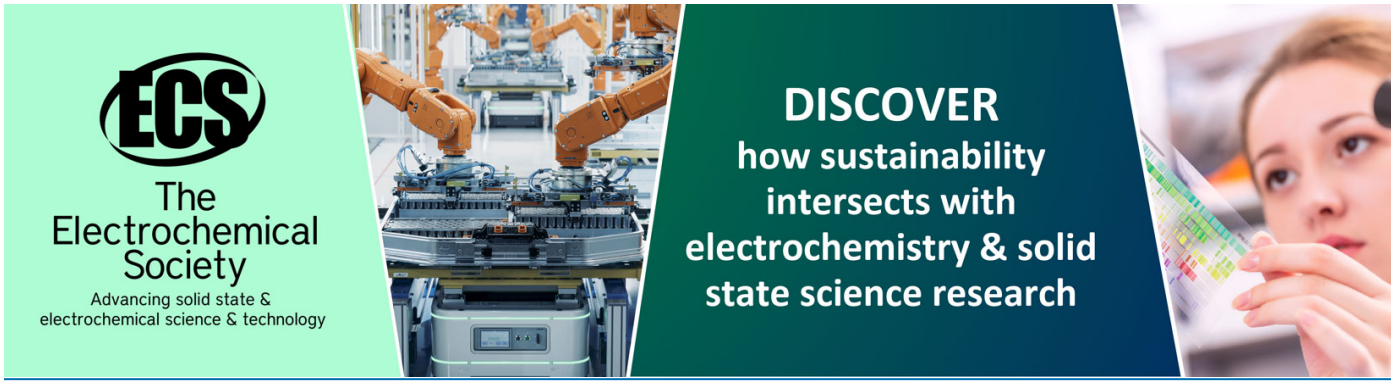
The presence of bromuconazole fungicide pollutant in organic waste anaerobic fermentation



The presence of bromuconazole fungicide pollutant in organic waste anaerobic fermentation

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Abstract. The presence of bromuconazole fungicide pollutant in organic waste anaerobic fermentation was carried out as well as the influence phenol and benzoate, and biodegradation of bromuconazole. Bromuconazole is a fungicide effective against *Ascomycetes*, *Basidiomycetes* and fungi imperfecti in cereals, grapes, top fruits and vegetables. It is also effective against *Alternaria* and *Fusarium sp*. The remaining fungicide in leaves might contaminates landfill. One month of organic waste added with bromuconazole was anaerobically incubated in 500 mL bottles at 30^{o}C without shaking in dark room. High-Performance Liquid Chromatography (HPLC) with UV detector and a 100 RP 185µm Lichrosphere column was used to determine bromuconazole concentration. Methane content was determined by Gas Chromatography (GC) method equipped with a flame ionization detector and a metal column packed with 5% neopentyl glycol sebacate and 1% H_3PO_4 on Chromosorb W-AW (mesh 80-100). After incubation for 225 days, bromuconazole of 200 mg/L inhibited the production of methane (99.5 mM) significantly, but did not inhibit the production of volatile fatty acids. The addition of 100 mg/L phenol or 146 mg/L benzoate increased the production of methane, 143 mM and 135.2 mM, respectively compared with control (121.8 mM). In anaerobic conditions, the presence of toxic pollutants such as fungicide bromuconazole in landfills sites may cause further problems with the accumulation of volatile fatty acids in leachate. Further study to determine the threshold, the presence of bromconazole in low concentration (less than 200 mg/L) on the methane production is recommended.

1. Introduction

Azole compounds, particularly triazole, has been used as the active ingredient in the manufacture of fungicides and pharmaceutical preparations since 1970^{s}. Although the production and its use has increased, but it is still lack of information about the nature of these compounds in environment either as well as landfill site and process effluent. The problem may arises when these potentially toxic compounds pollute the environment and threaten both the aquatic organism as well as human health. Activities of microorganisms might be disturbed by the presence of such compounds, and decrease the fermentation process performance. Therefore, the experiment to observe the presence of the compound in anaerobic process of the refuse is important. There are about 16 different fungicide triazole derivatives and one of them is bromuconazole (Figure 1). This compound has been developed by the Rhone Poulenc Agrochemie UK since 1991. Bromuconazole ($C_{13}H_{12}BrCl_2N_3O$, MW = 377) or 1[(2RS,4RS,2SR,4SR)-4-bromo-2-(2,4-dichlorophenyl) tetra- hydrofurfuryl]-1H-1,2,4-triazole is a heterocyclic-aliphatic compound belongs to the groups of systemic fungicide and it is active against *Ascomycetes*, *Basidiomycetes*, fungi imperfecti in cerealia and fruits as well as active against fungus *Alternaria sp* and *Fusarium sp*. Toxicity by 50% (LD_{50}) for rats and mice, respectively, 365 mg/kg and 1151 mg/kg. Towards fish such as Blugill and trout, toxicity of bromuconazole (LC_{50}) is 3.1 mg/L and 1.7 mg/L, respectively [1]. Bromuconazole is one of the 79 substances of the third stage Part A of the review program covered by Commission

Regulation (EC) No 1490/2002 [2]. Similar products such as Amitrole (3-amino-1 h -1,2 4-triazole) reported to be transformed into 3-(3-amino-1 h-triazole-1-yl) alanin by *E. coli* [3]. Benzotriazole transformed to 1-deoxyribosida by *Aerobacter aerogeneses* ATCC9621 supplementation with 5 '-thymidylic acid and *Aspergilus niger* (Van Teigh) was able to reducing Triadimefon became Triadimol [4].

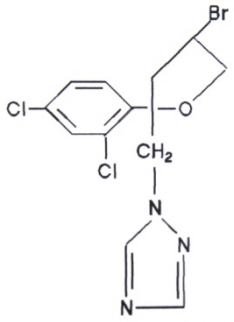

Figure 1. Chemical structure of bromuconazole.

As pollutant, bromuconazole might be degraded biologically in aerobic and anaerobic condition. In anaerobic fermentation of organic compounds, the resulted xenobiotic compounds such as pesticide will accumulate if not degraded into methane [5]. The production of methane, as a source of energy, is one of the advantages of anaerobic fermentation process. Anaerobic degradation occurs due to the role of either one type or mixture of microbes. For example, the benzoate was degraded by *Moraxella sp* or *Pseudomonas* to diethyl ester [6] and degradation of phenols by a community of microbes produced methane [7][8]. Xenobiotic compound can be degraded if the appropriate enzyme produced by microbes during the process of evolution. Degradation is influenced by the ability of the enzyme to accept a substrate with a structure similar (but not identical) to that of natural and less complex compound that also presence in media. The degradation is also influenced by the ability of substrate to stimulate synthesis of the necessary enzyme [9].

2. Experimental

2.1. Anaerobic experiment

This study was conducted in Waste Technology Laboratory Strathclyde University Glasgow. The presence of anaerobic bromuconazole was carried out using one month partially degraded waste substrate taken from local domestic waste disposal as source of microbes. As many as 25 gr of refuse was filled into 500 mL volume of bottle and added with water as much as 250 mL. Bottle sealed was then sealed with the rubber sub a seal No. 45. Prior to incubation at 30^{o}C without stirring in a dark room, the bottle purged with oxygen-free nitrogen gas assuming the anaerobic conditions was obtained. The experiment was conducted using three replicates. Bromuconazole is an aromatic compound with solubility in water is 50 mg/L, so for preparing a solution with a high concentration, organic solvent such as acetone needs to be used. Bromuconazole with final concentration of 100 mg/L of which dissolved with acetone was added each on day 0 and 31 (treatment 1). On the different bottle (treatment 2) on day 31 was added with 100 mg/L (final concentration) phenol. And bottle as treatment 3 was added with 146 mg/L benzoate (final concentration). Bottles with phenol and benzoate was then added with bromconazole 200 mg/L (final concentration) on day 103. Bottle without bromuconazole, benzoate, or phenol was used as treatment 4 or control. Sample was taken on a regular basis to determine the concentration of methane in the headspace bottle, volatile fatty acids, phenols, and benzoate.

2.2. Chemical analysis: bromuconazole, phenols and benzoate

Determination the concentration of bromuconazole, phenol and benzoate was carried out by High-performance Liquid Chromatography (HPLC) method. Bromuconazole standard was received from the Rhone-Poulence Agrochemie whereas HPLC solvents such as acetonitrile and other chemicals purchased from E-merk. The equipment consists of a pump Binary LC250 (Perkin Elmer); Lichrosphere 100 RP18 column um 5:150 x 4.6 mm (Altech); UV detector LC 90J (Perkin Elmer). An interface of Nelson and Nelson 900 Series system integrators software PC version 5.1 was used to process the data. The determination was carried out isocratically with mobile phase acetonitrile/water (65/35% v/v) on the conditions of flowrate, 1 mL/min; wavelength, UV 230 nm; response, 0.5; sensitivity, 1 AUFS; maximum pressure, 3500 psi; duration 15 min; injection volume 20 µl. Bromuconazole concentration in the sample was determined based on the area and the concentration of the standard of bromuconazole dissolved in mobile phase on the same analysis conditions. Concentration of phenols and benzoate in the sample was determined by the same method.

2.3. Methane and volatile fatty acid

The concentration of methane in the headspace bottle was determined using Gas Chromatography methods, GC8700 (Perkin Elmer) equipped with Flame Ionisation Detector (FID). The temperature of the injector, detector, and oven each set on 200, 210 and 80^{o}C, respectively. Pure methane (uL 10 x 5) and the triplicates sample separately injected through a gas syringe (Hamilton). Concentration of methane was calculated based on the standard temperature (^{o}K) and local pressure (mm). Oxygen-free nitrogen gas used as carrier with speed 20 lbf/sq-inc), column (Phase Sep) made of metal of 2 m x 2 mm. Type of packing column used was 5% neopentyl glycol sebacate and 1% H_3PO_4 on Chromosorb w-aw (80 – 100 mesh). For the concentration of volatile fatty acids (VFA), the temperature of the injector and detector were set at 200 and 300^{o}C. The oven was set at 100 ^{o}C for 2 min and then up 30 ^{o}C per min until the preserved 150^{o}C for 3.6 seconds. VFA standard (0-10 mm) consists of acetate, propionate, butyrate, hexanoate and valerate. Both standard and acidified samples with concentrated formic acid (0.7:0.3) as much as 1 µL were injected separately through the liquid syringe (Hamilton). Through comparison with a standard that has been analyzed at the same day, the VFA concentration in the sample was determined.

3. Result and discussion

By HPLC, bromuconazole was separated into two peaks with a proportion of 51 : 49% at 5.07 and 6.58 min, respectively. HPLC separated 2 sets of diastereomers and but not the 2 sets of enantiomers. These two peaks, corresponded to diastereomer consists of 1,2,4- and 1,3,4-bromuconazole and diastereomer which consists of 1,2,3- and 1,2,5-bromconazole, respectively. Figure 2 shows the production of methane from fermentation of refuse without carbon source as control. Total methane concentration in the headspace increased quickly after the first 2 months of incubation whereas the volatile fatty acid concentration reached maximum point (18 mM) on day 31 and then declined. After 100 days of incubation, volatile fatty acid was not detected. On day 173, the total methane concentration in the headspace reached 121.78 mM. In this experiment, benzoate and phenol has been selected as the additional carbon source or induced substrate due to the basic structure of the compound similar with one (phenyl ring) of the rings that formed bromuconazole. It was expected that with the additional carbon source depletion, microbes degraded bromuconazole through its phenyl ring.

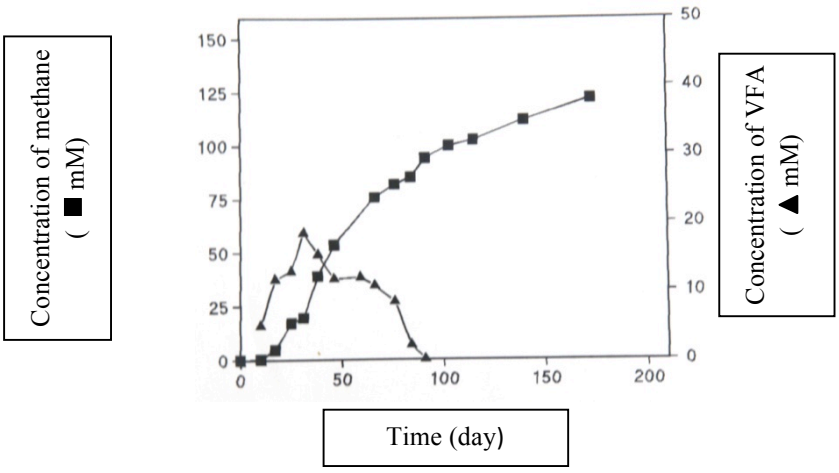

Figure 2. Production of methane and volatile fatty acid (VFA) from refuse fermentation.

Figure 3 and Figure 4 show the ability of the microbes to degrade phenol or benzoate as a source of carbon. Phenol or benzoate added separately on day 31 when the main carbon source of refuse has been depleted as a result of degradation by microbes and was indicated by maximum concentration of volatile fatty acids produced (Figure 2).

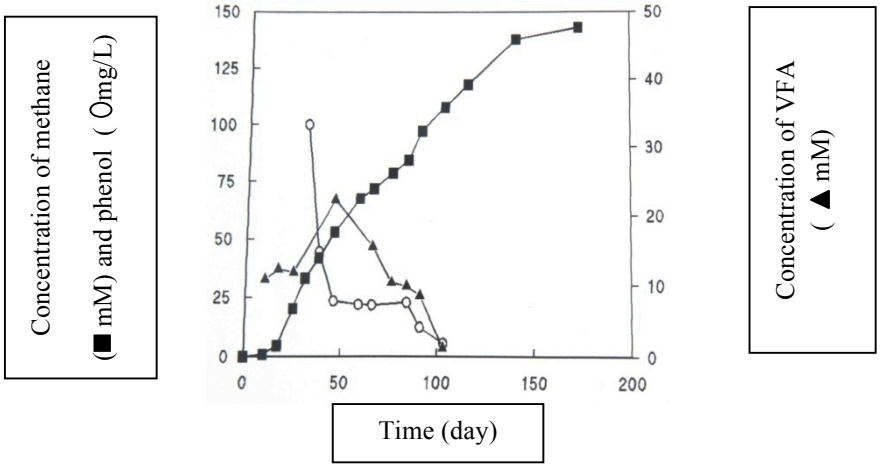

Figure 3. Effect of phenol addition on methane production at day 31.

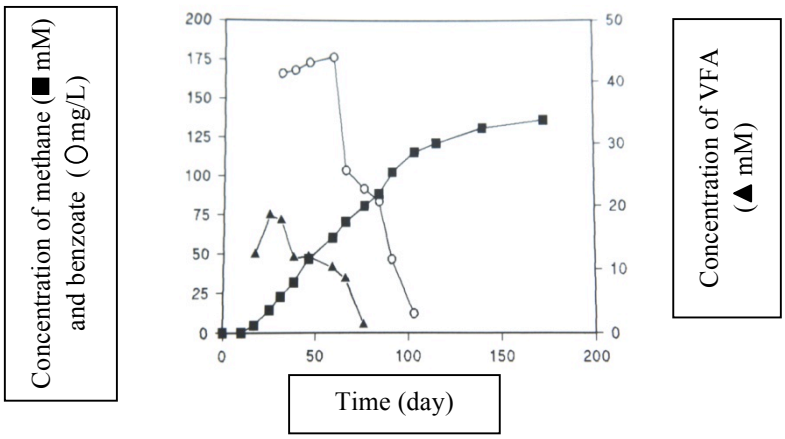

Figure 4. Effect of benzoate addition on methane production at day 31.

Figure 3 shows that by the addition of phenol on the substrate, volatile fatty acid production continued and reached a maximum of 22.30 mM on day 46 and then declined to 1.20 mM on day 103. At day 46, phenol concentration decreased rapidly to 23.46 mg/L whereas at day 103, phenol concentration was very low (5.64 mg/L). Meanwhile, methane production increased rapidly since day 17 and reached 142.98 mM/l at day 173, higher than control was of 121.78 mM . The effect of benzoate addition on methane production also showed similar pattern (Figure 4). At day 173, total accumulation of methane (135.15 mM) in the headspace of the bottle with the addition of benzoate was also higher than control, but lower than that of the addition of phenol (142.98 mM). On the other hand, the concentration of benzoate, of which was not declined initially, started to decrease quickly on day 59 and was very low (12.11 mg/L) on day 103. Effect of bromuconazole addition is in Figure 5.

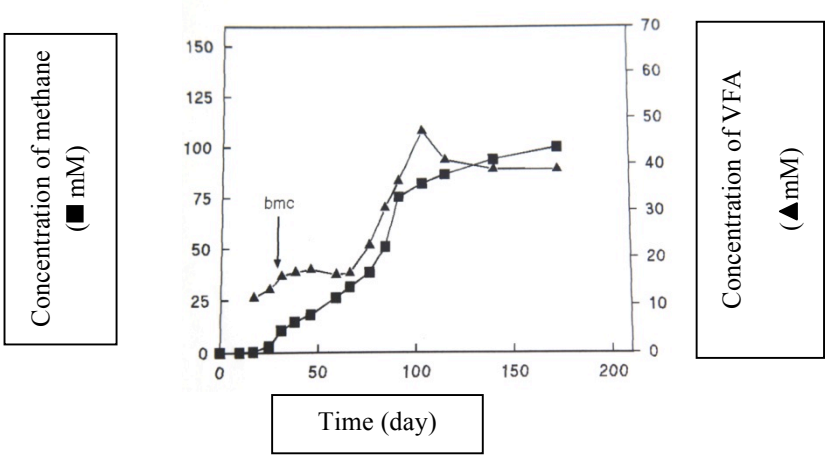

Figure 5. Effect of bromuconazole addition on methane and volatile fatty acid production at day 0 and 31.

In this experiment, bromuconazole added on day 0 and 31 so that the total concentration in the bottles (final concentrations) was 200 mg/L. The methane production after bromuconazole addition on day 0 was lower than the control (Figure 2) but did significantly influence against the volatile fatty acid concentration up to day 31. Since day 31, where as another 100 mg/L of bromuconazole were added, methane was produced but volatile fatty acid production was inhibited until day 66. The concentration of

volatile fatty acid continued to accumulate until 38.77 mM on the day 173, higher than control. Although methane was produced continuously but on the day 173 its concentration was 99.58 mM, lower than control and other treatments with phenol and benzoate. It suggested that bromuconazole had greater influence on methanogen activities rather than fermentative bacteria producing volatile fatty acid. The influence of bromuconazole inhibition against the production of methane is shown in Figure 6. Methane production stopped as so bromuconazole (200 mg/L final concentration) was added on the day 103, whereas concentration of phenol or benzoate was very low (each 5.64 mg/L and 12.11 mg/L). Inhibition of methane production continued until the end of the experiment at day 173. Methane was still generated on bottles that was not added with bromuconazole on day 103. This indicated that the contamination of bromuconazole highly influential on the production of methane and the concentration of 200 mg/L might be too toxic to methanogen bacteria. Further study to determine the threshold, the presence of bromconazole in low concentration (less than 200 mg/L) on the methane production from refuse and also its toxicity based on bacterial growth inhibition by 50% (IC_{50}) is recommended.

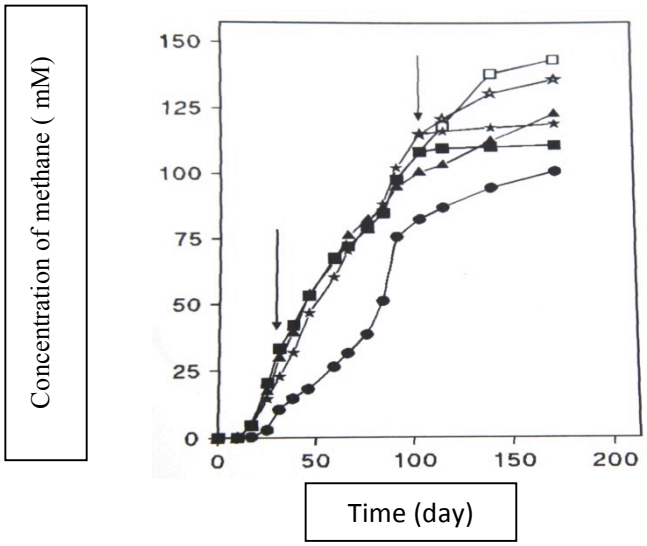

Figure 6. Methane production from refuse supplemented with:
● bromuconazole 100 mg/L at day 0 and 31.
■ Phenol 100 mg/L at day 31 and bromuconazole 200 mg/L at day 103; ★Benzoate 146 mg/L at day 31 and bromuconazole 200 mg/L at day 10□☆ Phenol or benzoate at day 31 and no added bromuconazole at day 103; ▲Control: without phenol, benzoate or bromuconazole

4. Conclusion

It was concluded that phenol or benzoate as a source of additional carbon improved methane production. By improving methane production, phenol or benzoate indicated no inhibition on methanogen bacterial activity. Therefore the presence of phenol or benzoate in refuse were in tolerant by fermentative bacteria as well as methanogen bacteria to degrade refuse. In contrast, bromuconazole indicated its toxicity against methanogen bacterial activity. The addition of phenol and benzoate improved adaptation of the anaerobic microbes to bromuconazole. The presence of bromuconazole in anaerobic process caused the refuse imperfectly fermented and so the production of methane was inhibited and volatile fatty acids accumulated. Accumulation of the volatile fatty acids may cause further pollution in aquatic environment around the landfills site. This phenomenon indicated the persistence of bromuconazole and this compound might not be degraded in the process.

Acknowledgement
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References
[1] Worthing C R 1991 *Pesticide Manual 9th ed*. The British Crop Protection Council.
[2] European Food Safety Authority 2010 *J. EFSA*. **8(8)** 1704–1788.
[3] Kieslich K 1976 Microbial transformation of nonsteroid cyclic compounds, John Wiley and sons.
[4] Deas A H B and Clifford D R 1982 *Pest. Biochem. and Physio.* **17** 120–133.
[5] Fewson C A 1981 *FEMS Symp*. **12** 141–179.
[6] Evans W C and Fuchs G 1988 *Ann. Review of Microb.* **42** 289–317.
[7] Balba M T and Evans W C 1977 *Biochemical Soc. Trans*. **5** 302–304.
[8] Fery J G and Wolfe R S 1976 *Arch. Microb.* **107** 33–40.
[9] Hutzinger O and Veerkamp W 1981. *FEMS Symp*. **12** 3–45.