Abstract The controlled biodegradation of ametryn and methomyl has been performed, in accordance with the OECD Zahn–Wellens/EMPA procedure, by use of an enriched mixture of activated sludge collected from three domestic waste-water-treatment plants (WWTP). During the process concentrations of ametryn and methomyl in the water samples were isolated by solid-phase extraction (SPE); recovery rates were 98.9 and 93.2 for methomyl and ametryn, respectively. Liquid chromatography–mass spectrometry (LC–MS) was used to determine final pesticide concentrations and for metabolite identification.

The efficiency of aerobic biodegradation of ametryn and methomyl was evaluated by measuring both the decrease in the concentration of the pesticides and global properties such as the chemical oxygen demand (COD).

Introduction

Methomyl was introduced in 1966 as a broad-spectrum insecticide. It is extensively used as an acaricide to control ticks and spider and it is employed for foliar treatment of vegetable, fruits and field crops, cotton, and commercial ornamental plants. This pesticide is effective as both a contact and systemic insecticide.

Methomyl is considered very toxic to mammals and it is classified by the EPA as a restricted-use pesticide (RUP) or Class I. Reported oral 50% lethal dose (LD50) values are 17 to 24 mg kg\(^{-1}\) in rats [1], 10 mg kg\(^{-1}\) in mice, and 15 mg kg\(^{-1}\) in guinea pigs [2]. Methomyl is moderately to highly toxic to fish, with reported LD50 values of 3.4 mg L\(^{-1}\) for rainbow trout exposed for 96 h and 0.8 mg L\(^{-1}\) for bluegill sunfish [1]; it is also highly toxic to aquatic invertebrates, with LD50=0.0287 mg L\(^{-1}\) for Daphnia magna [3].

Ametryn toxicity is Class III, which means slightly toxic [4]. It is relatively non-toxic to mammals and fish [5] but highly toxic to crustaceans and mollusks [6]. The LD50 is 8.8 mg L\(^{-1}\) for rainbow trout exposed for 96 h, 4.1 mg L\(^{-1}\) for bluegill sunfish, 14.1 mg L\(^{-1}\) for goldfish, 2.3 mg L\(^{-1}\) for mysid shrimp after exposure for 96 h, 28 mg L\(^{-1}\) for Daphnia magna, and 14 µg L\(^{-1}\) for green algae exposed for 72 h.

The high water-solubility (10 mg L\(^{-1}\)) of methomyl and its low soil organic carbon partition coefficient (Koc=0.16) make this pesticide a candidate groundwater contaminant. A study performed by the EPA revealed that methomyl was present in 25 out of 1023 groundwater samples; maximum concentrations were 10 µg L\(^{-1}\) [7].

Aqueous solutions of methomyl have been reported to decompose more rapidly on aeration, in sunlight, or when alkaline. The estimated aqueous half-life of the insecticide is about 6 days in surface water, i.e. with aeration and sunlight, but it is estimated that the half-life in groundwater can be more than 50 weeks.
Ametryn is a persistent compound. Loss from the soil is principally by microbial degradation. Ametryn moves both vertically and laterally in soil, because of to its high water solubility. It might be leached by high rainfall, floods, and furrow irrigation [8]. The wide application of s-triazine herbicides often leads to their presence in fresh waters [9, 10] and marine coastal environments [11]. Residues of ametryn and methomyl are introduced into the aquatic system either by diffuse pollution as a result of surface runoff, leaching, atmospheric deposition, and point sources such as WWTP. In this respect it has been found that effluents of WWTP contain high levels of herbicides that escape treatment [12, 13]. The degradation of triazine herbicides such as ametryn and insecticides such as methomyl has been well investigated and much work has been performed on the removal of pesticides from wastewater by special procedures such as oxidation or irradiation [14]. Few publications have dealt with elimination of pesticides in WWTP [15].

In this work mixtures of activated sludge from three different domestic WWTP were applied to the biodegradation of methomyl and ametryn. The biodegradation process was followed by use of routine methods, e.g., measurement of COD and TOC, and by chemical analysis. These techniques do not, however, enable assessment of any global effect of the compounds, nor the synergistic effects of their metabolites. To evaluate the hazard associated with their presence and that of their degradation products in an effluent, acute toxicity was evaluated during the microbial degradation process. Several biological tests and assays, using algae, protozoa, fish, and other organisms, have been developed for determination of toxicity in aquatic environments. Most of these bioassays, however, take a relatively long time (48 h to days), are expensive, and often require extensive preparation. In this work toxicity data were based on inhibition of the bioluminescence of V. fischeri. Although methomyl and ametryn are not highly toxic to V. fischeri, the test can provide a rapid and cost-effective information about the toxicity of the effluent mixture from the biodegradation process.

The objectives of this work were:

- to establish EC50 and TU for methomyl and ametryn by use of the ToxAlert100 biological test, which is based on inhibition of the bioluminescence of Vibrio fischeri;
- to study the toxicity of methomyl and ametryn, and their metabolites, during aerobic biodegradation with a mixture of activated sludge; and
- to identify metabolites formed during biodegradation by use of solid-phase extraction followed by liquid chromatography–mass spectrometry

**Experimental**

Chemical and reagents

Liquid-dried and freeze-dried photo bacteria reagent Vibrio fischeri NRRL B-111 77 was obtained from Merck (Darmstadt, Germany). Stock standard solutions (10 g L−1) of methomyl and ametryn were prepared in methanol from pure standards purchased as powders from Sigma (St Louis, MO, USA). A stock standard solution of a mixture of both compounds (200 mg L−1) was prepared in methanol by appropriate dilution of individual stock solutions.

Potassium biphthalate, sodium carbonate, and sodium bicarbonate were from Shimadzu. HPLC-grade water, acetonitrile, acetone, and methanol were obtained from Merck and were passed through 0.45-µm membrane filters before use.

**Microbial biodegradation**

Biodegradation of ametryn and methomyl was performed according to the Zahn–Wellens/EMPA procedure [16], which is recommended by the OECD as an intrinsic biodegradability test for xenobiotic substances. Biodegradation was performed with an enriched mixture (in equal parts) of activated sludge collected from three different waste-water-treatment plants receiving domestic effluent discharges.

Briefly, in the Zahn-Wellens/EMPA procedure a mixture of the test substance, mineral nutrients, and a relatively large amount of activated sludge, in aqueous medium, is agitated and aerated at 20–25°C in the dark or in diffuse light for up to 28 days. Blank controls, containing activated sludge and mineral nutrients but no test substance, are run in parallel. The biodegradation process is monitored by determination of the chemical oxygen demand (COD) or the total organic carbon (TOC) content. In this work the amount of biodegradation was evaluated periodically by determining the ratio of the COD in the test experiment to that in the blank. The amount of biodegradation (%) plotted against time is known as the biodegradation curve. Specific analysis is recommended when primary biodegradation of the analyte is known to occur.

The tests were performed in duplicate, for each pesticide (ame-tryn and methomyl) and for the blanks, in cylindrical glass vessels, each equipped with a stirrer and a glass tube for introduction of air, enabling aeration and agitation. The working volumes were fixed at 2.5 L.

**Sample collection**

Biodegradation of ametryn and methomyl was followed for 28 days. Samples were collected after 3 h on the first day and once a day thereafter.

**Sample-preparation procedures**

Water samples taken from the supernatant were filtered through 0.7-µm glass micro-fiber filters, to remove suspended matter, immediately after sampling. Every sample was subdivided into different fractions to apply the different analytical procedures. The COD and TOC of the filtered samples were measured directly, immediately after sampling.

Disposable 6-mL cartridge columns packed with 500 mg C18 adsorbent (Merck) were used for solid-phase extraction (SPE). For SPE of methomyl cartridges were conditioned by passage of HPLC water (10 mL), acetone (5 mL), and finally HPLC water (5 mL) at a flow rate of 1 mL min−1. Filtered samples of the supernatant (10 mL) were loaded at a flow rate of 5 mL min−1 and the column was then washed with HPLC water (5 mL) at 1 mL min−1. Methomyl was then desorbed with acetone (5 mL) and HPLC water (5 mL) was added to the extract. The acetone was evaporated with a stream of nitrogen at 25°C, the extract was diluted to 10 mL with water, and this solution was stored at 4°C until analysis. In previous spiking experiments recovery of methomyl was found to be 98.9% (n=3), standard deviation 3.4%.

For ametryn analysis SPE cartridges were conditioned by passage of HPLC water (10 mL), methanol (10 mL), and again HPLC water (10 mL) at a flow rate of 1 mL min−1. Filtered samples of the supernatant (10 mL) were then loaded at a flow rate of 5 mL min−1 and the column was washed with HPLC water (10 mL) at a flow rate of 1 mL min−1. The preconcentrated trapped compounds were