33 of the OECD screening test together with mass spectra corresponding to peaks **A**, **B** and **C**

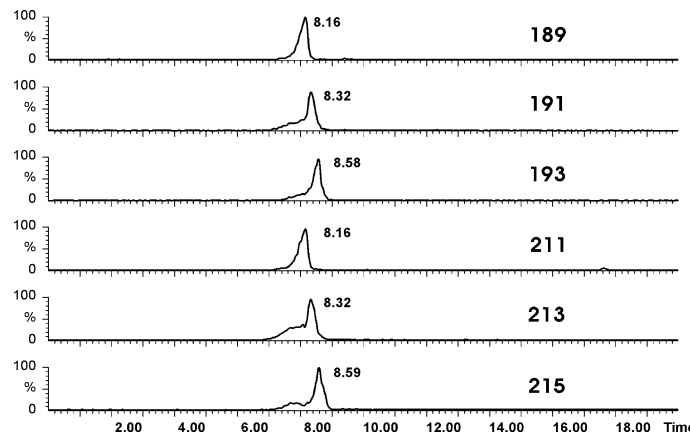

Fig. 6 The single ion chromatograms of chloroform extract of the sample from day 33 of the OECD screening test. The initial concentration of tripropylene glycol (mg l^{-1}): 5

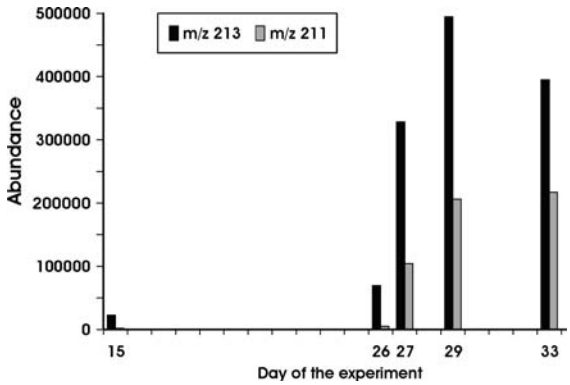

Fig. 7 The analytical signals of metabolites of tripropylene glycol at the period between day 15 and 33 of the OECD screening test, as determined by single ion chromatograms. The initial concentration of tripropylene glycol (mg l^{-1}): 5

activated sludge surface is probably responsible for the poisonous effect.

Biotransformation products of tripropylene glycol are metabolites with a single terminal aldehyde or ketone group and metabolites with two terminal aldehyde or ketone groups. Their concentrations are growing at the end of the OECD screening test.

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