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Biodegradation of N-methylmorpholine-N-oxide

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Abstract

N-Methylmorpholine-N-oxide (NMMO) is capable of dissolving cellulose without any further addition of chemicals. The solution can be used to produce cellulosic staple fibres by pressing it through spinning jets into an aqueous spinning bath. Because of results from conventional biodegradation tests using non-adapted activated sludge, the solvent is generally considered being persistent. The object of the described work was to show, whether and how activated sludge can be adapted to N-methylmorpholine-N-oxide and whether it is possible to purify NMMO-containing wastewaters in conventional wastewater treatment plants. The experiments showed that the sludge can be adapted within about 15–20 days. Adapted sludge can degrade the substance itself and its most important metabolites to concentrations below their detection levels and retain this ability even during limited periods without solvent being present in the wastewater. The main requirement for a successful adaptation is a high sludge age. The degradation takes place in several steps. First, NMMO is reduced to N-methylmorpholine. The next step is a demethylation of N-methylmorpholine to morpholine. This step is crucial for the adaptation process. Once morpholine has been formed, the adaptation proceeds very quickly until none of the substances in question can be detected any longer. So the next step must be the cleavage of the morpholine ring structure.

Abbreviations: COD – chemical oxygen demand; DOC – dissolved organic carbon; 96hEC50 – concentration resulting in a 50% decrease of the growth rate after 96 h; M – morpholine; NMM – N-methylmorpholine; NMMO – N-methylmorpholine-N-oxide; WWTP – wastewater treatment plant; SRT – solids retention time; SS – suspended solids

Introduction

N-Methylmorpholine-N-oxide (NMMO) is the only solvent capable of directly solving cellulose known so far, which is already being used for the production of cellulosic fibres in industrial scale. Lenzing AG has been dealing with this solvent for nearly a decade and has started to build the first large-scale plant in 1995. The plant has started operation in 1997 with a production capacity of about 15,000 tons per year.

Although the plant has to be run with the maximum recovery rate for NMMO for both economical as well as ecological reasons, aqueous emissions of NMMO and its reaction products N-methylmorpholine (NMM) and morpholine (M) cannot be totally avoided. NMMO as well as morpholine have been reported to be recalcitrant to biological degradation. For this reason various attempts have been made to develop physical-chemical degradation techniques, such as the TiO_2 photocatalytic degradation process for morpholine (Doherty et al. 1995) or the ozonation of NMMO-containing wastewater (Stockinger 1995; Stockinger et al. 1996).

The aim of this study was to investigate whether and how NMMO, NMM and M can also be degraded by microorganisms, and to develop a method for the treatment of NMMO-containing wastewaters.

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Material and methods

Test compounds

Without the exceptions noted below all chemicals used were analytical-grade and were obtained from Merck (FRG).

NMMO was technical-grade with an NMMO content of about 60% in water and was purchased from Huntsman (USA, former Texaco) or BASF (FRG). The actual NMMO content was determined before it was used and NMMO concentrations were always related to 100% NMMO.

NMM and M (analytical grade) were obtained from Fluka (Switzerland).

Wastewater

Wastewater was taken from the influent to the industrial WWTP of Lenzing AG (containing the effluents of primarily viscose fiber, pulp and paper production facilities). As the wastewater itself does not contain N- or P-containing compounds, it is necessary to add urea and phosphate before the biological treatment to ensure bacterial growth. In all the experiments described here the wastewater was taken after the addition of urea and phosphate. The wastewater also did not contain NMMO or related morpholine-like compounds. For experiments with lab- and pilot-scale WWTPs, the wastewater was mixed with NMMO to give the desired concentration.

For some lab-scale biodegradation tests wastewater from the lyocell pilot-plant ('lyocell wastewater') was used. As a result of optimization processes in the lyocell plant itself, the NMMO concentration in the wastewater varied between lower than 30 mg/l and more than 1,900 mg/l.

With the exception of lyocell wastewater, which was not supplemented with NMMO, the NMMO concentration in the influent was adjusted to 30 mg/l.

Activated sludge

For the biodegradation tests with non-adapted sludge, the inoculum was taken from Lenzing AG's WWTP which was then only partially completed. Between 1987 and 1991 only pulp and paper mill wastewaters (and a small portion of domestic wastewater) could be treated biologically. The plant was highly loaded, resulting in a sludge age of 2–3 days.

All other experiments were performed after the WWTP had been completed in 1991. Since then the plant is a 2-stage biological system and has also been treating the wastewaters of the viscose fibre production. The load is high in the first stage (sludge age of ca. 2–3 days) and low in the second stage, resulting in a sludge age of more than 14 days in the second stage. In biodegradability tests mixtures of equal portions of the sludges from the first and second stages were used.

Other microorganisms

Saccharomyces cerevisiae was taken from a commercially available baker's yeast cube (Ottakringer, Austria) and *S. carlbergensis* was obtained from a brewery (Zipf, Austria).

Anaerobic sludge was taken from the sludge digester of a WWTP which is treating municipal wastewater (situated in Lenzing, Upper Austria).

Examination of biodegradability

Biodegradability was tested according to the modified Zahn-Wellens test, as described in the OECD-Guideline 302 B (OECD 1981). The substance was mixed with an inoculum of activated sludge and the biodegradation process was followed via the loss of COD or DOC, respectively, in the solution.

The inoculum was taken from Lenzing AG's industrial WWTP. The sludge was mixed with the substance being investigated and a nutrient cocktail (KH_2PO_4, 85 mg/l; K_2HPO_4, 217.5 mg/l; $Na_2HPO_4.2H_2O$, 334 mg/l; NH_4Cl, 5 mg/l; $CaCl_2$, 27.5 mg/l; $MgSO_4.7H_2O$, 22.5 mg/l; $FeCl_3.6H_2O$, 0.25 mg/l), giving a final COD concentration in the supernatant of about 1,000 mg/l. The suspension was kept dark while being stirred for at least 28 days at room temperature. When necessary (i.e. the oxygen concentration was less than 2 mg/l) it was aerated. The samples were filtrated to separate the sludge from the liquid, and the biodegradation was monitored in the liquid phase via COD (in some cases also DOC) analysis.

Differing from the standard test method the degradation of NMMO was also studied in the presence of 600 mg/l glucose. In this case the biodegradation was observed by analysis of the substances themselves (HPLC) instead of their loss of COD/DOC.

Laboratory-scale biological treatment

Simulations of the activated sludge process were undertaken with small (7.5 l) laboratory WWTPs consisting of a 1-stage aeration basin with a built-on sedimentation unit, where the settled sludge could

Table 1. Pilot-plant studies: concentrations of NMMO, nitrate and nitrite added to the influents

	Time period [days]	NMMO concentration in influent [mg/l]		Nitrate added to influent [mg N/l]		Nitrite added to influent [mg N/l]	
		Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	Plant 2
First test series	1–48	25–35	25–35	0	0	0	0
Second test series	1–60	25–35	25–35	5	0	1	0
	61–65	0	0	0	0	0	0
	66–91	25–35	25–35	0	0	0	0
	92–112	1	0	0	0	0	0
	113–119	1	25–35	0	0	0	0
	120–136	25–35	25–35	0	0	0	0

flow back to the aeration basin. Excess sludge was removed about twice a week, the suspended solids (SS) concentration varied between 2 g/l and 4.5 g/l with an average concentration of about 3.5 mg/l. The plant was inoculated with sludge from the large-scale WWTP. The COD load was between 1 and 2 $g.l^{-1}.d^{-1}$. The devices were kept at 35-37 °C in a water bath.

For the experiments with lyocell wastewater, the wastewater was supplemented with urea and phosphate to ensure a ratio of ΔCOD:N:P of about 100:5:1.

Pilot-scale biological treatment

For investigations in a larger scale a 2-stage pilot-plant was used, which consisted of 2 identical lines with aeration basins of 300 and 1,000 l for the first and second biological stages, respectively. The plants were operated at a temperature of 22 ± 2 °C. The inoculum was taken from the large-scale WWTP, and the SS concentrations were adjusted to about 3–4 mg/l and 2.5–3 mg/l in the first and second stages, resp. The COD load was between 6 and 10 $g.l^{-1}.d^{-1}$ in the first stage and lower than 1 $g.l^{-1}.d^{-1}$ in the second stage.

The NMMO concentration was adjusted to about 30 mg/l in the influent (exceptions are given in Table 1).

To study the influence of nitrogen-containing salts on the degradation of NMMO, 5 ppm NO_3^--N and 1 ppm NO_2^--N were added to the influent of one line of the pilot-plant.

Table 1 shows the concentrations of NMMO, NO_3^--N and NO_2^--N adjusted during the different periods of the pilot-plant trials.

Tests with microorganisms other than activated sludge

Degradation experiments were also conducted with a laboratory system for anaerobic wastewater treatment and with 2 yeast cultures, *Saccharomyces cerevisiae* (obtained from commercially available baker's yeast) and *Saccharomyces carlsbergensis* (from a nearby brewery).

The anaerobic treatment took place in a completely mixed 7 l-reactor at 37 °C with a COD load of 1 $g.l^{-1}.d^{-1}$. Besides pure solutions of NMMO also mixtures of NMMO and glucose were investigated because of the possible need to have an easily accessible carbon source.

The 2 yeast strains were cultivated at room temperature in batch cultures with 500 and 1,000 mg/l of NMMO, respectively, without any addition of other nutrients. The cells were incubated in closed flasks under anaerobic conditions. In addition the influence of varying concentrations of saccharose was investigated: one batch was without saccharose, a second one with equal amounts of sugar and NMMO and a third approach with 10 g/l of saccharose.

Chemical analysis

NMMO, NMM and M were analysed by HPLC (Hewlett-Packard). The column was Hypersil, 5μm, 125 mm by 4 mm internal diameter (Seibersdorf, Austria). The mobile phase was a mixture of 52% acetonitrile and 48% of a 10 mM KH_2PO_4 solution, with a pH of 6.7. The flow rate was set to 1 $ml.min^{-1}$. The substances were detected by UV absorption at 192 nm. The detection levels were 1 mg/l for all 3 substances.

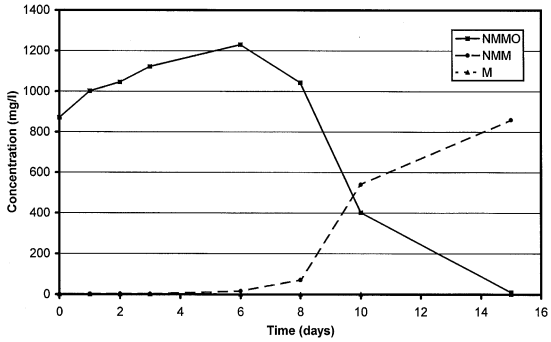

Figure 1A. Time course of NMMO, NMM and M concentrations during treatment with *Saccharomyces carlsbergensis*. Initial concentrations: NMMO: 1 g/l; saccharose: 10 g/l.

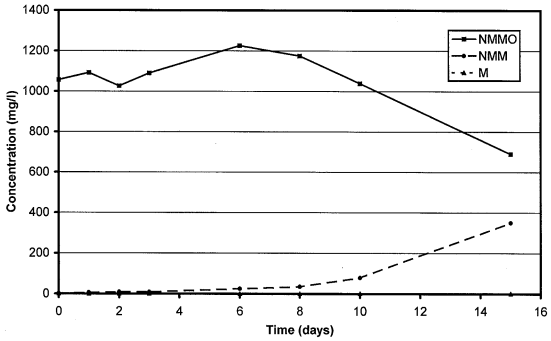

Figure 2. Time course of NMMO, NMM and M concentrations during treatment with *Saccharomyces cerevisiae*. Initial concentrations: NMMO: 1 g/l; saccharose: 10 g/l.

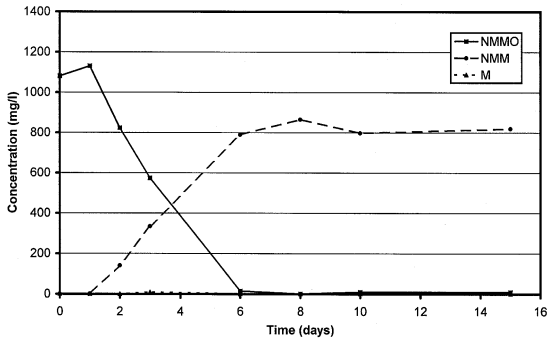

Figure 1B. Time course of NMMO, NMM and M concentrations during treatment with *Saccharomyces carlsbergensis*. Initial concentration: NMMO: 1 g/l; no sugar added.

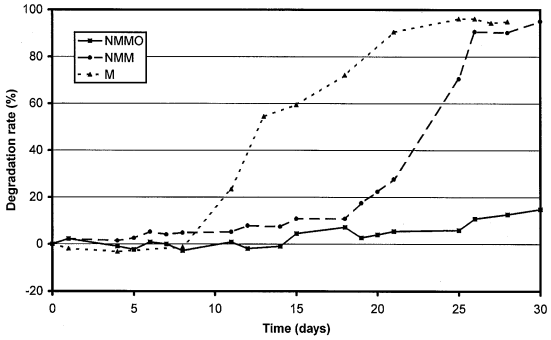

Figure 3. Zahn-Wellens test for biodegradation of NMMO, NMM and M with unadapted activated sludge.

Results

Tests with microorganisms other than activated sludge

Anaerobic wastewater treatment

Under anaerobic conditions NMMO was completely converted to NMM, but at this point the reaction stopped and no further metabolization took place. The addition of glucose had no influence on the capability of anaerobic sludge to metabolize NMMO.

Yeasts

The two yeast strains behaved differently, but none was capable of degrading NMMO within 14 days. Whereas *S. carlsbergensis* could reduce NMMO to NMM (completely), *S. cerevisiae* was not able to do even this.

The addition of saccharose did not alter the metabolic capabilities of the yeast strains, but had an influence on the kinetics of the formation of NMM by *S. carlbergensis*. Saccharose concentrations of 10 g/l significantly decreased the reduction rate of NMMO to NMM, it took about one week until NMMO concentrations began to decrease and NMM concentrations started to increase (Figure 1A). On the contrary, without saccharose the reduction process started after ca. 2 days (Figure 1B). The addition of saccharose in equal concentrations as NMMO did not alter the reduction kinetics.

S. cerevisiae did not reduce NMMO although a slight decrease of the NMMO and a slight increase of the NMM concentrations could be observed in the presence of high NMMO (1 g/l) and sacharose (10 g/l) concentrations (Figure 2). When no saccharose was added, no morpholine could be detected in the culture broth. When saccharose was present, also small concentrations of M (up to 2 mg/l) could be found after about 10 days.

Tests with activated sludge

Biodegradability

As shown in Figure 3, in the first test with non-adapted sludge (in 1989, with sludge from the first erection

state of the WWTP) NMMO was to be classified as a persistent substance according to the OECD guidelines. In 28 days, less than 20% of the initial COD had been degraded.

The results of the biodegradability test of N-methyl-morpholine (NMM) are also shown in Figure 3. It is of interest that NMM was almost completely degraded in 28 days, but showed a long lag period of about 20 days until the degradation started indicating the necessity of an adaptation phase.

Morpholine was degraded much better than NMMO or NMM. The COD-removal started after a lag period of about one week and reached 90% within 3 weeks (Figure 3).

Lab-scale experiments

The data of the static degradation tests indicated that a complete biodegradation of NMM can be achieved when the sludge is allowed to adapt and the sludge age is high enough to ensure that the adapted organisms cannot be washed out.

The experiments with anaerobic sludge and *S. carlsbergensis* showed a quantitative reduction of NMMO to NMM, but no further degradation could be achieved.

As a consequence the complete biodegradation of NMMO seemed to be possible, because NMMO can be reduced to NMM on one hand, and NMM again can be completely biodegraded on the other, both steps carried out via biological reactions.

This hypothesis could first be substantiated by laboratory-scale experiments with small activated sludge plants, in which the sludge age was ensured not to be shorter than 15 days. After an adaptation period of about 14 days, the NMMO, NMM and M contents in the effluent could be reduced to concentrations below their detection levels.

However, since NMMO had been added only in minor concentrations to the wastewater used for these tests, it was not possible to tell if there was an influence of the wastewater itself on the adaptation or if there even was a need for additional wastewater streams to get the process working. To investigate this question parallel tests were run with

a) wastewater from the lyocell pilot-plant with adapted sludge,
b) wastewater from the lyocell pilot-plant with non-adapted sludge (from a domestic WWTP) and
c) a mixture of wastewater from the lyocell pilot-plant and domestic wastewater (in ratios of 1:4–1:6), inoculated with non-adapted sludge.

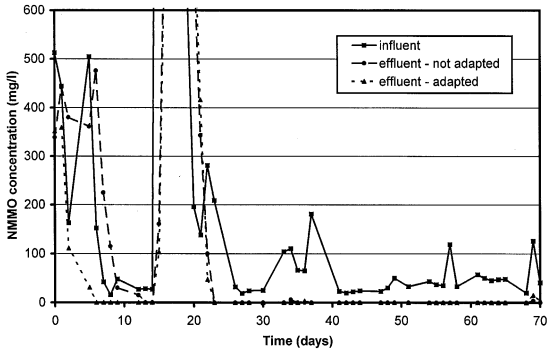

Figure 4. Lab-scale treatment of Lyocell wastewater. Two plants were run parallel with the same influent, one using adapted, the other one unadapted sludge.

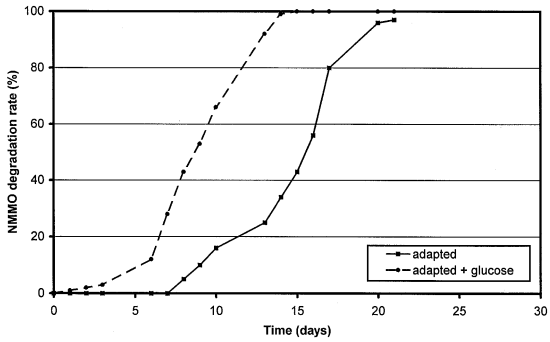

Figure 5. Zahn-Wellens test for biodegradation of NMMO, NMM and M with adapted activated sludge. The degradation can be accelerated by the addition of glucose (600 mg/l).

The results for plants a) and b) are shown in Figure 4. Plant a) and b) differed only during the first 14 days. After the adaption period the plants ran exactly parallel showing that the adaptation is independent of other wastewater streams, because plants a) and b) were equally efficient in reducing the NMMO concentration as plant c).

Biodegradation tests with adapted sludge

Applying the same test method that had shown a NMMO biodegradation below 20% COD with unadapted sludge, it could be shown that NMMO can be completely biodegraded with adapted sludges (Figure 5). The only difference to the initial test was that it was inoculated with sludge having been adapted to NMMO in a small (laboratory-scale) activated sludge plant prior to the test.

Although the biodegradation can be accelerated by the addition of glucose as a readily biodegradable co-substrate, NMMO was also completely biodegraded when it was the sole carbon source. Differing from

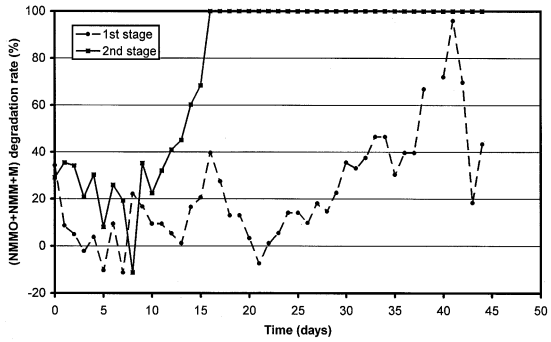

Figure 6. Time course of the biodegradation rate of NMMO, NMM and M in pilot-scale treatments of NMMO-containing wastewater in each of the two biological stages. The degradation rate refers to the sum of the 3 compounds.

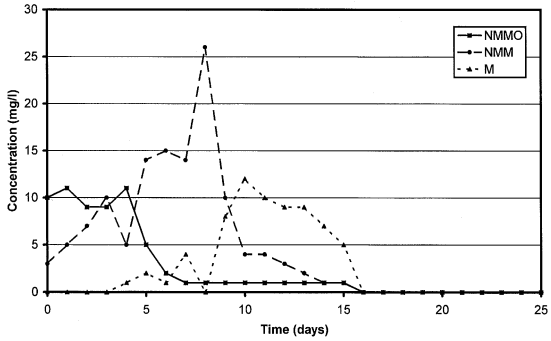

Figure 7. Close-up of the adaptation phase to NMMO-containing wastewater of activated sludge in the second biological stage of a pilot-WWTP.

standard degradation tests, the degradation was followed by substrate-specific analysis of NMMO, NMM and M via HPLC in addition to COD and TOC measurements to ensure not to determine the glucose degradation instead (Figure 5).

Pilot-plant studies

The biodegradation of NMMO was examined in 2 long-term studies lasting several months each.

The first series of experiments was undertaken to confirm the results of the benchscale studies in a larger scale and under conditions being closer to the large-scale plant. After an adaptation period of about 2–3 weeks with varying amounts of NMMO, NMM and M being measured in the effluent, the biodegradation of NMMO and its metabolites was very stable. Figure 6 shows the overall degradation rate for NMMO, NMM and M. Once the sludge had been adapted, measurable amounts of NMMO, NMM and M could only be detected in exceptional cases.

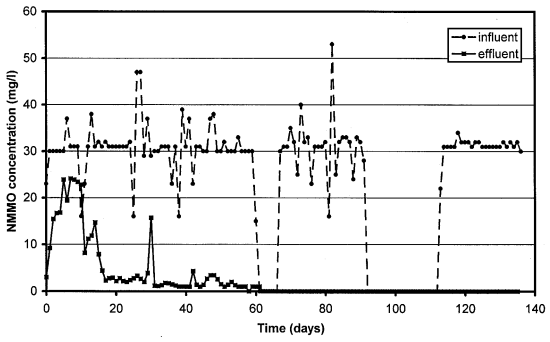

Figure 8. Time course of the NMMO concentrations in the influent and effluent (2^{nd} stage) of a pilot-WWTP treating NMMO-containing wastewater (plant 1).

It is interesting to have a closer look upon the adaptation period, especially the effluent of the second biological stage (Figure 7): only a few days after start-up the NMMO concentration decreased while there was an increase in NMM concentrations. Some days later the NMM concentrations decreased with a parallel increase of M, until after completion of the adaptation none of the 3 compounds could be measured any longer.

The second set of experiments was aimed to examine the ability of the sludge to retain the adapted status over a period of several weeks without any NMMO or its metabolites being present in the wastewater, thus simulating a short-term cessation of the fiber production (for repair, service, etc.). In addition, it was intended to study the fate of NMMO in the presence of other nitrogen containing compounds. To answer the second question, nitrate and nitrite were added to the influent of one line. As the wastewater itself, which was used as influent, contained practically no nitrogen the second line could be used as a reference.

The existence of nitrogen salts in the influent also had an influence on the degradation rates of NMMO. With nitrate and nitrite present the concentration of NMM in the first biological stage was constantly lower (and the NMMO concentration higher) than in the absence of nitrogen salts. The relatively high concentrations of nitrite, which were in some cases even higher than the nitrate concentrations, indicated that nitrate had been reduced instead of NMMO.

To examine the stability, both lines were run parallel without any addition of N-compounds except NMMO (and urea) (Figures 8 and 9). First, the addition of NMMO was interrupted for 5 days after the plant (both lines) had been adapted to NMMO. Then the sludges were allowed to recover for 25 days (with

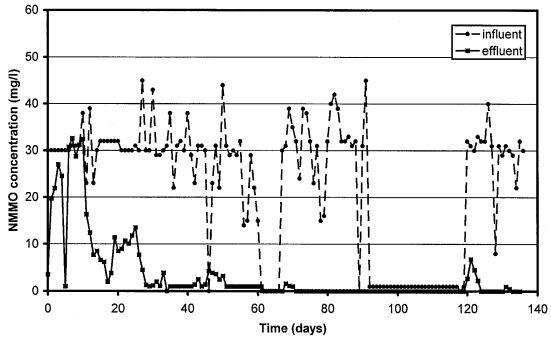

Figure 9. Time course of the NMMO concentrations in the influent and effluent (2^{nd} stage) of a pilot-WWTP treating NMMO-containing wastewater (plant 2).

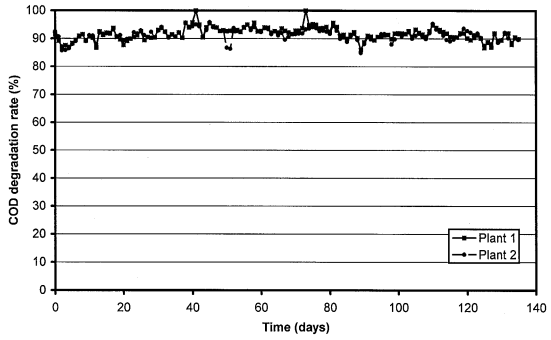

Figure 10. Time course of the COD degradation rate of two pilot-WWTPs treating NMMO-containing wastewater.

the normal NMMO dosage), and after that the NMMO addition was completely stopped in one line for 3 weeks and decreased to 1 mg/l for 4 weeks in the other line.

After a complete interruption of the NMMO addition for 3 weeks, no increase of the NMMO, NMM and M effluent concentrations could be observed when the NMMO influent concentration was elevated to its normal value (30 mg/l). Even the sludge of the first biological stage (which had a short sludge age) was immediately capable to reduce NMMO to NMM again.

The other line, in which the NMMO influent concentration had been decreased to 1 mg/l for 4 weeks showed just a slight and short-term increase of the NMMO and NMM effluent concentrations after the raising of NMMO in the influent to its starting level.

COD removal

For all the pilot-plant and most of the lab-scale tests mainly wastewater from the viscose fibre, pulp and paper production was used. It was therefore of interest to see if the overall COD degradation was influenced by the NMMO added (Figure 10). The COD decrease was slightly lowered during the first, say, 3 weeks, because of the adaption process. During this phase, NMMO and/or its metabolites were not mineralized completely, leading to higher effluent COD concentrations. After completion of the adaptation, the COD removal efficiency was $> 90\%$ in both lines of the pilot-plant indicating that there was no negative influence of NMMO on the other wastewater compounds.

Discussion

Toxicity of NMMO, NMM and M on activated sludge and aquatic organisms

Concentrations of NMMO, NMM and M, as used in the tests, showed no significant adverse effects on the degrading biocoenosis. Concerning morpholine, this finding is in accordance with the results of Strotmann et al. (1993). Knapp et al. (1982) also did not observe any toxic effects of morpholine on bacteria, even in concentrations up to 8.7 g/l. Mazure and Truffaut (1994) concluded from their experiments that the toxicity threshold of morpholine would lie between 5 and 7 g/l for *Mycobacterium aurum* MO1 in a fed-batch culture, but the observed decrease of the degradation rate was rather caused by the accumulated ammonia than by morpholine.

On the other hand, Calamari et al. (1980) showed that there is some toxic effect of morpholine on aquatic organisms, especially algae (*Selenastrum capricornutum*, 96hEC50: 28 mg/l), indicating the need for the elimination of morpholine from wastewaters prior to their emission to a receiving river.

Adaptation to NMMO

The investigations showed that conventional aerated sludge plants can completely degrade NMMO and its ring-structured metabolites NMM and M, provided that the sludge has a sufficient sludge age and had been adapted to NMMO.

The adaptation takes place during the first three weeks of operation, leading to a constant biodegradation of NMMO and its metabolites to levels below their detection limits. Having been adapted, the sludge organisms retain their ability to degrade NMMO for several weeks, even if no NMMO is present in the wastewater to be treated.

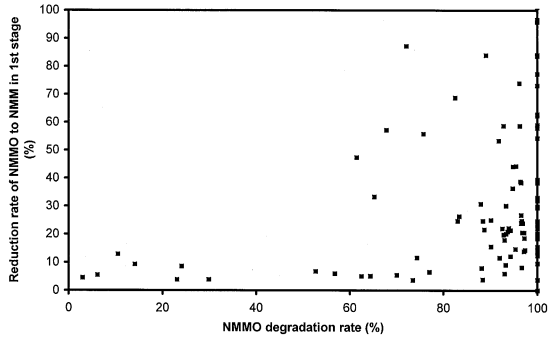

Figure 11. Dependence of the overall NMMO degradation rate in a 2-stage WWTP on the reduction rate of NMMO to NMM in the first biological stage.

The presence of NMM in the effluent of the first biological stage does not seem to be necessary for already adapted sludges to retain their biodegrading capabilities. High efficiencies in the biodegradation of NMMO were also observed in cases when nearly no NMMO had been reduced to NMM in the first stage (Figure 11). Therefore, it is not necessary to have a separated zone for the reduction of NMMO to NMM, the reduction and the subsequent steps of the biodegradation process can also run simultaneously. This corresponds to the fact that also 1-stage completely mixed WWTPs (lab-scale) could be adapted to the biodegradation of NMMO.

The most important prerequisite for a successful adaptation is a high enough sludge age to ensure that also slowly growing microorganisms can establish themselves in the biocoenosis. Similar observations were made by Watson (1993), who studied the effect of two different acclimation procedures on the biodegradation of substances known not to be degraded rapidly or easily. The enrichment procedure, which is a standard procedure for acclimating pure cultures of microorganisms to problematic substances, produced sludges much worse in their biodegradation capacities than the single-flask method. One reason for this phenomenon is that, due to repeated dilutions every 2 to 3 days, slowly growing microbes are eliminated in the enrichment procedure.

The adaptation process could be studied in the (2-stage) pilot-plant experiments. It took only a few days ($<$ 1 week) until more than 95% of the added NMMO was eliminated, nearly all of it in the first biological stage. This elimination was not yet a biodegradation, but only a reduction to NMM, which was followed by an adaptation of the slowly growing organisms of the second biological stage to NMM, resulting in a true biodegradation of NMMO, NMM and M.

Once adapted, the sludge is capable of biodegrading NMMO, NMM and M even after a few weeks cessation of the NMMO dosage. It is not necessary to have at least a small amount of NMMO in the influent to keep the adaptation intact over periods of standstills. Of course, the time during which the sludge loses this capability depends on the actual sludge age, but the investigation of this dependence was not a subject of this study.

Adaptation to M

Static biodegradation tests showed that even activated sludges with a low sludge age were able to degrade M without any or with only a very short adaptation period. At first sight this result seems to contradict the findings by Strotmann et al. (1993) that an adaptation period of 1 to 3 weeks was necessary before degradation occurred. As Strotmann et al. (1993) could not observe any difference between adapted and unadapted sludges in the OECD screening test which uses very low concentrations of the inoculum, one possible reason for the lag period could be that even the adapted sludges contained only very low absolute counts of morpholine degraders and made an increase of their relative portion necessary, before the degradation could become visible.

Knapp and Whytell (1990) tested river waters and activated sludges from different sources and found morpholine degrading microbes in almost all of these probes although with varying numbers and lag times in die-away tests. The results indicate that morpholine degraders are present almost ubiquitously, but due to their low growth rate, their counts very much depend on the environmental conditions, especially the solids retention time. As a consequence, it should be rather easy to establish an activated sludge capable of morpholine biodegradation if there is enough substrate and the sludge age is high enough to ensure that the organisms cannot be washed out. Our experiments confirmed this assumption because in every case the adaptation succeeded and took only about 2–3 weeks when the environmental connditions met the above requirements.

Biodegradation of NMMO, NMM and morpholine

Laboratory-scale experiments and, more clearly, the first trials in the pilot-plant suggested that the biodegradation is a sequential process including first

the reduction of NMMO to NMM, then the demethylation of NMM to M and finally the cleavage of the morpholine ring.

The first clear indication of the biodegradability of this type of chemical structures was the fact that – after an adaptation period – NMM could be degraded in a static biodegradibility test. The demethylation of NMM turned out to be the most critical step (see below). Once NMM has been demethylated, the further biodegradation should take place without further problems.

The first step is the reduction of NMMO. In some cases this first step took place even when influent solutions stood at room temperature without agitation. A reduction of NMMO could easily be achieved with anaerobic bacteria and some yeast strains. Yet, they stopped at NMM and no further biodegradation was obtained under anaerobic conditions.

Although it could be assumed that the NMMO reduction takes place in anoxic areas of the first biological stage, the existence of anoxic regions in the WWTP is not a necessary requirement for a complete degradation of NMMO, because NMMO was also degraded in 1-stage completely mixed WWTPs or when it was injected directly into the first biological stage of the pilot-plant. The fast-growing bacteria of this stage are capable of reducing NMMO, so it does not seem to be necessary to have sludge ages longer than ca. 1–2 days for this first biodegradation step.

The most critical step is the demethylation of NMM. The first indications of the need for a long adaptation phase, which could be concluded from the static biodegradation tests, were supported by the pilot-plant experiments. NMM was the only substance (out of NMMO, NMM and M), which was found in measurable concentrations in the effluent of the first biological stage over the whole test period. The sludge age has to be high enough to enable the organisms which are capable of performing that step to survive in the system. It was not intended to determine the exact sludge age needed, but it should not be shorter than about two weeks.

Once morpholine has been produced the further biodegradation takes place rather rapidly. The biodegradability of morpholine has been shown by various authors (see below).

This 3-step biodegradation process could be clearly seen during the adaptation period in the second biological stage of the pilot-plant. During the first few days NMMO still occurred in the effluent, followed by a decrease of the NMMO concentration and an increase of NMM. Adaptation to NMM resulted in decreasing NMM concentrations and the appearance of morpholine, which again disappeared within a few days. It is obvious that microbiological adaptation to a certain substance can only take place, when this substance is present in concentrations high enough to be worth the adaptation. That is why the adaptation to NMM could not start immediately after beginning of the NMMO dosage, but only after NMMO had been reduced to NMM. For the same reason the adaptation to M was only possible after the sludge had been adapted to NMM, so that M appeared in the aeration basin. As a consequence, the adaptation to M began with a delay of a couple of days, but was concluded within a few days.

Morpholine is much better biodegradable than NMM or NMMO, its biodegradability has been examined extensively by various authors. It seems that only Gram-positive bacteria can use morpholine as a sole source of carbon, most of these are mycobacteria (*Mycobacterium* spp.) (Cech et al. 1988; Knapp & Brown 1988; Brown & Knapp 1990), another one is thought to be an *Arthrobacter* sp. (Dmitrenko et al. 1985). The ability of activated sludge organisms to degrade morpholine has been shown by Brown (1988). It could be shown by Mazure (1993) and Knapp et al. (1996) that also Gram-negative bacteria, most of them members of the genus *Pseudomonas*, are able to degrade morpholine, but they can use it only as a nitrogen, not as a carbon source.

Lamant and Jaffrin (1996) established a model for the biodegradation of morpholine using a mixed culture of nine bacteria and two yeasts. The model was first deduced from discontinuous and continuous cultures and then also extended to a membrane bioreactor. The authors concluded that the coupling of a microfilter (for biomass separation) with a bioreactor could raise the rate of morpholine biodegradation because of the possibility to maintain a higher biomass concentration and to avoid the washout of organisms growing more slowly. A similar acceleration of the degradation rate can also be achieved by the immobilization of the biomass (Mazure & Truffaut 1994; Poupin et al. 1996).

Starting with sludge from an industrial biological wastewater treatment plant, Knapp et al. (1982) isolated two microorganisms capable of utilizing morpholine as the sole source of carbon, nitrogen and energy, called Mor G and Mor D. Strain Mor G was also tested for growth on some heterocyclic secondary

amines, among them N-methylmorpholine, but no growth occurred with this substance.

Contrary to the results presented here and by many other authors (see above), Calamari et al. (1980) could not observe any biodegradation of morpholine.

Degradation of morpholine should result in a cleavage of the ring structure. Knapp et al. (1982) showed that growth of the *Mycobacterium* strain Mor G, which seems to be identical to *Mycobacterium chelonae*, on morpholine also induced the catabolism of pyrrolidine and postulated that ethanolamine, acetaldehyde and glycolate were likely intermediates of the degradation of morpholine. Based on the pathway for pyrrolidine catabolism, which was elucidated for *Pseudomonas fluorescens* by Jacoby and Fredericks (1959) and seems to be the same in M. chelonae, Swain et al. (1991) proposed a hypothetical pathway for morpholine degradation. At the later stages the pathway divides into two different routes, one leading to acetyl-CoA via ethanolamine, the other one goes to glycerate via glycolate. Mazure and Truffaut (1994) tested growth of *Mycobacterium aurum* MO1 on different compounds of the two pathways and concluded that the glycolate route seems to be the first involved in a culture starting with a high morpholine concentration, but switches towards the ethanolamine route at lower morpholine concentrations (0.1 g/l).

Influence of sludge age

The results presented here demonstrate the dependence of NMMO-, NMM- and M-degrading organisms on the sludge age or solids retention time (SRT). While it was not possible to adapt activated sludge with a mean sludge age of about 2–3 days to the degradation of NMMO, the adaptation could be achieved not only in the 2-stage pilot plant with a low-loaded second biological stage, but also in 1-stage lab-scale plants after increasing the sludge age to about 15 days or longer. As morpholine-degraders and other microorganisms responsible for the degradation of various xenobiotics are slowly growing organisms (Chudoba et al., 1989), they cannot be established in high-loaded systems with low sludge ages.

Similar results were achieved by Cech and Chudoba (1988), who investigated the role of SRT on the biodegradation of substances known from the literature to be non-biodegradable or only slowly biodegradable, among them also morpholine. Cech and Chudoba (1988) observed a minimum SRT of 3 days to ensure that morpholine-degrading organisms are not washed out, and the authors were never successful when they tried to adapt sludge with an SRT of 5 days or shorter to morpholine. By comparing the degradation in a system containing only morpholine with a second system, which additionally contained peptone, Cech and Chudoba (1988) also observed that morpholine was degraded by organisms different from those degrading peptone and that the SRT has to be above 10 days if morpholine is used as a single substrate. According to Chudoba et al. (1989), the critical solids retention time, below which the maximum volumetric removal rate of a substance is lower than the volumetric loading and a tested compound behaves as only partially degradable or nondegradable, is 3.8 days for morpholine.

Maintenance of the ability to degrade NMMO

Mycobacteria, which are the most prominent morpholine degraders, are known to lose this ability when morpholine is deleted from the influent to an activated sludge plant (Brown & Knapp 1990). This phenomenon, which seems to be due to phenotypic instability, was investigated by Brown et al. (1990). They observed that the ability to degrade morpholine is lost at a high rate suggesting that the ability could be plasmid encoded. The involvement of plasmids in coding for morpholine degradation by mycobacteria was also concluded from the studies of Waterhouse et al. (1991). On the other hand, Brown and Knapp (1990) reported that the phenotype which is able to degrade morpholine was rather stable under the conditions of an activated sludge plant, because the sludge could degrade morpholine even during a 220 d period when morpholine was absent from the influent. But due to a decline in the specific population of morpholine-degrading microorganisms the lag period increased before morpholine degradation was detected in a die-away test.

The results of this study fully correspond to the findings by Brown and Knapp (1990). It could be shown that sludge organisms can retain their ability to degrade NMMO - and therefore also morpholine - during periods of several weeks without any or with only very low concentrations of NMMO present in the influent.

Necessity of cosubstrates

Although NMMO can be degraded when no other biodegradable substances are present, static biodegradation tests showed a significant increase in the

biodegradation rate when an easily biodegradable substrate (glucose) was present in the test solution. According to the studies by Knapp et al. (1996) a group of microorganisms, which is present in many samples of water, can degrade morpholine but only when provided with an additional carbon source. Thus, the degradation can be accelerated when also microbes, which are not able to use morpholine as a carbon source, take part in the biodegradation process. It could be shown by Mazure (1993) and Knapp et al. (1996) that also Gram-negative bacteria, most of them members of the genus *Pseudomonas*, are able to degrade morpholine, but they can use it only as a nitrogen, not as a carbon source. Knapp et al. (1996) suggested that co-metabolism of morpholine may occur with environmental microorganisms.

The second set of experiments in the pilot-plant, when nitrate and nitrite were added to the influent of just one of the two lines, showed that there is an influence of nitrogen compounds on the biodegradation of NMMO and its metabolites.When nitrate was present, the reduction rate of NMMO to NMM decreased. Obviously, nitrate was reduced instead of NMMO, which could also be concluded from the elevated nitrite levels, which were in many cases higher than the nitrate concentrations. It seems obvious that nitrate inhibits or slow down the reduction of NMMO to NMM.

Conclusions

NMMO has been shown to be a well suited solvent for the production of lyocell fibres. This study has demonstrated that the biological degradability does not restrict the use of NMMO because conventional WWTPs can easily be adapted to the biodegradation of NMMO. If the sludge age is higher than about 10–14 days (at least in the second biological stage), the complete biodegradation of NMMO can be expected.

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