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Effect of 3,3',4',5-Tetrachlorosalicylanilide on Reduction of Excess Sludge and Nitrogen Removal in Biological Wastewater Treatment Process

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Abstract A metabolic uncoupler, 3,3',4',5-tetrachlorosalicylanilide (TCS), was used to reduce excess sludge production in biological wastewater treatment processes. Batch experiments confirmed that 0.4 mg/l of TCS reduced the aerobic growth yield of activated sludge by over 60%. However, the growth yield remained virtually constant even at the increased concentrations of TCS when cultivations were carried out under the anoxic condition. Reduction of sludge production yield was confirmed in a laboratory-scale anoxic-oxic process operated for 6 months. However, it was found that ammonia oxidation efficiency was reduced by as much as 77% in the presence of 0.8 mg/l of TCS in the batch culture. Similar results were also obtained through batch inhibition tests with activated sludges and by bioluminescence assays using a recombinant *Nitrosomonas europaea* (pMJ217). Because of this inhibitory effect of TCS on nitrification, the TCS-fed continuous system failed to remove ammonia in the influent. When TCS feeding was stopped, the nitrification yield of the process was resumed. Therefore, it seems to be necessary to assess the nitrogen content of wastewater if TCS is used for reducing sludge generation.

Keywords: Excess sludge, metabolic uncoupler, 3,3',4',5-tetrachlorosalicylanilide (TCS), nitrification

Nowadays, biological wastewater treatment processes are being widely used for treatments of municipal and industrial wastewaters [3, 16, 25]. Biological wastewater treatment processes convert organic pollutants in wastewater to cell mass and respiration products, such as CO_2, CH_4, N_2, and H_2S [14, 17, 18]. The cells can be separated from the treated water and disposed of in a concentrated form called excess sludge [15]. Activated sludge processes are known to generate excess sludge of around 0.5 kg SS (suspended solid)/kg BOD_5 removed [15]. It is also generally accepted that treatment of excess sludge accounts for 40–60% of the total capital and operation costs of wastewater treatment plants [4]. However, the disposal methods of the excess sludge including incineration, landfill, and ocean dumping are facing social and technical difficulties in many densely populated nations because environmental regulations are becoming stringent [4, 28].

In order to solve excess sludge problems, various innovative approaches have been proposed [3]. These include physical and chemical methods, such as ozonation, chlorination, thermal treatment, and so on [3, 15, 32]. It has also been proposed that metabolic uncouplers can reduce sludge production [4, 19]. Among them, use of metabolic uncouplers could be easier and less expensive than the posttreatment of the excess sludge produced, because a metabolic uncoupler can be easily fed to the oxic tank of a wastewater treatment plant without constructing new basins or installing expensive facilities. Therefore, many researchers have attempted to use metabolic uncouplers in the activated sludge process for reducing excess sludge production [3, 15, 18, 22].

Metabolic uncouplers dissociate the energy coupling between catabolism and anabolism, thereby a part of energy extracted from substrates is wasted through futile cycles, which leads to less production of bacterial cell mass [4]. Generally, the oxidative phosphorylation for generating adenosine-5'-triphosphate (ATP) is driven by the proton gradient built up across the cell membrane [21]. Metabolic uncouplers disconnect oxidative phosphorylation from electron transport by dissipating the proton gradient across the cell membrane. Therefore, the energy released from the oxidation of substrate is given off as heat rather than as ATP [20, 24]. As a result, the growth yield is much lowered in microbial culture with metabolic uncouplers. Therefore, the dissipation of energy via uncoupling processes can promote sludge reduction, and the effectiveness of metabolic uncouplers in reducing excess sludge production has been reported [22].

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Metabolic uncouplers that have been used to reduce excess sludge production yield in the activated sludge processes include 2,4-dichlorophenol (dCP), 3,3',4',5-tetrachlorosalicylanilide (TCS), 2,4-dinitrophenol (dNP), *p*-nitrophenol (pNP), gramicidin, 2,4,5-trichlorophenol (TCP), etc. [4, 22, 27]. However, it should be noted that most metabolic uncouplers are xenobiotic and potentially harmful to the environment [15]. Considering that TCS had been often used for formulations of soaps, rinses, and shampoos, TCS could be environmentally sound compared with other metabolic uncouplers [2, 15]. Use of TCS to reduce the microbial growth was also reported by Cook and Russell [6] for the pure culture of *Streptococcus bovis*. Chen *et al.* [4] reported that excess sludge production was reduced as much as 40% by 0.8 mg/l of TCS. However, most previous works focused only on effects of metabolic uncouplers on excess sludge reduction; research of its impact on the removal of particular substrate, such as nitrogen compounds, is hardly found. Only a few studies have discussed the technical problems, such as unexpected dissolved oxygen consumption and the microbial acclimation to metabolic uncouplers [19, 27].

Among the particular substrates, nitrogen is one of the most important nutrients for organisms [7, 31]. However, discharge of the nitrogen to receiving waters leads to significant impacts on water quality, resulting in eutrophication of water bodies and algal blooms that are short-lived and whose decay imposes a heavy oxygen demand in water [8]. When this happens, the dissolved oxygen is depleted, and such would result in the death of the higher forms of life. With more stringent effluent discharge standards, removal of nitrogen as well as BOD has become obligatory for both municipal and industrial wastewater treatment plants [7].

In municipal and industrial wastewater treatment plants, ammonia-oxidizing bacteria (AOB), such as *Nitrosomonas europaea*, play an important role in the removal of ammonia [23]. In *N. europaea*, ammonia is initially oxidized to hydroxylamine by ammonia monooxygenase (AMO). The subsequent oxidation of hydroxylamine to nitrite is catalyzed by hydroxylamine oxidoreductase (HAO) [1, 10]. Compared with nitrite oxidizers and other heterotrophs, AOB grow very slowly and are known to be extremely susceptible to a wide variety of inhibitors at low concentrations [12, 23, 30]. Some chemical compounds inhibit AMO activity at lower concentrations than other enzymes such as HAO [9]. It is generally accepted that inhibition of ammonia oxidation by AOB results in a total failure of nitrogen removal [11]. Thereupon, we investigated the effects of TCS on nitrification efficiency as well as excess sludge production. In addition, we investigated the long-term effects of TCS on process parameters including substrate removal efficiency, nitrification efficiency, process stability, and so on.

Materials and Methods

Batch Test Under Oxic and Anoxic Conditions

Batch tests under oxic condition were carried out in a 5-l reactor containing 2 l of a synthetic wastewater (1.4 g/l of Na_2HPO_4, 0.6 g/l of KH_2PO_4, 0.1 g/l of NaCl, 0.8 g/l of glucose, 0.022 g/l of $CaCl_2$, and 0.24 g/l of $MgSO_4$) to evaluate sludge production and substrate consumption in the presence of TCS ranging from 0 to 0.8 mg/l. Initial mixed liquor suspended solids (MLSS) and chemical oxygen demand (COD_{Cr}) were 500 mg/l and 800 mg/l, respectively. Temperature was controlled at 20°C, and dissolved oxygen (DO) concentration was maintained at around 6 mg/l. MLSS and soluble COD (SCOD) were measured every 2 h. In order to examine the effect of TCS on the growth of a bacterial pure culture, *Escherichia coli* DH5α was cultivated at 25°C and 130 rpm in a 1-l flask containing 500 ml of Luria Bertani (LB) medium (0.53 g/l of trypton, 0.26 g/l of yeast extract, and 0.53 g/l of NaCl) with 0–0.8 mg/l of TCS. Cell concentration was estimated every 2 h by measuring optical density at 600 nm (OD_{600}) using a spectrophotometer (Smart Plus 3255, Korea).

Anoxic batch tests were carried out in a 5-l reactor for evaluating the effect of TCS on sludge growth in the absence of oxygen. Operation conditions were identical to those of the oxic experiments except that no air was supplied. SCOD, pH, and MLSS were measured every 2 h.

Respirometric Assay Using *N. europaea* ATCC 19178

The respirometric assay is a simple method of determining the effect of inhibitors on nitrifying bacteria [13]. *N. europaea* ATCC 19178, the best studied ammonia-oxidizing bacterium, was grown in a minimal P-medium (0.7 g/l of KH_2PO_4, 13.5 g/l of Na_2HPO_4, 2.5 g/l of $(NH_4)_2SO_4$, 0.5 g/l of $NaHCO_3$, 0.1 g/l of $MgSO_4 \cdot 7H_2O$, 5 mg/l of $CaCl_2 \cdot 2H_2O$, and 1 mg/l of Fe-EDTA, pH 8.0–9.2). Cultivated *N. europaea* was centrifuged at 7,000 rpm and 4°C for 10 min and resuspended in 0.1 M phosphate buffer (pH 7.8). Air-saturated P-medium (without cell) was added into a 50-ml beaker, and an oxygen electrode (YSI 5000, YSI, U.S.A.) was placed into the 50-ml beaker to measure the DO value of the P-medium. After the DO of P-medium was stabilized, 1 ml of cell suspension of *N. europaea* was added into the P-medium and left to stand until a new steady state of DO was reached. Finally, TCS solution was added into the P-medium containing cells of *N. europaea*. The DO was measured for about 10–15 min. Ammonia oxidation efficiency was calculated as Eq. (1),

$$\text{Ammonia oxidation efficiency (\%)} = (OUR_N/OUR_i) \times 100 \quad (1)$$

where OUR_i and OUR_N refer to the oxygen uptake rates before and after TCS addition, respectively. The strength of inhibition of ammonia oxidation by the inhibitor was

expressed as the AIC_{50} (ammonia oxidation inhibitory concentration), defined as the concentration of inhibitor causing 50% reduction in ammonia oxidation efficiency.

Batch Tests for Nitrification and Denitrification by Activated Sludge

Batch tests were carried out using activated sludge obtained from the Seonam domestic wastewater treatment plant (Seoul, Korea) for examining the effect of TCS on the nitrification rate. The above synthetic wastewater was diluted except for NH_4Cl to adjust concentrations of COD_{Cr} and NH_3-N to be 300 and 100 mg/l, respectively. The initial biomass concentration was set to 3,000 mg/l as MLSS, and TCS was added to be 0–0.8 mg/l. The other conditions were the same as above. MLSS, SCOD, NH_3-N, NO_3^--N, NO_2^--N, and pH were measured every 2 h during 24 h of experiments.

Anoxic batch experiments were carried out in order to examine the effect of TCS on the denitrification rate. Initial MLSS, NO_3^--N, and COD_{Cr} were set to about 500 mg/l, 100 mg/l, and 800 mg/l, respectively. NH_3-N, NO_3^--N, NO_2^--N, SCOD, pH, and MLSS were measured every 2 h. The strength of inhibition of nitrification by the inhibitor was expressed as the NIC_{50} (nitrification inhibitory concentration), defined as the concentration of inhibitor causing 50% reduction in nitrification rate.

Bioluminescence Assay Using Recombinant *N. europaea* (pMJ217)

A recombinant bacterium, *N. europaea* (pMJ217) carrying the *luxAB* genes of *Vibrio harveyi*, was constructed by this research group for the rapid and sensitive detection of ammonia-oxidizing inhibition by measuring decreased luminescence in the presence of inhibitors [26]. The *luxAB* genes constructed under the control of the promoter *hao* (hydroxylamine oxidoreductase gene) and the completed plasmid pMJ217 were transferred into the *N. europaea*. The recombinant *N. europaea* (pMJ217) was cultivated in a 500-ml flask containing 100 ml of P-medium and 100 μg/ml of ampicillin at 30°C in a shaking incubator. A 500-μl volume of culture cells (ABS_{600} 1.2–1.4) was added into a 1-ml polypropylene vial followed by the addition of 500 μl of TCS, which made the final TCS concentration range from 0 to 0.8 mg/l. The luminescent reaction was started by the injection of 2 μl of 0.1% (v/v) n-decanal solution dissolved in 20 mM Tween 80. All measurements were performed at 25°C. The strength of inhibition of bioluminescence by the inhibitor was expressed as the LIC_{50} (luminescence inhibitory concentration), defined as the concentration of inhibitor causing 50% reduction in light output from that in the control reaction.

Long-Term Application of TCS in Anoxic-Oxic Process

In order to investigate the effect of TCS on the sludge reduction and ammonia removal for a long period of time, a lab-scale anoxic-oxic reactor was operated in the presence of TCS. The system was composed of 3.37 l of an anoxic tank, 6.71 l of an oxic tank, and 5.07 l of a settler. An agitator was provided for each of the anoxic and oxic tanks to provide mixing. DO concentration in the oxic tank was maintained at 5 mg/l and temperature was kept at 20°C with a temperature-controllable water jacket. The synthetic wastewater was fed to the reactor at a rate of 1 l/h. TCS was continuously added into the oxic tank using a syringe pump (KD Scientific 210, U.S.A.) to be 0.4 mg/l. The system was operated for 6 months and was divided into 4 periods (Table 1). During the period A (days 1–22), TCS was not added to the system. Then, 120 ml of excess sludge was withdrawn daily from the system. For the period B (days 23–70), TCS was fed to the system at a rate of 768 μl/day (which made the TCS concentration in the oxic tank to be 0.4 mg/l), and 240 ml/day of excess sludge was continuously withdrawn from the system. In the period C (days 71–98), TCS was continuously fed to the system, but no sludge was wasted. During the period D (days 99–161), TCS feeding was ceased, and excess sludge wasting was resumed at a rate of 480 ml/day.

In order to investigate the nitrification efficiency and operational characteristics of the system, MLSS, COD, pH, NH_3-N, NO_3^--N, NO_2^--N, and SVI (sludge volume index) were measured.

Analysis

MLSS, SVI, and COD_{Cr} were measured following the Standard Methods [5]. Samples were centrifuged at 5,000 rpm for 10 min and supernatants were used for measuring pH and concentrations of SCOD, NH_3-N, NO_3^--N and NO_2^--N. NH_3-N concentration was measured by direct Nesslerization and a spectrophotometer at 450 nm [29]. Concentrations of NO_3^--N and NO_2^--N were measured using an ion

Table 1. Operation conditions of the anoxic-oxic system.

Period	Operation time (day)	Feed rate of synthetic wastewater (l/h)	TCS feed rate (μl/day)	Rate of sludge wasting (ml/day)	External recycle rate (l/h)	HRT (h)	SRT (day)
A	1–22	1	Without TCS	120	0.24	10	25
B	23–70	1	768 (0.4 mg/l)	240	0.4	10	14
C	71–98	1	768 (0.4 mg/l)	0	4	10	-
D	99–161	1	Without TCS	480	0.4	10	10

chromatograph (DX-120, Dionex, U.S.A.). DO and pH were measured with a YSI-58 DO meter and Orion-370 pH meter, respectively.

RESULTS AND DISCUSSION

Effects of TCS on Sludge Growth Under Oxic and Anoxic Conditions

TCS at different concentrations of 0–0.8 mg/l was tested in the oxic and anoxic batch reactors. Observed growth yield (Y_{obs}) of sludge was calculated from experimental data using Eq. (2),

$$Y_{obs}=(X-X_o)/(S_o-S) \quad (2)$$

where S and X represent concentrations of SCOD and MLSS at time t, and S_o and X_o are initial concentrations of SCOD and MLSS, respectively. As shown in Fig. 1A, Y_{obs} of the reactor with 0.4 mg/l of TCS decreased 60% compared with the control culture that was free of TCS. This result was basically identical to that of Chen *et al.*, who observed 40% sludge reduction at 0.8 mg/l of TCS [4]. However, the growth yield remained virtually constant even at the increased concentrations of TCS when cultivations were carried out under anoxic condition (Fig. 1A). It is generally known that the energy generated from the oxidation of an organic substrate by oxidative phosphorylation would be lost as heat rather being captured in ATP when a metabolic uncoupler such as TCS is present [15]. As a result, the growth yield is lowered in a metabolic uncoupler-containing microbial culture. However, in the anoxic condition, the energy uncoupling might be negligible, probably because ATP is generated through different mechanisms from oxidative phosphorylation.

In order to reconfirm the efficacy of TCS on reduction of cell growth, the pure culture of *E. coli* DH5α was aerobically grown in LB medium containing TCS. As shown in Fig. 1B, the relative specific growth rate of *E. coli* decreased significantly as TCS concentration increased. The specific growth rate was decreased by 40% at 0.4 mg/l of TCS. From these results, it was again confirmed that TCS was effective for limiting cell growth.

Effect of TCS on Nitrogen Removal

In order to investigate the effect of TCS on ammonia oxidation, the pure culture of an ammonia-oxidizing bacterium, *N. europaea*, was selected to perform batch

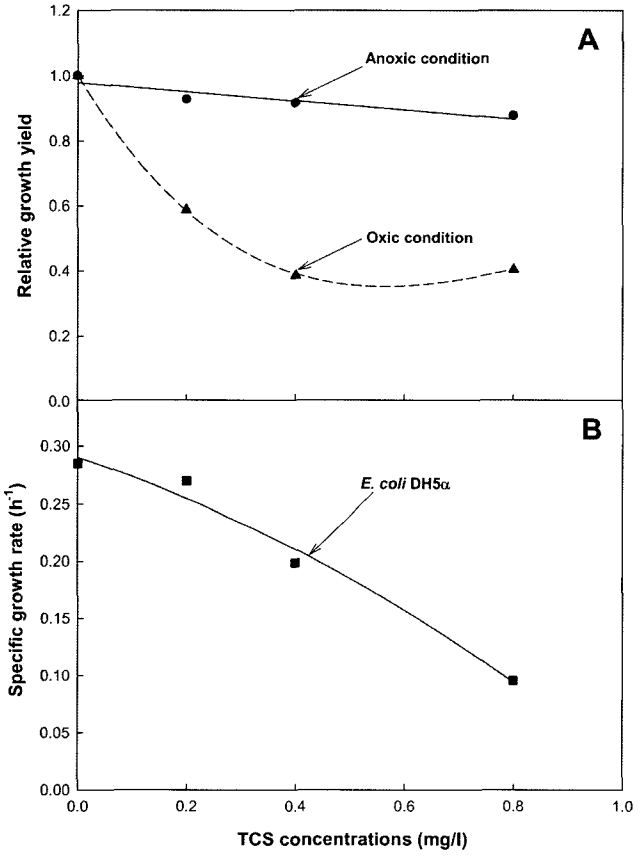

Fig. 1. Effect of TCS on relative growth yield of activated sludge under oxic and anoxic conditions and the specific growth rate of *E. coli*.

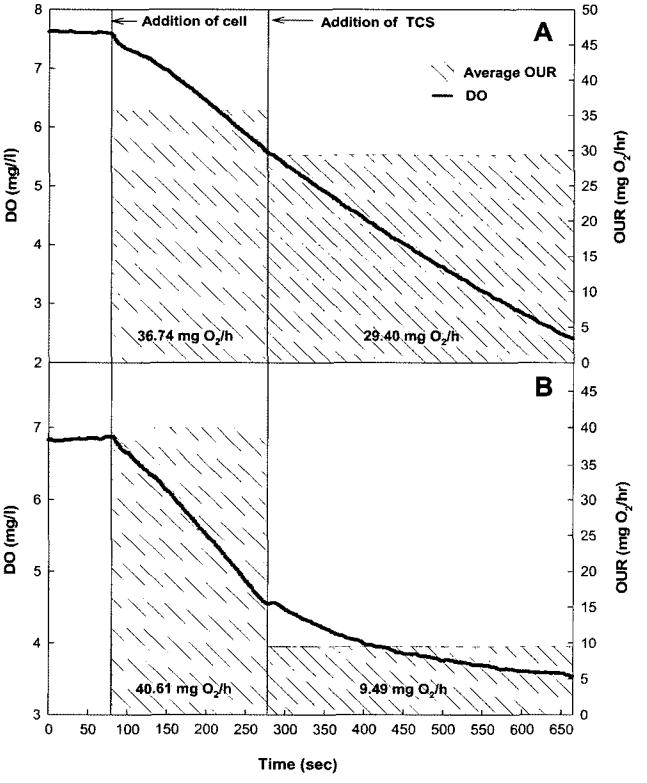

Fig. 2. DO and oxygen uptake rate (OUR) in batch ammonia oxidation inhibition experiments with suspended culture of *N. europaea*.
A. 0.2 mg/l of TCS. **B.** 0.8 mg/l of TCS.

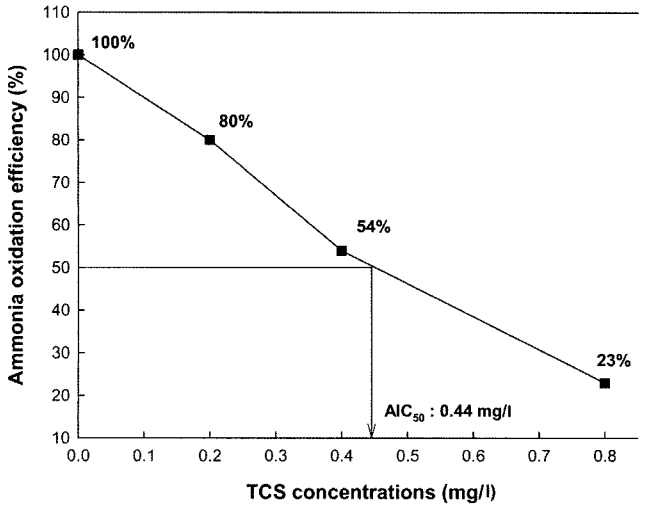

Fig. 3. Ammonia oxidation efficiency curve for TCS concentrations in cultivation of *N. europaea* and determination of the AIC_{50}. Ammonia oxidation was monitored by measuring DO value of the suspension of *N. europaea* after TCS addition.

ammonia oxidation inhibition experiment by measuring oxygen uptake rates, which reflect the ammonia oxidation rates. As shown in Fig. 2, the DO consumption rate of *N. europaea* decreased when TCS was added, which indicated that ammonia oxidation rate decreased. Furthermore, ammonia oxidation efficiency significantly decreased as the TCS concentration increased (Fig. 3). Specifically, ammonia oxidation efficiency was only 23% of the control (without TCS) when 0.8 mg/l of TCS was present in the medium.

Ammonia is oxidized to hydroxylamine by ammonia monooxygenase (AMO) in *N. europaea* as follows [1, 10],

$$NH_3+O_2+2H+2e^-\rightarrow NH_2OH+H_2O \quad (3)$$

As a reactant of Eq. (3), oxygen is consumed in the ammonia oxidation. Moreover, because addition of metabolic uncouplers can significantly increase oxygen uptake rate (OUR) as a result of uncoupling, the DO consumption rate must be increased in the normal conditions [19, 27]. Nevertheless, the DO consumption rate and ammonia oxidation efficiency of *N. europaea* significantly decreased as the TCS concentration increased in our results. This meant that ammonia oxidation was directly inhibited by TCS.

Inhibition of ammonia oxidation was reconfirmed by measuring the concentrations of ammonia and NO_x^- (nitrate and nitrite) in batch cultivations of activated sludge with TCS. As shown in Fig. 4A, there was not a significant impact of TCS on nitrification when its concentration was lower than 0.2 mg/l. However, ammonia consumption rate and NO_x^- formation rate decreased significantly by TCS concentration increase up to 0.8 mg/l. The relative nitrification rates are shown in Fig. 4B. In the TCS concentration range of 0.4–0.8 mg/l, these were only 5–16% of the control, which meant that a TCS concentration

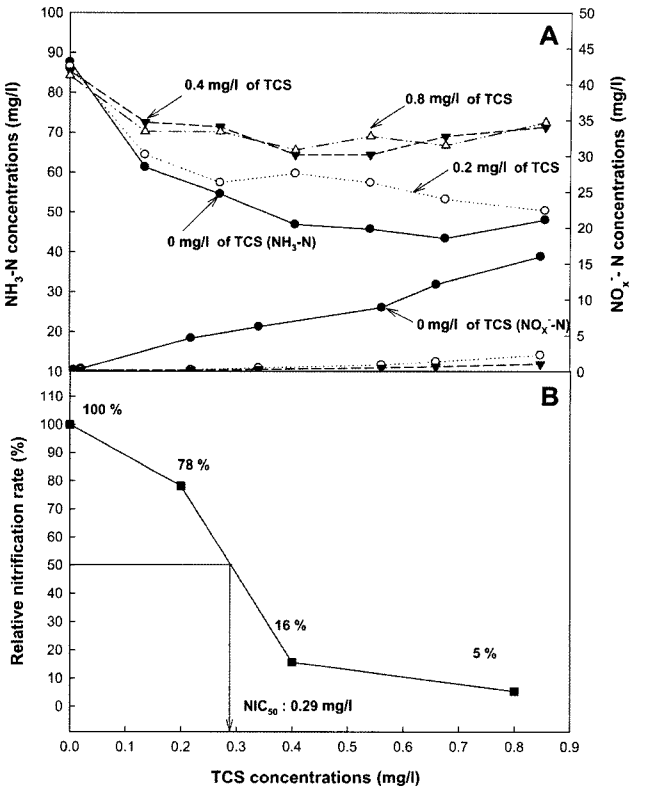

Fig. 4. Batch nitrification experiments using activated sludge in the presence of TCS and comparison of relative nitrification rate for TCS concentrations with the determination of NIC_{50}. Nitrification was monitored by measuring concentrations of NH_3-N and NO_x^--N.

higher than 0.4 mg/l could completely stop the nitrification reaction.

The effect of TCS on bioluminescence in recombinant *N. europaea* (pMJ217) was examined. When TCS was added to the culture broth of *N. europaea* (pMJ217), the intensity of the light emission significantly decreased, as shown in Fig. 5. The inhibition response appeared dose dependent. At the concentration of 0.4 mg/l of TCS, the intensity of the light emission was reduced to 26%, and only 10% of the light emission remained at a concentration of 0.8 mg/l. The LIC_{50}, AIC_{50}, and NIC_{50} of TCS obtained from these data were 0.17 mg/l (Fig. 5), 0.44 mg/l (Fig. 3), and 0.29 mg/l (Fig. 4B), respectively. The bioluminescence inhibition assay was the most sensitive for detection and evaluation of ammonia oxidation inhibition. Comparing these results, it was clear that TCS strongly inhibited the ammonia oxidation of *N. europaea* and activated sludge. Hooper and Terry [9] reported that ammonia oxidation efficiency was reduced as much as 60% by 3.51 mg/l of TCS. However, ammonia oxidation efficiency was reduced as much as 77% by 0.8 mg/l of TCS in our studies. From these results, it was concluded that nitrification is inhibited by adding TCS at a low concentration.

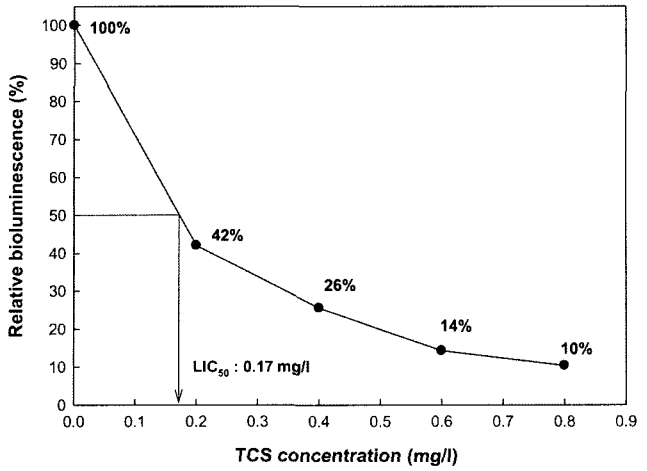

Fig. 5. Relative bioluminescence curve for TCS concentrations using a recombinant *N. europaea* (pMJ217) and determination of the LIC_{50}.

Batch denitrification experiments were also carried out in the anoxic condition. It was found that there was no difference in nitrate reduction at different TCS concentrations as in sludge growth yield in anoxic condition (data not shown).

Effect of TCS on the Long-term Operation of Anoxic-Oxic Process

In order to monitor the effect of TCS on sludge production and nitrogen removal in a long-term continuous cultivation, the anoxic-oxic process was operated for 161 days (Fig. 6). As shown in Fig. 6A, during the period A (from day 1 to day 22), the MLSS of anoxic and oxic tanks were maintained at approximately 8,000 mg/l, and the MLSS of the return stream and effluent were 36,000 mg/l and 10 mg/l, respectively. During the period B (from day 23 to day 70), while TCS was added to be 0.4 mg/l in the oxic tank, the anoxic and oxic MLSS concentrations decreased from about 8,000 mg/l to 4,000 mg/l, and MLSS in the settler (so in the recycle stream) rapidly decreased from 36,000 to 15,000 mg/l. These findings supported a feasibility of TCS for achieving excess sludge minimization. Since the sludge wasting was stopped during the period C (all of the excess sludge was recycled to the anoxic tank), the MLSS in the anoxic and oxic tanks increased. During the period D (from day 99 to day 161), the MLSS concentrations of all parts of the process increased. Sludge production rapidly decreased while TCS was continuously fed to the oxic tank (period B). On the other hand, sludge production increased again when TCS feeding was stopped (period D). This result showed that TCS was effective in reducing sludge production as in the batch experiments.

SVI, TCOD (total COD), and SCOD are also shown in Figs. 6B and 6C. SVI rapidly increased when TCS was

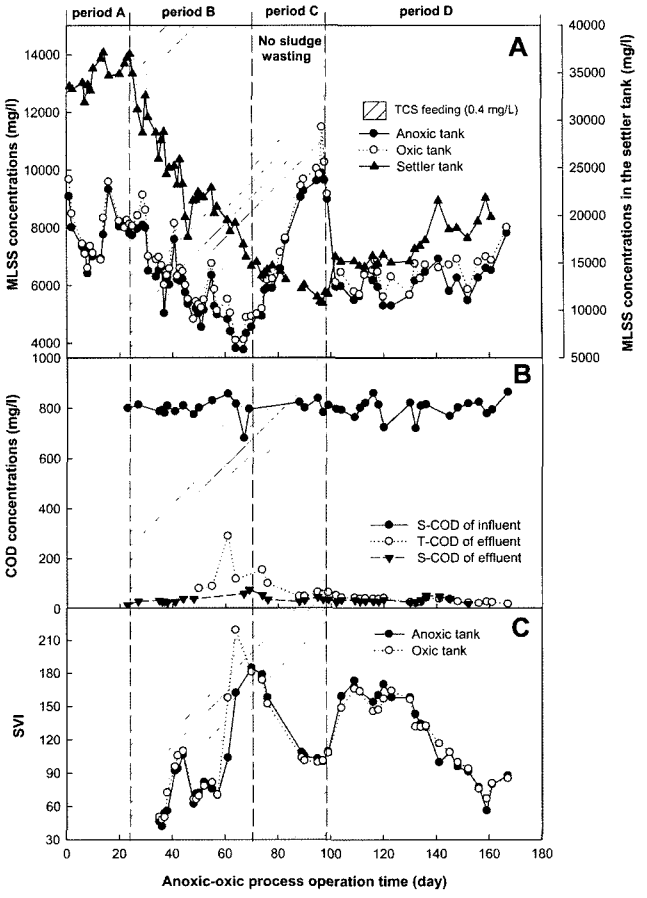

Fig. 6. MLSS, COD, and SVI of the anoxic-oxic process during 161 days of operation with 0.4 mg/l of TCS.

added to the system (period B). As a result, effluent TCOD increased because settleability became poor in the settler. However, increase of SCOD in the effluent was not significant, which suggested that the organic removal efficiency in the anoxic-oxic system was not negatively affected by TCS.

The concentrations of NH_3-N and NO_3^--N in the influent, effluent, and anoxic and oxic tanks of the anoxic-oxic system during the last 120 days are shown in Fig. 7. During TCS-fed periods (periods B and C with 0.4 mg/l of TCS), only a little ammonia was removed owing to the inhibitory effect of TCS on nitrification. By stopping feeding of TCS, nitrification activity was gradually resumed, by which the effluent ammonia concentration decreased from 80 to 40 mg/l. However, there was no nitrate accumulation observed during the entire period of the experiment, which was thought to be because generated nitrate, if any, was totally denitrificated, because TCS did not affect the denitrification reaction as seen in the batch experiments.

This study was aimed to evaluate the feasibility of using 3,3',4',5-tetrachlorosalicylanilide (TCS) for reducing excess

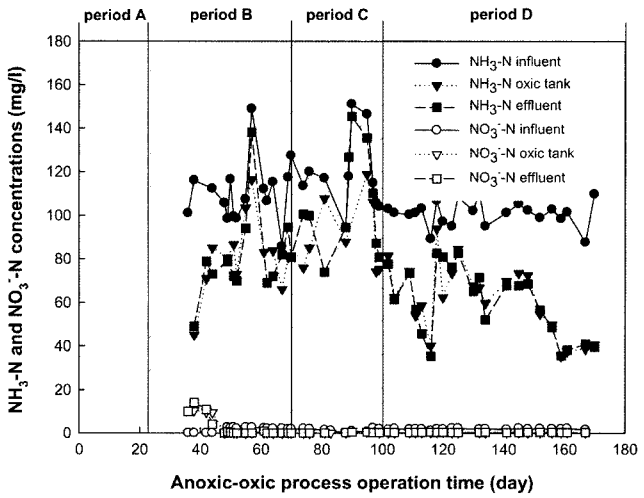

Fig. 7. Nitrogen concentrations (NH_3-N and NO_3^--N) of the anoxic-oxic process during 161 days of operation with 0.4 mg/l of TCS.

sludge production and to investigate its impact on nitrogen removal. TCS was very effective in reducing sludge production from the activated sludge process. It was found that TCS reduced sludge production yield by over 40% with 0.4 mg/l of TCS for activated sludge and pure cultivation of *E. coli* DH5α. Nonetheless, substrate removal capability was not significantly affected. However, TCS appeared noneffective for reducing sludge growth under anoxic condition. In an anoxic-oxic process operated for a long period of time, TCS was effective in reducing sludge production as in the batch experiments. In the nitrogen removal experiments, nitrification efficiency was reduced by as much as 80% when 0.4 mg/l of TCS was added in the batch reactor. This result was also reproduced in the TCS-fed anoxic-oxic system. On the other hand, denitrification efficiency was not affected by TCS. From these results, it was concluded that nitrification was inhibited by adding TCS, and excess sludge production was reduced. In conclusion, although TCS was effective for reducing sludge production, TCS inhibited nitrogen removal in the biological process. Therefore, it seems to be necessary to assess the nitrogen content of wastewater if TCS is to be used for minimizing sludge production. Wastewaters with a nitrogen level below the discharge limit would be a good target for TCS application. It is also necessary to find novel metabolic uncouplers that can be used for wastewaters containing nitrogen compounds of high concentrations.

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