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Nitrification Inhibition by Naphthalene Derivatives and Its Relationship with Copper

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The QSAR (Quantitative Structure Activity Relationships) method is based on the premise that the activities and properties of chemicals might be related to their molecular constituents and structures. It has been used successfully to interpret and predict the physical-chemical properties of a wide range of organic chemicals (Hansch and Leo 1995). It has also found uses in environmental toxicology, which is regarded a more challenging area. Extending the same methodology for predicting the inhibitory behaviors of chemicals in sewage treatment may offer practical help to the water industry to cope with increasing numbers of chemicals being discharged with the wastewater, which has to be treated to meet stringent discharge standards.

Nitrification of ammonia in wastewater treatment by *Nitrosomonas* and *Nitrobacter* is particularly susceptible to inhibition by organic substances. As nitrification becoming more commonly used in secondary treatment of sewage, the significance of this issue grows. Considerable efforts have been made in monitoring and measurement of inhibition in recent years. These have been reported or reviewed in a number of papers (Richardson 1985; Blum and Speece 1991). Less attention has been paid to the possible relationship between both the molecular structure and physical-chemical properties of potential inhibitors and their inhibition behaviors.

Naphthalene derivatives are widely used as intermediates for dyestuffs. As a group of polycyclic aromatic hydrocarbons (PAHs), they are recognized as being toxic to microorganisms present in sewage treatment processes. The objective of this research was to investigate whether there was any correlation between the chemical structure of a selected group of PAHs and their inhibitory effects on nitrification in activated sludge. The level of inhibition was measured by the value of IC_{50}, determined using a respiratory technique. Quantitative relationship between inhibition and the chemical structure of the inhibitor was established following a QSAR approach. It was expected that the results of this research could be used to predict the maximum acceptable loading limit of the selected PAHs contained in trade effluents at sewage treatment works.

MATERIALS AND METHODS

Seven naphthalene derivatives were obtained from Sigma-Aldrich Co Ltd with purity as follows: 2-naphthol, 99+%; sodium 2-naphthalenesulfonate, 90%; 2-amino-1-naphthalenesulfonic acid, 98%; 5-amino-1-naphthol, 97%; 1-amino-4-chloronaphthalene, 98%; 7-amino-4-hydroxy-2-naphthalenesulfonic acid, 97% and 1-naphthylamine. The stock solutions containing 1000mg/L naphthalene derivatives were prepared by dissolving each chemical in deionized water. Diluted sulfuric acid solution and sodium hydrate solution were drip added to help dissolve where necessary.

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A 100-fold concentrated solution of OECD/EEC (The Organization for Economic Cooperation and Development/European Economic Community) synthetic sewage was prepared by dissolving the following in 1 L of deionized water: 16 g peptone, 11 g meat extract, 3 g urea, 0.7 g sodium chloride, 0.4 g calcium chloride dihydrate, 0.2 g magnesium sulphate heptahydrate, 2.8 g dipotassium hydrogen phosphate.

Sample of actively-nitrifying sludge mixed liquor was obtained from a pilot activated sludge plant at Water Research Center, Swindon, UK. The mixed liquor had a suspended solids (SS) concentration of 1500 - 2000 mg/L. It was settled and some supernatant was decanted so that the SS concentration of the sludge reached about 4000 mg/L.

A solution of allylthiourea (ATU) of 2500 mg/L was prepared by dissolving 0.25g ATU in 100ml deionized water.

In the standard test procedure for assessment of inhibition, the respiration rate of activated sludge was determined from the rate of oxygen depletion measured by an oxygen electrode in a cell containing a mixture of the test solution and nitrifying sludge. Part way through each run ATU was injected into the cell to inhibit nitrification. A reduction in respiration rate indicated nitrifying activity, whilst no change indicated inhibition of nitrification.

Measurement of respiration rates was conducted as follows: 100 ml nitrifying sludge with SS concentration around 4000 mg/L was introduced to a flask and kept aerated with an air dispenser. A given volume of naphthalene derivative stock solution, 2 ml concentrated synthetic sewage solution and deionized water were added to a second flask to make the total volume of mixture 100 ml (volume of naphthalene derivative stock solution was calculated according to the expected concentration in the final mixed liquor). pH value of the mixture was adjusted, where necessary, to the range between 7.0 and 8.0, using alkali or acid. (pH of the mixed liquor was measured again after respiration rate test and found always stable between 7.0 and 8.0. Therefore no buffer was used in this study.) The mixture in the second flask was aerated for 10 minutes to raise its dissolved oxygen level to the saturation concentration. It was then mixed with 100 ml aerated nitrifying sludge in the first flask. The suspended solid concentration in the final mixture was around 2000 mg/L. The final mixture was aerated for 5 minutes and then placed in a Rank Cell (Rank Brothers Ltd) which was then sealed with a lid designed to vent trapped bubbles of air. Mixing of the cell contents was by a magnetic stirrer. The respiration rates were measured by the decline of dissolved oxygen in the Rank Cell which was output continuously to a chart recorder. Each experiment was conducted in duplicates.

Prior to determining the respiration rates of each mixture, the dissolved oxygen probe of the Rank Cell was calibrated. Calibration procedure comprised tilling the cell with deionized water which was saturated with air, starting the stirrer and setting the chart recorder response to 100%. An excess amount of anhydrous sodium sulphite was then added to the water and the cell lid closed. The dissolved oxygen concentration was allowed to fall and bottom-out. The chart recorder response was then calibrated to 0%. The cell was emptied, rinsed and refilled with deionized water and left on standby to receive the test samples.

Control tests were carried out in a similar manner but without the addition of the naphthalene derivative solution.

For those naphthalene derivatives proved to be nitrification inhibitors, remedial reagent $CuSO_4$ solution was added to the Nitrifying sludge-Synthetic sewage-Naphthalene derivative mixture to make final Cu^{2+} concentration 3 mg/L. Respiration rates were then measured as mentioned above.

The respiration rate for each test (including the controls) was derived from the slope of the dissolved oxygen curve from the recorder trace. The slope was measured for the longest linear portion of each trace drawn between 25 and 65% saturation. The rate was calculated as follows:

$$Rate(R) = slope \times \frac{\%SaturationDO}{100} \times 60 mgO_2 / L \times \frac{1}{mgSS / L}$$

The following notation was adopted, based on that given in the standard method for determining the extent of inhibition of nitrification and/or carbonaceous oxidation:

R_C the mean measured respiration rate in unamended controls, the sum of base nitrification and carbonaceous components (mg O/g.SS.h);

R_{C+ATU} the mean measured respiration rate in controls where the nitrification component was eliminated by the addition of ATU, leaving the base carbonaceous rate (mg O/g.SS.h);

R_N the mean base rate due to nitrification determined by the difference R_C- R_{C+ATU} (mg O/g.SS.h).

To compare nitrification rates in different tests, relative respiration rate (r) was calculated by

$$r\ (\%) = 100 \times R_{N\ Sample} / R_{N\ Control}$$

logKo, of each naphthalene derivative was estimated by program WSKOWWIN Vl.26 from William Meylan.

E_{HOMO} of each naphthalene derivative was calculated by program MOPAC V6.0 based on MNDO method obtained from Frank J. Seiler Research Laboratory, US Air Force Academy.

RESULTS AND DISCUSSION

The relative respiration rates (r) of microorganisms in nitrifying sludge to various concentrations of naphthalene derivatives (C) in the mixture were plotted in Figure 1.

This figure showed that all of the seven naphthalene derivatives were potential nitrification inhibitors. The extent to inhibition caused by three compounds with electron-withdrawing sulfo group (2-naphthalenesulfonic acid, 2-amino-1-naphthalenesulfonic acid and 7-amino-4-hydroxy-2-naphthalenesulfonic acid) was not as high as four compounds without sulfo group in naphthalene ring.

Experimental data of r were correlated with concentrations of naphthalene derivatives and the regression equations established along with correlation coefficients R^2 were given in Table 1.

Table 1. Correlation between relative respiration rates (r) and concentrations of naphthalene derivatives (C)

Compound	Regression equations	R^2
2-Naphthol	logr = -1.133logC + 3.06	0.988
1-Naphthylamine	logr = -1.632logC + 3.61	0.936
5-Amino-1-naphthol	logr = -0.290logC + 2.04	0.995
1-Amino-4-chloronaphthalene	logr = -0.243logC + 2.01	0.990
2-Naphthalenesulfonic acid	r = -0.041C + 88.0	0.957
2-Amino-1-naphthalenesulfonic acid	r = -0.092C + 102	0.984
7-Amino-4-hydroxy-2-naphthalenesulfonic acid	r = -0.067C + 88.6	0.992

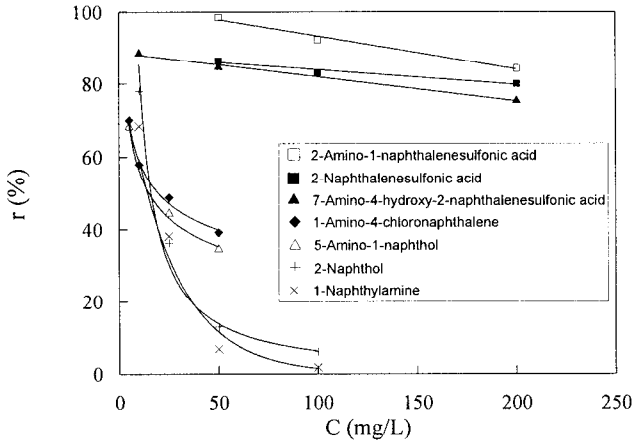

Figure 1. Inhibition of nitrifiers by naphthalene derivatives.

It was observed from the regression equations in Table 1 that for naphthalene derivatives with and without electron-withdrawing sulfo group, different relations were presented between r and C. This phenomena may be attributed to the difference in physical and chemical properties such as electron density in naphthalene ring and lipophilic property which were resulted from the different substituting groups.

Curves in Figure 1 implied that nitrification inhibition caused by naphthalene derivatives varied from compound to compound. To compare inhibitory effect, median inhibitory concentrations IC_{50} were calculated according to the equations from Table 1 and were listed in Table 2.

Nitrification inhibition concentration of 1-naphthylamine was reported to be 15mg/L for 50% reduction in nitrifying rate (Hockenbury and Grady 1977). This concentration was very closed to the above-predicted IC_{50} 14.8 mg/L. Unfortunately relevant literature of nitrification inhibition by naphthalene derivatives was far from sufficient and data of IC_{50} were hardly available.

It has been widely accepted that the rate at which an organic molecule is degraded depends on a number of factors such as, (i) the ease with which the molecule can penetrate the cell (of microorganisms) and reach the appropriate enzyme site; (ii) the extent to which electronic effects either interfere with enzyme bonding or alter the energy required to break the critical bonds in the molecule (Pitter and Chudoba 1990). In treatment of sewage and wastewater, the extent to which an organic molecule inhibits the activity of either aerobic or anaerobic microorganisms could be supposed to depend on similar factors mentioned above, i.e., lipophilic property and electronic effect, based on the knowledge that molecule of inhibitor will interact with the membrane and oxidizing enzyme as well.

Energy of highest occupied molecular orbit E_{HOMO} is thought to be an appropriate parameter describing the tendency of a molecule to donate electrons, while partition coefficient in the 1-octonol-water system K_{OW} is most frequently used as a measure of lipophilic properties. Therefore the values of E_{HOMO} and logKo, of seven naphthalene derivatives were estimated respectively by program MOPAC and WSKOWWIN V1.26 and shown in Table 2.

Table 2. IC_{50}, $logK_{OW}$ and E_{HOMO} of naphthalene derivatives.

Compound	IC_{50} (mg/L)	$logK_{OW}$	E_{HOMO} (eV)
2-Naphthol	15.9	2.70	-8.4180
1-Naphthylamine	14.8	2.25	-8.3210
5-Amino-1-naphthol	15.0	1.77	-8.2493
1-Amino-4-chloronaphthalene	19.1	2.90	-8.8496
2-Naphthalenesulfonic acid	926	0.01	-9.3894
2-Amino-1-naphthalenesulfonic acid	568	-1.16	-9.1272
7-Amino-4-hydroxy-2-naphthalenesulfonic acid	573	-1.39	-8.9428

Regression analysis was then conducted between $logIC_{50}$ and $logK_{OW}$ along with E_{HOMO} and two equations obtained were listed as follows, showing previous assumption was reasonable that inhibition caused by naphthalene derivatives was related to both their lipophilic property and electronic effect.

$logIC_{50} = 0.016\ logK_{OW} - 0.138\ E_{HOMO}$ $R^2 = 0.9999$, for the first four strong inhibitors

$logIC_{50} = 0.069\ logK_{OW} - 0.3.15\ E_{HOMO}$ $R^2 = 0.9997$, for the last three weak inhibitors

A review of general biochemistry of nitrifying bacteria may provide an insight into the mechanism of inhibition. Nitrification in the activated sludge process is due to the presence of *Nitrosomonas* and *Nitrobacter*. *Nitrosomonas* oxidizes ammonia to nitrite via hydroxylamine, nitroxyl, (NOH), and nitric oxide intermediates. A copper protein is essential for the oxidation of both ammonia (Anderson 1965) and hydroxylamine (Nicholas et al. 1962). Oxidation of nitrite to nitrate by *Nitrobacter* also involves a copper containing protein (Campell and Aleem 1965).

Copper is present in enzymes as a Cu(I)-Cu(I1) electrocouple (Peisach et al. 1966) and complexation of either oxidation state prevents the couple from operation. The inhibitor competes with enzyme for copper and probably becomes incorporated into the overall enzyme structure thus modifying its properties detrimentally (Wood et al. 1981). It is supposed that to achieve this the inhibitor molecule may be adsorbed on the cell membrane, accumulate in some of its layers, transport across the membrane and reach the appropriate enzyme site. Although further laboratory work is needed, it might be deduced that remarkable lipophilic property is a must. The transport across cell membranes can be inhibited by the ionization of certain functional groups (e.g. the sulfo group) in the molecule of a given compound and thus leads to weaker inhibition to nitrification by three naphthalene derivatives with sulfo group vs nonionic stronger inhibitors.

With molecule successfully penetrating the cell the extent of inhibition should be then proportional to the tendency to donate electrons as the mechanism of inhibition is predominantly by complexation of organic molecule with Cu (Wood et al. 1981). This is consistent with the above-obtained equations in which log-linear relation was presented between IC_{50} and E_{HOMO}.

Interference with respiration rate will become manifest in disruption nitrification, with adverse effect on ammonia removal efficiency of the treated effluent. Thus it is necessary to determine the highest concentrations of naphthalene derivatives discharged into treatment plant. From an operational point of view a 50% reduction in removal efficiency is not an acceptable proposition and the lab-derived IC_{50}s are not appropriate concentrations, thus determining the highest no-effect concentration (max IC_0) would seem more useful. However, respiration curves in Figure 1 exhibited that all seven naphthalene derivatives were strong nitrification inhibitors even at low concentrations, it may not be of much significance to determine IC_0s or even IC_{05}s as the values would be much close to zero.

Taking into consideration that in-service treatment plant normally operates with certain anti-shock capacity and will keep on running even if respiration of microorganisms is slightly inhibited, in this case IC_{20} (the effective concentration that causes a 20% reduction in respiration rate, i.e. relative respiration rate r = 80%) may be used to indicate the maximum acceptable loading limit. Values of IC_{20} were calculated for seven naphthalene derivatives and Table 3 gave the results. This IC_{20} maximum acceptable loading limit is only a suggestion rather than regulatory and can be replaced by IC_{10} or IC_{30} in accordance with the practical operation experience at works.

Table 3. Maximum acceptable loading limit of naphthalene derivatives.

Compound	IC_{20} (mg/L)
2-Naphthol	10.5
1-Naphthylamine	11.1
5-Amino-1-naphthol	3.0
1-Amino-4-chloronaphthalene	2.8
2-Naphthalenesulfonic acid	195
2-Amino-1-naphthalenesulfonic acid	238
7-Amino-4-hydroxy-2-naphthalenesulfonic acid	128

As mentioned above copper played an important role in nitrification and Cu^{2+} increased *Nitrosomonas* growth as well as enzymatic activity at low concentration (Loveless and Painter 1968; Skinner and Walker 1961). On the other hand, many known nitrification inhibitors acted by complexing the copper enzyme, while inhibition was reported could be counteracted via Cu(II) addition (Vandevivere et al. 1998). The mechanism of action of Cu(II) may involve an interaction either directly with the bacterial cells or rather with the organic inhibitors present in the wastewater since copper ions were known for their very strong affinity for organic compounds (Vandevivere et al. 1998). With this knowledge research was carried out to remedy the inhibited nitrifying system by four compounds 1-naphthylamine, 2-naphthol, 5-amino-l-naphthol and 1-amino-4-chloronaphthalene with addition of Cu(II). $CuSO_4$ solution was added to the Nitrifying sludge-Synthetic sewage-Naphthalene derivative mixture to make final Cu^{2+} concentration 3 mg/L and respiration rates were measured.

Respiration curves before and after Cu(II) addition were illustrated in Figure 2. It was observed that respiration rates increased at the given dose of Cu(II). Relative respiration rates r were again correlated to concentrations of naphthalene derivatives and IC_{50}s were obtained from the regression equations and listed in Table 4.

Table 4. Correlation between r and C and IC_{50}s after Cu(II) addition.
(Concentration of Cu(II) in the mixed liquor = 3mg/L)

Compounds	Regression equations	R^2	IC_{50}(mg/L)
2-Naphthol	logr = -0.704 logC + 2.70	0.935	26.4
1-Naphthylamine	logr = -0.511 logC + 2.41	0.999	24.6
5-Amino-1-naphthol	logr = -0.455 logC + 2.39	0.932	33.0
1-Amino-4-chloronaphthalene	logr = -0.121 logC + 1.96	0.967	143.6

Ail of the IC_{50} values were obviously higher than their counterparts before Cu(II) addition. It could be expected that nitrification inhibition by naphthalene derivatives was reduced when remedial reagent Cu(II) was added.

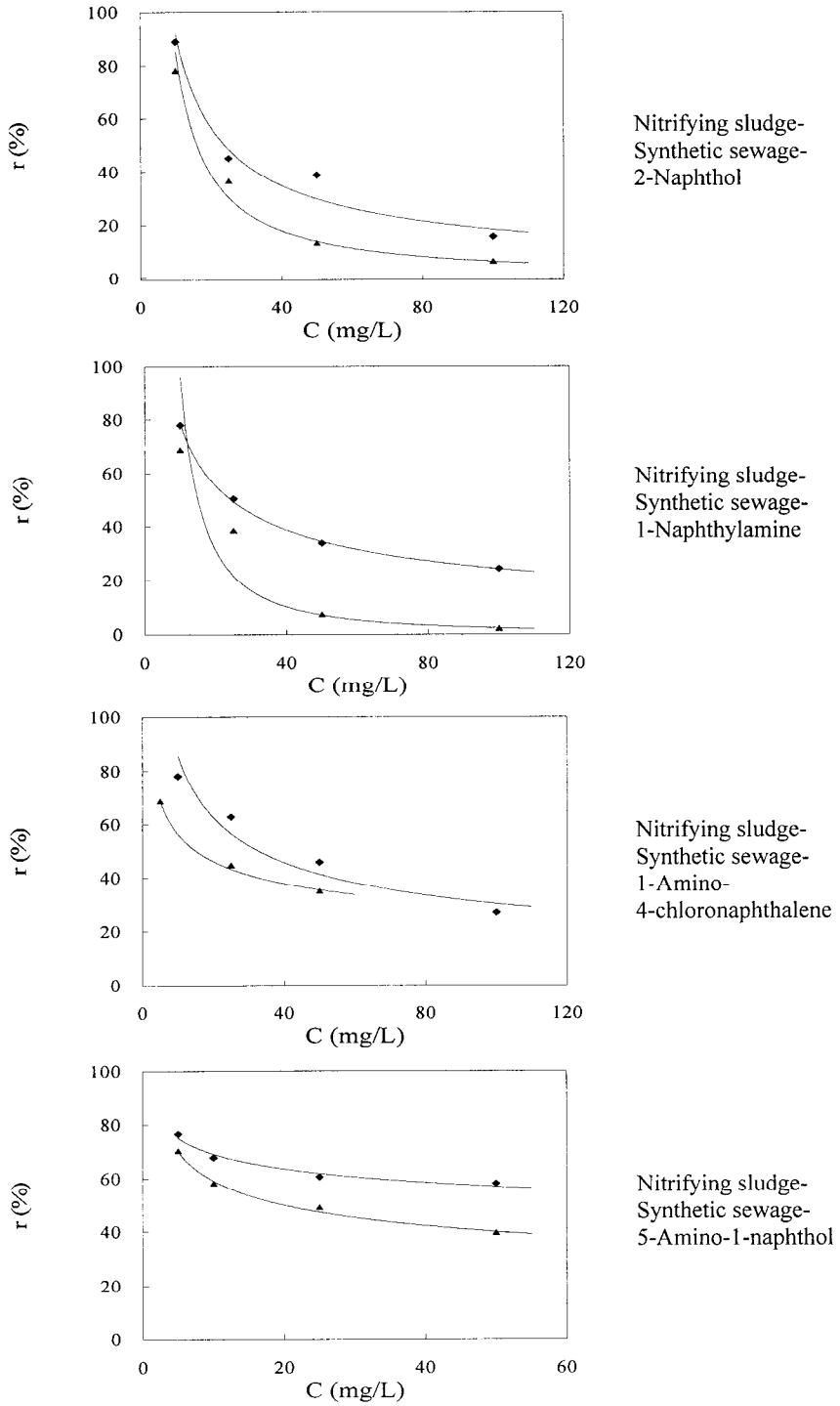

Figure 2. Relative respiration rates of nitrifiers before and after Cu(II) addition.
▲ Before Cu(II) addition; ◆ After Cu(II) addition.

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