The disappearance of bromuconazole fungicide in domestic waste anaerobic fermentation

H R Hariyadi
Research Unit for Clean Technology, Indonesian Institute of Sciences (LIPI)
Komplek LIPI Bandung, Jalan Sangkuriang, Gedung 50, Bandung, 40135, Indonesia
*Email: hari.rom.haryadi@lipi.go.id, hariyadi_hr@yahoo.com

Abstract. A previous study recommended observing degradation of bromuconazole in waste anaerobic fermentation. This research, therefore, was carried out to study the disappearance of bromuconazole in waste anaerobic fermentation. Bromuconazole is a fungicide which is effective against Ascomycetes, Basidiomycetes and fungi imperfecti in cereals, grapes, top fruits and vegetables. It is also effective against Alternaria and Fusarium sp. In this study, one-month old refuse added with bromuconazole was anaerobically incubated in the 500 ml bottles at 30°C with no shaking in the dark room. Bromuconazole with total concentration of 200 mg/L was added in day 0 and 31. As a control, a bottle with refuse and bromuconazole at concentration of 200 mg/L was autoclaved and incubated at the same condition. High-performance Liquid Chromatography with UV detector and a 100 RP18 5μm Lichrosphere column was used to determine bromuconazole concentration. Methane content was determined by Gas Chromatography method equipped with a flame ionization detector and a metal column packed with 5% neopentyl glycol sebacate and 1% H₃PO₄ on Chromosorb W-AW (mesh 80-100). The disappearance of bromuconazole in anaerobic enrichments can be observed from mass balance determination after incubation for 225 days. The result showed that there was no change in bromuconazole mass balance. Bromuconazole was mostly adsorbed by the refuse. Bromuconazole inhibited the production of methane (99.58 mM), less than that of the control bottle with no added bromuconazole (121.78 mM). This phenomenon indicated the persistence of bromuconazole and its toxicity against methanogen bacterial activity. The presence of bromuconazole in the anaerobic process caused the refuse to be imperfectly fermented and so the production of methane was inhibited and volatile fatty acids were accumulated. Accumulation of the volatile fatty acids may cause further pollution in aquatic environment around the landfills site.

1. Introduction
Azole compounds, particularly triazole, have been used as the active ingredient in the manufacture of fungicides and pharmaceutical preparations since the 1970s. Although the production and its use have increased, there is still a lack of information about the nature of these compounds in the environment as well as landfill site and process effluent. The problem may arise 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_{2}N_{2}O, MW = 377) or [\{(2R,4R,2SR,4SR)\}-4-bromo-2-(2,4-dichlorophenyl)tetrahydrofuranyl]-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 are 365 mg/kg and 1151 mg/kg, respectively. Towards fish such as Bluegills and trout, the toxicity of bromuconazole (LC_{50}) are 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) was reported to be transformed into 3-(3-amino-1 h-triazole-1-yl) alanine by E. coli [3]. Benzotriazole transformed to 1-deoxyribosida by Aerobacter aerogeneses ATCC9621 supplementation with 5\'-thymidyllic acid and Aspergillus niger (van Teigh) was able to reduce Triadimefon to become Triadimol [4].

**Figure 1.** Chemical structure of bromuconazole.

As a pollutant, bromuconazole might be degraded biologically in aerobic and anaerobic conditions. 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 compounds can be degraded if the appropriate enzyme are produced by microbes during the process of evolution. The degradation is influenced by the ability of the enzyme to accept a substrate with a structure similar but not identical to that of a natural and less complex compound that is also present in media. The degradation is also influenced by the ability of substrate to stimulate synthesis of the necessary enzyme [9]. A previous study [10] recommended to observe the degradation of bromuconazole in waste anaerobic fermentation. This research, therefore, was carried out to observe the disappearance of bromuconazole in waste anaerobic fermentation.

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. An amount of 25 g of refuse was filled into 500 ml volume of bottle and added with 250 ml of water. The bottle was then sealed with rubber sub-seal No. 45. Prior to incubation at 30°C without stirring in a dark room, the bottle was 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 had to be used. Bromuconazole dissolved in acetone with final concentration of 100 mg/L was added each on day 0 and 31 (treatment 1). A different bottle (treatment 2) on day 31 was added with 100 mg/L of phenol (final concentration). Another bottle as treatment 3 was added with 146 mg/L of benzoate as
final concentration. The bottles with phenol and benzoate were then each added with 200 mg/L of bromuconazole as final concentration on day 103. A bottle added with 100 mg/L of bromuconazole at each on day 0 and 31 was then autoclaved as treatment 4 or sterile control. Samples were taken on a regular basis to determine the concentration of bromuconazole in liquid and methane in the headspace bottle.

2.2. Chemical analysis

2.2.1. Bromuconazole. Determination of the concentration of bromuconazole 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 were purchased from E-Merck. The equipment consisted of a Binary LC250 pump (Perkin Elmer), Lichrosphere 100 RP18 column um 5:150 x 4.6 mm (Altech), and 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 of acetonitrile/water (65/35% v/v) on the following conditions: flow rate of 1 ml/min, wavelength UV at 230 nm, response of 0.5, sensitivity of 1 AUFS, maximum pressure of 3500 psi, duration of 15 min, and injection volume of 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.

2.2.2. Methane. The concentration of methane in the headspace bottle was determined using Gas Chromatography method, GC8700 (Perkin Elmer) equipped with Flame Ionization Detector (FID). The temperature of the injector, detector, and oven was each set on 200, 210, and 80°C, respectively. Pure methane (ul 10 x 5) and the triplicates sample was separately injected through a gas syringe (Hamilton). Concentration of methane was calculated based on the standard temperature (K) and local pressure (mm). Oxygen-free nitrogen gas was used as the carrier with speed 20 lbf/sq-inc, column (Phase Sep) made of metal of 2 m x 2 mm. The type of packing column used was 5% neopentyl glycol sebacate and 1% H₃PO₄ on Chromosorb w-aw (80 – 100 mesh).

3. Results and discussion

By HPLC, bromuconazole was separated into two peaks with a proportion of 51:49% at 5.07 and 6.58 min, respectively. Bromuconazole was present in four absolute compounds that comprised two sets of diastereomers and two sets of enantiomers. HPLC separated the 2 sets of diastereomer but not the 2 sets of enantiomers. These two peaks, corresponded to diastereomer 1, called as bromuconazole 1, consisted of 1,2,4- and 1,3,4-bromuconazole and diastereomer 2, called as bromuconazole 2, consisted of 1,2,3- and 1,2,5-bromuconazole. Bromuconazole is a lipophilic compound with the solubility in water as low as 50 mg/l and may attach to the solid particle easily. Therefore, the disappearance of bromuconazole domestic waste anaerobic fermentation with refuse as inoculum source could be observed from mass balance determination. The mass balance of bromuconazole was determined after 225 days (An6) and 122 days (An7 and An13) of incubation. The result shows that there were no changes in the total bromuconazole mass balance (Figure 2).

Bromuconazole 1 (Figure 3) and bromuconazole 2 (Figure 4) in all enrichments and sterile control were, mostly, adsorbed by refuse particle. No net disappearance of bromuconazole, therefore, was observed in refuse anaerobic fermentation. This result suggested that bromuconazole was not degraded by microbes present in the refuse. Although one month partially degraded refuse was used as the source of organic carbon and microbes, this refuse contained cellulosic compounds did not support the capabilities of microbes to consume the bromuconazole. Based on this result, it was suggested that the properties of bromuconazole, such as lipophilicity, determine the effective treatment of wastes containing this compound. The presence of solid particles or biomass might remove bromuconazole by adsorption. However, the adsorption of bromuconazole by refuse might affect the production of methane in anaerobic fermentation.
Figure 2. Mass balance of bromuconazole in domestic waste anaerobic fermentation.

**An6:** Bromuconazole (100mg/L) was added on day 0 and day 31.

**An7:** Bromuconazole (200mg/L) was added on day 103 in media supplemented with phenol (100mg/L) on day 31.

**An13:** Bromconazole (200 gm/L) was added on day 103 in media supplemented with benzoate (146 mg/L) on day 31.

Figure 3. Mass balance of bromuconazole 1 in domestic waste anaerobic fermentation.

**An6:** Bromuconazole (51mg/l) was added on day 0 and day 31.

**An7:** Bromuconazole (102mg/l) was added on day 103 in media supplemented with phenol (100mg/l) on day 31.

**An13:** Bromconazole (102gm/l) was added on day 103 in media supplemented with benzoate (146 mg/l) on day 31.
Figure 4. Mass balance of bromuconazole 2 in domestic waste anaerobic fermentation.
An6: Bromuconazole (49 mg/l) was added on day 0 and day 31.
An7: Bromuconazole (98 mg/l) was added on day 103 in media supplemented with phenol (100 mg/l) on day 31.
An13: Bromconazole (98 mg/l) was added on day 103 in media supplemented with benzoate (146 mg/l) on day 31.

Figure 5. 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 103; □☆ phenol or benzoate at day 31 and no added bromuconazole at day 103; ▲ control: without phenol, benzoate or bromuconazole.
In this experiment, benzoate and phenol had been selected as the additional carbon source or induced substrate due to the basic structure of the compounds was 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 5 shows the effect of bromuconazole addition on the methane production in refuse fermentation.

In this experiment, bromuconazole was added on day 0 and 31 so that the total concentration in the bottles as final concentrations was 200 mg/L. The methane production after bromuconazole addition on day 0 was lower than the control. At day 31, another 100 mg/L of bromuconazole was added, methane was continuously produced. Although methane was produced continuously but on the day 173 its concentration was 99.58 mM, lower than the control. It suggested that bromuconazole had a greater influence on methanogen activities. As shown in Figure 5, methane production stopped as so bromuconazole 200 mg/L as inal concentration was added on the day 103. Inhibition of methane production continued until the end of the experiment at day 173. Methane was still generated on bottles without bromuconazole addition on day 103. Presumably, the bromuconazole attached to the surface of refuse particles protected the refuse from being degraded by the microbes to obtain energy. Therefore, high concentrations of co-substrates (bromuconazole) could starve the methanotrophs of the energy that was needed to survive [11]. This experiment indicated that the contamination of bromuconazole was highly influential on the production of methane and the concentration of 200 mg/L might be too toxic to the methanogen bacteria. Bromuconazole is a lipophilic compound with rings of benzyl, triazole, and phenyl. According to a study, there was a significant correlation between the toxicity of phenyl compounds and their hydrophobicity, but no correlation was found for benzyl compounds. This is the reason that the more hydrophobic or lipophilic a molecule is, the more readily it crosses the cell membrane and becomes highly toxic and vice versa [12]. Anaerobic fermentation showed ineffective degradation of bromuconazole and therefore, it is interesting to conduct the experiments on aerobic treatment.

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. The presence of phenol or benzoate in refuse was tolerated 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 were accumulated. Accumulation of the volatile fatty acids may cause further pollution in aquatic environment around the landfill site. This phenomenon indicated the persistence of bromuconazole and this compound was not degraded in the domestic waste anaerobic fermentation process.

References
[1] Worthing C R 1991 The Pesticide Manual: A World Compendium 9th ed. (Suffolk: British Crop Protection Council)
[2] European Food Safety Authority 2010 J. EFSA 8 1704–88
[3] Kieslich K 1976 Microbial Transformation of Non-Steroid Cyclic Compounds (New York & London: John Wiley & Sons)
[4] Deas A H B and Clifford D R 1982 Pest. Biochem. Physiol. 17 120–33
[5] Fewson C A 1981 FEMS Symp. 12 141–79
[6] Evans W C and Fuchs G 1988 Ann. Review Microbiol. 42 289–317
[7] Balba M T and Evans W C 1977 Biochem. Soc. T. 5 302–4
[8] Fery J G and Wolfe R S 1976 Arch. Microbiol. 107 33–40
[9] Hutzinger O and Veerkamp W 1981 FEMS Symp. 12 3–45
[10] Hariyadi H R 2017 IOP Conf. Ser.: Earth Environ. Sci. 60 012022
[11] Strong P J, Clarke W, Karthikeyan O P, Zhu J and Wu W 2017 Agro-Environmental Sustainability ed J S Singh and G Seneviratne (Cham: Springer) pp 19-40

[12] Kayembe1 K, Basosila L, Mpiana P T, Sikulisimwa P C and Mbuyu K 2013 Int. Res. J. Pure Appl. Chem. 3 48-58