Effects of exposure times on the toxic response of ammonia oxidizing mixed culture (AOMC) to phenol and chlorinated phenols

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Abstract This study investigated the effect of exposure times on the response of ammonia oxidizing mixed culture (AOMC) to phenolic compounds while having the future goal to develop a biosensor using AOMC for toxicity monitoring. AOMC was used instead of purified nitrifying culture because of the ease of culture development. The oxygen utilization rate (OUR) was measured during three exposure periods; 0–15, 25–40 and 50–65 min. It was found that phenolic compounds have a strong inhibitory effect on AOMC. The percentage of OUR reduction increased with higher concentrations and the extended exposure times improved the toxic response of AOMC, especially to the lower concentrations (0.25, 0.50, 1.0 mg/L). Further, AOMC detoxifying mechanisms might result in the reduction of toxic response when the longest exposure time was applied. However, at the higher concentrations (2.5 and 5.0 mg/L), the extended exposure times did not have a critical effect on the response pattern of AOMC, especially for phenol and mono-chlorinated phenols. It was illustrated that AOMC is very sensitive to phenolic compounds and its sensitivity is high enough for the detection of phenolic compounds at the level of effluent standard in Thailand and Japan with a rapid response time of 15 min. To improve the sensitivity of AOMC to low phenolic compound concentrations, an extended exposure time of 25–40 min would be recommended.
Keywords Ammonia oxidizing mixed culture; biosensor; chlorinated phenols; nitrifiers; oxygen utilization rate; phenol; phenolic compounds

Introduction

Phenol and chlorinated phenols are discharged to the environment in the waste streams of several industrial operations, through its use as biocides, or as by-products of other industrial operations, such as pulp bleaching with chlorine, water disinfection, or even waste incineration. They finally reach the receiving water bodies and cause an adverse effect on the environment and the eventually ecosystem (WHO, 1989, 1994). Because of their toxic effects, it is necessary to set up proper countermeasures to control the emission of these toxic chemicals. One of the most effective measures is to set up regulations, and another is the introduction of effective wastewater treatment processes. In both cases, the monitoring of toxic chemicals in water and wastewater is essential. Without monitoring methods, regulation cannot be implemented, and the effectiveness of wastewater treatment processes cannot be achieved.

Several monitoring methods are available for the detection of toxic chemicals in water and wastewater. Biosensor is one of these methods and is based on biological mechanisms being used for toxicity monitoring (Grunditz *et al.*, 1998; Eggins, 2002; Gheewala

et al., 2003). Nitrifiers are known to be sensitive to toxic chemicals (Blum and Speece, 1991). The effects of toxic chemicals on nitrifiers have been reported by several research-ers (Ensign *et al.*, 1993; Lee *et al.*, 1997; Inui *et al.*, 2002; Tanaka *et al.*, 2002; Juliastuti *et al.*, 2003; Eilersen *et al.*, 2004; Noophan *et al.*, 2004), but only a few studies have been performed seriously on the response of nitrifiers to toxic chemicals. Inui *et al.* (2002) developed a biosensor using *Nitrosomonas europae*, and reported its response to different kinds of chlorinated phenols. Blum and Speece (1991) also reported the effect of chlorinated phenols on the inhibition of ammonia consumption of *Nirosomonas*. Both studies used pure culture as the biosensing element. While the results from single species may be repeatable and unambiguous, it is difficult to extrapolate to other species. Further, it could be possible that the use of several species rather enhances the accuracy of the bioassay test. Therefore, it is of great interest to use mixed nitrifying culture as the biosensing element. A new effort in biosensor development needs to be extensively conducted to realize biosensor using nitrifiers.

In this study, ammonia oxidizing mixed culture (AOMC) was developed in the labora-tory and then applied to toxicity tests in which it was exposed to phenolic compounds. The reduction of oxygen utilization rate (OUR) was monitored as the indicator of the toxic response. As it has been found that exposure time affects the response of ammonia oxidizers to toxic chemicals (Inui *et al.*, 2002), the effect of exposure times was investi-gated. Moreover, the sensitivity of AOMC to each phenolic compound at different exposure time lengths was defined in terms of EC_{10} and EC_{50} values.

Materials and methods

Experimental approach

In this study, AOMC was acclimatized using ammonia as the nitrogen source. The enriched culture was then subjected to the toxicity test, or a batch experiment, to observe the response to different phenolic compounds with different exposure times. In the tox-icity test, the effect of phenolic compound on AOMC was detected as the reduction of OUR in the presence of phenolic compounds.

Ammonia oxidizing mixed culture (AOMC) enrichment

A 5 L reactor continuously supplied with air was employed for the acclimatization of AOMC. The composition of the enrichment solution for AOMC acclimatization was as follows: $(NH_4)_2SO_4$ 6.6 g, K_2HPO_4 0.44 g, $MgSO_4.7H_2O$ 1.9 g, $CaCl_2.2H_2O$ 0.1 g, $Na_2MoO_4.2H_2O$ 0.5 g (Madigan *et al.*, 1999). Every day, 30 mL of the enrichment solution was supplied to the reactor. The pH of the enrichment solution was adjusted to 7.5 with 0.5 M K_2CO_3, which also had a role as the carbon source. The reactor was operated under a room temperature of 27–30 °C. Once a week, aeration was stopped, the biomass was allowed to settle, supernatant of 4 L was removed by decantation, and the reactor was filled with the same volume of deionized water. In the reactor, pH was maintained at 8.5 by the addition of 10% $NaHCO_3$ solution. pH, temperature and dissolved oxygen were routinely monitored. After more than one month of operation, biomass from the reactor was collected and used for the toxicity test. The biomass concentration was 1 500 mg/L at the start of enrichment, and was about 1 800–2 000 mg/L after one month of enrichment.

The microbial population used as the seed was obtained from the mixed liquor of an activated sludge process located in Thammasat University, Rangsit Campus in Thailand that treated domestic wastewater from the university campus. The enrichment was per-formed several times with seed sludge from the treatment process obtained at different timings. The acclimatization period of biomass was 31–48 days.

Tested toxic chemicals

Toxic chemicals considered in this study were phenol and eight kinds of chlorinated phenols; 2-Chlorophenol (2-CP), 3-Chlorophenol (3-CP), 4-Chlorophenol (4-CP), 2,3-Dichlorophenol (2,3-DCP), 2,4-Dichlorophenol (2,4-DCP), 2,5-Dichlorophenol (2,5-DCP), 2,6-Dichlorophenol (2,6,-DCP), and 3,4-Dichlorophenol (3,4-DCP). All toxic chemicals were obtained from Merck KGaA (analytical grade).

Toxicity test

The toxicity test to observe the effect of phenolic compound on AOMC was carried out with a sealed 100 mL bottle installed with an oxygen electrode connected to a voltmeter. The toxicity test was conducted as follows. Added to the bottle were 10–20 mL of AOMC containing final biomass of 100 mg dried weight, ammonia solution of final concentration of 70 mg/L NH_4-N, and different concentrations of phenolic compound. Deionized water was added to eliminate head space in the bottle to a final volume of 105 mL, then the bottle was closed with a cap equipped with an oxygen electrode. 10% $NaHCO_3$ was added as inorganic carbon source and also to maintain pH of biomass solution at 8.5 during biomass preparation for the toxicity test. A magnetic stirrer at 120 rpm was employed for mixing, and the testing temperature was between 24–26 °C. The inert OUR was determined in a similar test without the addition of phenolic compound and ammonia. The maximum OUR was determined in a similar test with ammonia but without phenolic compound. The OUR was calculated based on the linear range of oxygen consumption curve where the maximum and minimum OUR during the toxicity tests were observed to be 0.044 and 0.000 mg O_2/100 mg biomass min, respectively.

To observe the effect of exposure times in the toxicity tests, phenolic compound and ammonia were added at time 0, then the OUR was monitored during 0 to 15 min. The mixture was reaerated for 10 min, the OUR was monitored for 25 to 40 min reaerated again for 10 min, and the OUR was monitored for 50 to 65 min. At the beginning of the test, the pH was adjusted to 8.5 by using 0.1 N NaOH and 0.1 N H_2SO_4. The pH during the test was not controlled, but was observed to be between 8.0 and 8.5.

The inhibition of OUR was calculated as follows.

$$\%\text{inhibition} = \frac{\text{OUR}_{\text{max}} - \text{OUR}_{\text{tested solution}}}{\text{OUR}_{\text{max}}} \times 100$$

where OUR_{max}: oxygen utilization rate in the test with ammonia but without the addition of phenolic compound.

$\text{OUR}_{\text{tested solution}}$: oxygen utilization rate of tested solution with the addition of ammonia and phenolic compound.

The toxicity tests conducted are listed in Table 1.

Results and discussion

Effect of exposure times on the response to phenol and chlorinated phenols

The toxic effect of phenol and eight kinds of chlorinated phenols on AOMC was studied with different concentrations and exposure times. Six different concentrations, 0, 0.25, 0.50, 1.0, 2.5, and 5.0 mg/L, of each phenolic compound were tested. The OUR was measured after the addition of each phenolic compound during three periods: 0–15, 25–40 and 50–65 min. In the present study, it was observed that OUR_{max} reduced as a longer exposure time was applied. As the assumption of inorganic carbon should have been the most active in the control experiment where no toxic compound was added, the shortage of inorganic carbon should have caused reduction of OUR_{max}, and thus the reduction of % inhibition. In the interpretation of the results of the toxicity tests, this

Table 1 Toxicity tests

Toxic chemicals	Toxicity test number	Day of acclimatization	Toxic chemicals	Toxicity test number	Day of acclimatization
Phenol	Ph-1	31	2,4-DCP	2,4-DCP-1	31
	Ph-2	32		2,4-DCP-2	43
2-CP	2-CP-1	33	2,5-DCP	2,5-DCP-1	46
	2-CP-2	47		2,5-DCP-2	38
3-CP	3-CP-1	35	2,6-DCP	2,6-DCP-1	32
	3-CP-2	38		2,6-DCP-2	37
4-CP	4-CP-1	41	3,4-DCP	3,4-DCP-1	35
	4-CP-2	45		3,4-DCP-2	40
2,3-DCP	2,3-DCP-1	36			
	2,3-DCP-1	48			

possibility has to be taken into consideration. The toxicity tests for each phenolic compound were performed with two replications, and the results of the tests are given Figure 1.

Figure 1 illustrates that percentage of OUR reduction (% inhibition) increased with higher in concentrations of phenolic compounds. The reduction of OUR of AOMC after exposure to phenol and chlorinated phenols was high. The toxic effect of more than 10% of OUR reduction was detected at the presence of 0.25 mg/L within 15 min exposure time, except for in phenol and 2,4-DCP. Even such cases, the reduction of OUR was clearly observed as the higher concentration was dosed. This indicated that phenolic compounds have a strong inhibitory effect on AOMC, although the reduction of OUR of the tests with 2,4-DCP and 2,6-DCP was low in comparison to the others.

Generally, the sensitivity of AOMC to phenolic compounds was improved by extending exposure times to 25–40 and 50–65 min. As can be seen clearly in the tests with 2,4-DCP, it was found that the longest exposure time gave the highest % inhibition. In other cases, the extended exposure times increased % inhibition in comparison to the tests at 0–15 min, but the highest % inhibition was observed at 25–40 min. In addition, when the higher concentrations of phenol and mono-chlorinated phenols (2-CP, 3-CP, 4-CP) were tested, the reduction of OUR at the longer exposure times of 25–40 or 50–65 min were not significantly different from the tests at 0–15 min. The possible explanation could be that di-chlorinated phenols might need longer time than phenol and mono-chlorinated phenols to penetrate through the extracellular substances, cell wall and cell membrane, to cause a toxic effect on AOMC. On the other hand, in the tests with 3,4-DCP, the longest exposure time rather reduced % inhibition than at 0–15 min when the lower concentrations were tested. The mode of action of phenolic compounds on the inhibition of oxygen utilization is generally limited. Substantial evidence has shown the hydrophobic nature of compounds is an important determinate of affinity and ability to inhibit the activity of ammonia monooxygenase (AMO) of ammonia oxidizers (McCarty, 1999).

Further, in the tests with phenol (Ph-1, Ph-2), 2-CP (2CP-1, 2CP-2), 3-CP (3CP-1, 3CP-2), 4-CP (4CP-1), 2,3-DCP (23DCP-1, 23DCP-2), 2,5-DCP (25DCP-1, 25DCP-2), 2,6-DCP (26DCP-1, 26DCP-2) and 3,4-DCP (34DCP-1, 34DCP-2), the reduction of OUR at the longest exposure time of 50–65 min was rather lower than that found at 25–40 min. The possible explanation could be that the detoxifying mechanisms of AOMC were initiated and reduced the toxic effect of phenolic compounds to AOMC, especially when the lower concentrations, 0.25, 0.50, 1.0 mg/L, were dosed. Presumably, this might have been caused by the re-establishment of AMO (McCarty, 1999). Another explanation is that the degradation of phenolic compounds by the cultures (Murialdo *et al.*, 2003) might have reduced the concentration of the compounds and further resulted in the reduction of % inhibition when the longest exposure time was applied. However,
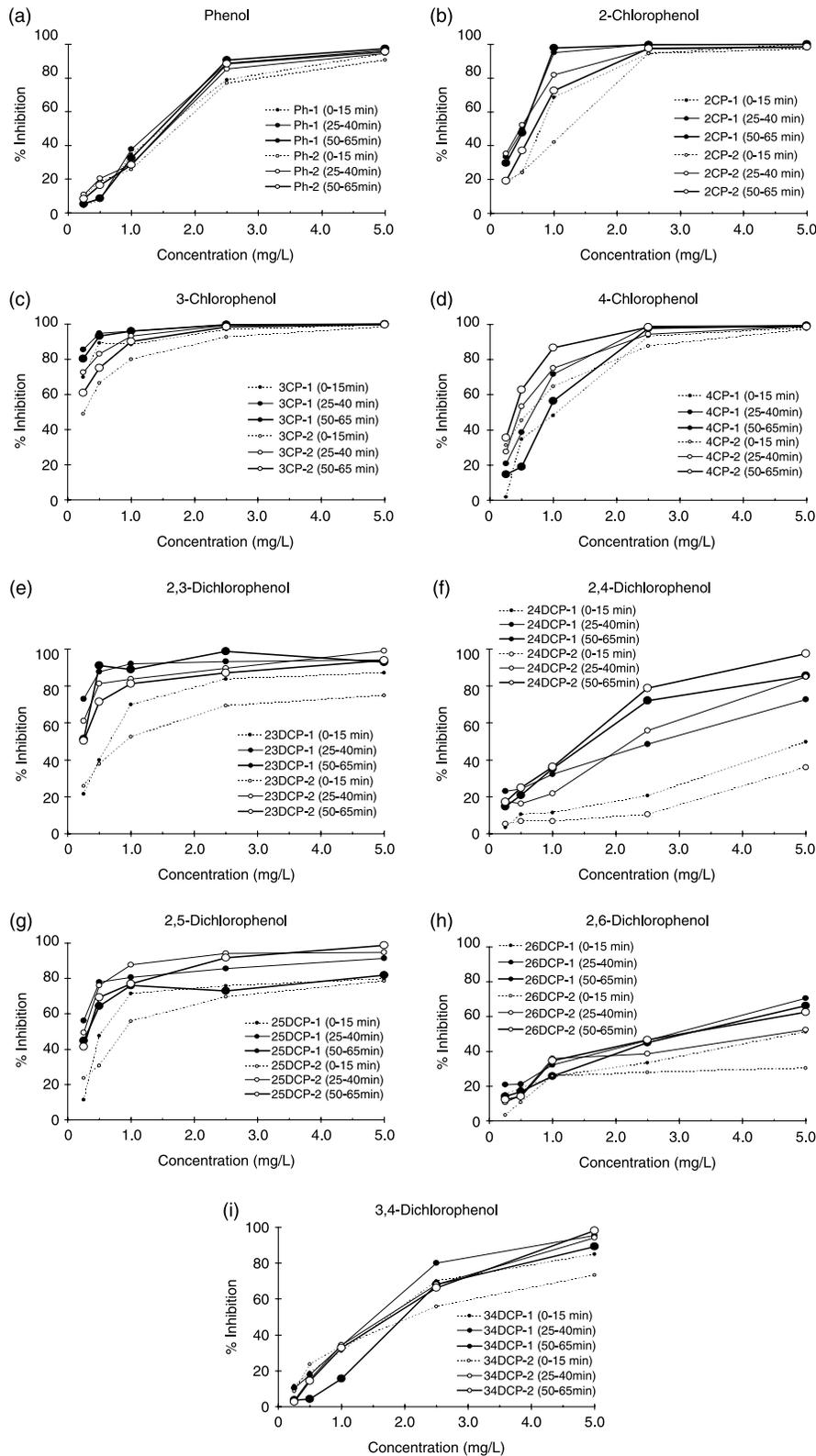
Figure 1 The effect of exposure times on the response of AOMC to phenol and chlorinated phenols

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at higher concentrations of 2.5 and 5.0 mg/L, the reduction of OUR at the extended exposure times, 25–40 min and 50–65 min, did not have a critical effect on the response pattern of AOMC, especially for phenol and mono-chlorinated phenols.

These results show that extended exposure times would be needed for tests with low phenolic compound concentrations to allow toxic chemicals to penetrate through and cause a toxic effect on AOMC. While the longest exposure time rather resulted in the reduction of % inhibition therefore, the extended exposure time of 25–40 min would be recommended for the improvement of the sensitivity of AOMC to low phenolic compound concentrations.

The sensitivity of AOMC to phenol and chlorinated phenols

The sensitivity of AOMC to phenolic compounds, or the EC_{10} and EC_{50} values, was calculated from the results of the study with different exposure times. The results are tabulated in Table 2 with their comparison to other studies.

The sensitivity of AOMC was comparable to the sensitivity of *N. europaea* reported by Inui *et al.* (2002). It was found that, generally, AOMC gave higher sensitivities than *N. europaea* reported by Inui *et al.* (2002). Inui *et al.* (2002) reported that *N. europaea* embedded in the membranes gave less sensitivity to more chlorinated phenols, pointing out the possibility that chlorinated phenols could not penetrate through the membrane and did not reach *N. europaea*. In the present study, as AOMC was suspended in the solution that contained phenolic compounds, there was no such interference that Inui *et al.* (2002) reported. Blum and Speece (1991) reported the concentration that inhibits 50% ammonia consumption (EC_{50}) of *Nitrosomonas* for phenol, 2-CP, 3-CP, 4-CP, 2,3-DCP, 2,4-DCP, 2,5-DCP and 2,6-DCP. Except for phenol, the EC_{50} values reported by them were in line with the results of the present study.

The results found in this study showed that the toxic effect of phenolic compounds on AOMC was very high and clearly detected with a rapid response time of 15 min. The authors observed a more complicated situation than that reported by Blum and Speece (1991) and Inui *et al.* (2002). It was found that the extended exposure times improved the sensitivity of AOMC to phenolic compounds. As can be seen, EC_{10} and EC_{50} at 25–40 min and 50–65 min exposure times were lower than those found at 0–15 min. However, the values of EC_{10} and EC_{50} at 25–40 min and 50–65 min exposure time were not significantly different.

Table 2 The values of EC_{10} and EC_{50} with different exposure times and their comparison with other studies

Toxic chemicals	0–15 min exposure time		25–40 min exposure time		50–65 min exposure time		Inui *et al.* EC_{10} (mg/L)	Blum and Speece EC_{50} (mg/L)
	EC_{10} (mg/L)	EC_{50} (mg/L)	EC_{10} (mg/L)	EC_{50} (mg/L)	EC_{10} (mg/L)	EC_{50} (mg/L)		
Phenol	0.3–0.5	1.5–1.7	0.2–0.5	1.4–1.6	0.3–0.5	1.5	2.2–2.9	21
2-CP	0.1	0.8–1.2	0.1	0.5	0.1	0.5–0.7	0.9–1.3	2.7
3-CP	0.1	0.2–0.3	0.1	0.2	0.1	0.2	1.4–2.8	0.20
4-CP	0.1–0.3	0.6–1.1	0.1	0.5–0.7	0.1–0.2	0.4–0.9	4.1–5.4	0.73
2,3-DCP	0.1	0.7–0.9	0.1	0.2	0.1	0.2–0.3	12–15	0.42
2,4-DCP	0.5–2.3	>5.0	0.1	2.2–2.7	0.1–0.2	1.5–1.6	31–40	0.79
2,5-DCP	0.1–0.2	0.6–0.9	0.1	0.2–0.3	0.1	0.3	7.6–9.1	0.61
2,6-DCP	0.2–0.5	4.8*	0.1–0.2	2.9–4.6	0.2	3.0–3.1	59–90	8.1
3,4-DCP	0.2–0.3	1.7–2.1	0.2–0.4	1.5–1.7	0.4–0.8	1.8–2.0	32–37	n.r.

EC_{10}, EC_{50}: effective concentration reducing 10% and 50% oxygen utilization rate, *: in one of the experiments EC_{50} was not determined. n.r.: not reported

When the outcomes shown in Table 2 are compared with the effluent standard in Thailand (1 mg/L for phenol and crezols) and in Japan (5 mg/L for phenolic compounds), if EC_{10} is to be taken as the criteria, AOMC is sensitive enough for the detection of the toxicity caused by phenolic compounds. Therefore, it is of great interest to develop a biosensor using AOMC for the detection of phenolic compounds. The inhibitory effects (EC_{50}) of AOMC to phenol and chlorinated phenols at 25–40 min exposure time can be ranked in the following order: 3-CP = 2,3-DCP > 2,5-DCP > 2-CP > 4-CP > Phenol > 3,4-DCP > 2,4-DCP > 2,6-DCP.

Conclusions

The effect of phenolic compounds on the toxic response of AOMC in terms of the reduction of OUR was studied with six different concentrations, 0, 0.25, 0.50, 1.0, 2.5, and 5.0 mg/L for each. The OUR was measured during three exposure periods; 0–15, 25–40, and 50–65 min. It was found that, higher concentrations increased the reduction of OUR and extended exposure time length improved the toxic response of AOMC to low phenolic compound concentrations (0.25, 0.50, 1.0 mg/L). Further, the detoxifying mechanisms of AOMC might have been affected, causing the reduction of the toxic response when the longest exposure time of 50–65 min was applied. However, at the higher concentrations (2.5 and 5.0 mg/L), the extended exposure times did not have a critical effect on the response pattern of AOMC, especially for phenol and mono-chlorinated phenols.

The results from this study illustrate that AOMC can be used as well as purified culture to monitor phenolic compounds (Blum and Speece, 1991; Inui et al., 2002). It is sensitive enough for the detection of phenolic compounds at the level of effluent standard in Thailand and Japan within a 15 min response time. To improve the sensitivity of the test with low phenolic compound concentrations, an extended exposure time of 25–40 min would be recommended.

References

Blum, D.J.W. and Speece, R.E. (1991). A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. *Research Journal of the Water Pollution Control Federation*, **63**(3), 198–207.

Eggins, B.R. (2002). *Chemical Sensors and Biosensors*, John Wiley & Son Ltd., West Sussex, UK.

Eilersen, A.M., Arvin, E., and Henze, M. (2004). Monitoring toxicity of industrial wastewater and specific chemicals to a green algae, nitrifying bacteria and an aquatic bacterium. *Wat. Sci. Tech.,* **50**(6), 277–283.

Ensign, S.A., Hyman, M.R. and Arp, D.J. (1993). In vitro activation of ammonia monooxygenase from *Nitrosomonas europaea* by copper. *Journal of Bacteriology*, **175**(7), 1971–1980.

Grunditz, C., Gumaelius, L. and Dalhammar, G. (1998). Comparison of inhibition assays using nitrogen removing bacteria: application to industrial wastewater. *Wat. Res.*, **32**(10), 2995–3000.

Gheewala, S.H., Babel, M.S., Gupta, A.D. and Babel, S. (2003). Rapid assessment techniques for chemicals in raw water sources. *Journal of Water Supply and Technology-AQUA*, **52.7**, 521–527.

Inui, T., Tanaka, Y., Okayasu, Y. and Tanaka, H. (2002). Application of toxicity monitor using nitrifying bacteria biosensor to sewerage systems. *Wat. Sci. and Tech.*, **45**(4–5), 271–278.

Juliastuti, S.R., Baeyens, J., Creemers, C., Bixio, D. and Lodewyckx, E. (2003). The inhibitory effects of heavy metals on the net maximum specific growth rate of the autotrophic biomass in activated sludge. *Journal of Hazardous Materials*, **B100**, 271–283.

Lee, Y.W., Ong, S.K. and Sato, C. (1997). Effects of heavy metals on nitrifying bacteria. *Wat. Sci. and Tech.*, **36**(12), 69–74.

Madigan, M.T., Martinko, J.M. and Parker, J. (1999). *Brock Biology of Microorganisms*, 8th edn, Prentice Hall, New Jersey, USA.

McCarty, G.W. (1999). Modes of action of nitrification inhibitors. *Biology Fertility of Soils*, **29**, 1–9.

Murialdo, S.E., Fenoglio, R., Haure, P.M. and Gonzalez, J.F. (2003). Degradation of phenol and chlorophenols by mixed and pure cultures. *Water SA*, **29**(4), 457–463.

Noophan, P., Figueroa, L.A., and Marr, J.M. (2004). Nitrite oxidation inhibition by hydroxylamine: experimental and model evaluation. *Wat. Sci. Tech.*, **50**(6), 295–304.

Tanaka, Y., Taguchi, K. and Utsumi, H. (2002). Toxicity assessment of 255 chemicals to pure cultured nitrifying bacteria using biosensor. *Wat. Sci. and Tech.*, **46**(11–12), 331–335.

World Health Organization (1989). *Environmental Health Criteria 93: Chlorophenols*, Geneva, Switzerland.

World Health Organization (1994). *Environmental Health Criteria 161: Phenol*, Geneva, Switzerland.