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Mechanisms and Pathways of Aniline Elimination from Aquatic Environments†

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The fate of aniline, a representative of arylamine pollutants derived from the manufacture of dyes, coal liquefaction, and pesticide degradation, was comprehensively evaluated by use of unpolluted and polluted pond water as model environments. Evaporation plus autoxidation proved to be minor elimination mechanisms, removing ca. 1% of the added aniline per day. Instantaneous binding to humic components of a 0.1% sewage sludge inoculum removed 4%. Biodegradation of aniline in pond water was accelerated by the sewage sludge inoculum. A substantial portion of the degraded aniline carbon was mineralized to CO_2 within a 1-week period, and microbial biomass was formed as a result of aniline utilization. Biodegradation was clearly the most significant removal mechanism of polluting aniline from pond water. A gas chromatographic-mass spectrometric analysis of biodegradation intermediates revealed that the major pathway of aniline biodegradation in pond water involved oxidative deamination to catechol, which was further metabolized through *cis,cis*-muconic, beta-ketoadipic, levulinic, and succinic acid intermediates to CO_2. Minor biodegradation pathways involved reversible acylation to acetanilide and formanilide, whereas *N*-oxidation resulted in small amounts of oligomeric condensation products.

Aniline and other aromatic amine pollutants originating from pesticides (3, 5), the manufacture of dye (1), and coal liquefaction (29) are subject to unusually complex environmental transformations. In addition to ultimate biodegradation, their microbial metabolism also results in acylation (20, 21, 30, 32, 35), polymerization (5), and nitro products (19, 20). Physicochemical processes, such as evaporation, autoxidation (25, 26), photooxidation (24, 28, 36), and chemical binding reactions (4), further complicate their fate.

Several microbial cultures have been found to be capable of growing on aniline (2, 11, 27, 33) and 4-chloroaniline (9, 14, 30). 3,4-Dichloroaniline is mineralized by *Pseudomonas putida* in the presence of aniline as a primary substrate (35). Both aniline (2, 33) and 3,4-dichloroaniline (35) were subject to dioxygenase attack, resulting in oxidative deaminations to the corresponding catechols. The subsequent biodegradation steps followed that of catechol or chlorocatechols, respectively. In addition to the biodegradation pathway described above, numerous side reactions were reported to affect various substituted anilines. These included acylation (20, 30, 32) and oxidation of the aniline to phenylhydroxylamine (19, 20), nitrosobenzene, or nitrobenzene (19, 20). Dimerization and polymerization reactions of some of the oxidation products described above, with a subsequent formation of azo (5, 36), azoxy (19, 20), and phenoxazine (9, 14) products, have been reported.

Studies on pesticide-derived substituted anilines have elucidated how the metabolic transformations described above and the reactions of the substituted anilines with soil humus influence the overall fate of these residues in the soil environment. However, with the exception of two recent studies on photodegradation (24, 36), the mechanisms of aniline elimination from natural surface waters have not been explored. Using unsubstituted aniline as a test compound and pond water or pond water with a sewage sludge inoculum as models of uncontaminated or contaminated surface waters, respectively, we conducted tests to assess the roles of evaporation, autoxidation, chemical binding, and microbial metabolism in the removal of this industrial pollutant.

MATERIALS AND METHODS

Chemicals. Aniline was purchased from Aldrich Chemical Co. and was purified by distillation. Other compounds that served as standards had a purity of 97 to 99% and were also obtained from Aldrich. Solvents were pesticide grade or high-performance liquid chromatographic quality.

Measurements of aniline removal. Water was collected during the months of August and September immediately before the experiments from a shallow eutrophic pond (pH 6.9 to 7.1) located on the campus of Cook College, New Brunswick, N.J. Aniline or acetanilide (1.25 or 12.5 mg) were added in small amounts of acetone to dry 250-ml Erlenmeyer flasks with steel cap closures. The solvent was evaporated under a stream of N_2 before the addition of 50 ml of fresh pond water. Before use, the pond water was strained through cheesecloth to remove filamentous algae and larger pieces of detritus. Microbial biomass was determined as protein by the procedure of Lowry et al. (22). For this measurement and for some biodegradation experiments, biomass was concentrated twofold by passing the pond water through a 0.45-μm pore-size Millipore filter. Some flasks also received 0.05 ml (0.1% v/v) of fresh, settled, activated sewage sludge (Raritan Valley Sewerage Authority Treatment Plant, Bridgewater, N.J.), adding 0.4 mg of protein (1.6 mg [dry wt]) to the original biomass of the pond water sample. The final concentrations of aniline or acetanilide in pond water were 25 or 250 μg ml^{-1}. The flasks were incubated on a rotary shaker (200 rpm) at 20°C and in the dark to exclude the effect of photodegradation. Steam-sterilized pond water with filter-sterilized aniline served as

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the control for aniline disappearance by autoxidation, evaporation, and chemical binding.

At time intervals, triplicate flasks were removed for analysis of residual aniline, acetanilide, and metabolites that were formed. Each flask was extracted three times with 50-ml fractions of *iso*-octane. After acidification to pH 1.0, the aqueous phase was similarly extracted with three portions of diethyl ether. Each solvent phase was pooled, dried over anhydrous Na_2SO_4, evaporated to near dryness on a flash evaporator at 45°C, and subsequently analyzed by gas chromatography (GC). Time zero extractions yielded a 96 ± 4% recovery efficiency for aniline by this procedure. Autoxidation plus evaporation losses were measured in a similar manner, but sterile deionized water or a mineral salts solution (35) were used as aseptic incubation environments.

Evolution of CO_2 during aniline biodegradation was measured in 1-liter Gledhill flasks (17) containing 250 ml of pond water, with or without 0.1% sewage sludge, and aniline at 250 μg ml^{-1}. CO_2 evolution was measured daily as described by Gledhill (17) and was corrected for by determination of CO_2 evolution from controls with no aniline added. Incubation was at 20°C with shaking and in the dark.

Aniline evaporation was measured under similar conditions, but the Gledhill flasks contained 250 ml of sterile distilled water containing 1 mg of filter-sterilized aniline per ml. Evaporating aniline was trapped in 10 ml of 1 N H_2SO_4, which replaced the alkali in these experiments. The trapping solution was retrieved for analysis by the Bratton-Marshall reaction (8) at 2-day intervals.

Removal of aniline by binding to dissolved or suspended humic materials was determined by the distillation technique of Bleidner et al. (6). After solvent extractions were carried out as described above, an entire 50-ml water sample was transferred to a 500-ml round-bottom flask with an equal volume of 12.5 N NaOH (31) and processed in the Bleidner apparatus (6) for 9 h. The *iso*-octane fraction was dried over anhydrous Na_2SO_4 and concentrated on a flash evaporator to a final volume of 1 ml, and its aniline content was measured by GC.

Chromatographic and spectrophotometric determinations. GC analyses were performed on a model 5700A Hewlett Packard Instrument equipped with flame-ionization detectors and dual stainless steel columns (ca. 0.31 by 183 cm) packed with 3% OV-17 on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Operating conditions were as follows: N_2 carrier, 30 ml min^{-1}; detector, 250°C; oven: linear temperature program of 50 to 200°C at 4°C min^{-1}. A Hewlett Packard model 5985 gas chromatograph-mass spectrometer interfaced with a computer was used for obtaining mass spectra at an electron impact of 70 eV. *iso*-Octane and ether extracts were analyzed directly or were methylated before chromatographic separations and mass spectrometric (MS) analyses. Methylation was performed with ethereal diazomethane, generated from *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) by using an MNNG-diazomethane kit (Aldrich) (16).

Preparative thin-layer chromatography was performed on precoated 500-μm silica gel plates (Fisher Scientific Co., Pittsburg, Pa.). Solvent systems were (i) chloroform-ethanol (9:1 [vol/vol]), (ii) hexane-benzene-acetone (7:3:1 [vol/vol]) or (iii) *iso*-octane–ethyl acetate–acetic acid–water (11:5:2:10 [vol/vol/vol/vol]). In some cases, when solvent system (i) was used, the spots eluted from the plate were dissolved in 0.1 ml of diethyl ether and were analyzed by GC. If not homogeneous, they were subjected to an additional two-dimensional thin-layer chromatography separation step by solvent systems (ii) and (iii). Spots were visualized under UV light (366 nm) or by a 0.1% ethanolic bromocresol purple spray. If the spots were to be purified further, parallel chromatograms were run, one-half of the plate was sprayed, and the areas corresponding to the visualized spots were scraped from the other half of the plate.

In a few cases, preliminary separation of metabolites by thin-layer chromatography with solvent system (i) was followed by high-performance liquid chromatography. In such cases, spots from the thin-layer chromatographic plates were eluted with a small amount of diethyl ether. High-performance liquid chromatographic separations were performed on a model SP 8000 chromatograph (Spectra Physics, Santa Clara, Calif.) equipped with SP 8310 double beam UV-visible detectors set at 254 nm. All runs were made on a Spectra Physics RP-8 column (250 by 4.6 nm) by gradient elution with methanol-water (15:85) and acetonitrile-water (40:60) solvent systems.

RESULTS

Mechanisms of aniline elimination. In sterile and nonsterile sewage sludge-supplemented pond water, a small part (3 to 5%) of the added aniline became solvent inextractable by binding to humic substances in the sewage sludge inoculum. No removal of aniline by binding was detected in pond water without added sewage sludge. The binding to sewage sludge humus occurred during the first day of incubation and showed no clear trend of increase or decrease during the subsequent 6 days of the experiment (Table 1). The fact that this disappearance occurred by binding rather than by some other mechanism was demonstrated by the recovery of the bound aniline by Bleidner distillation (6, 34).

Aniline was removed from sterilized pond water, a mineral salts solution, or distilled water at apparently linear rates by the two processes of evaporation and autoxidation. Over a 2-week period, a daily average of 0.4 and 0.5% of the added aniline was removed by evaporation and autoxidation, respectively (Table 1). As compared with biodegradation, each of the mechanisms of aniline removal from pond water described above was of minor significance.

The time course of biodegradation of 250 μg of aniline per ml in pond water, with and without a sewage sludge inoculum, is shown in Fig. 1. A strong acceleration of aniline biodegradation was evident as a consequence of the sewage sludge inoculum. During the rapid initial biodegradation phase in sewage sludge-inoculated pond water, acetanilide was synthesized, but this acylation product decreased to very low levels by day 5. At 25 μg ml^{-1}, the time course of aniline biodegradation was very similar (data not shown). The evolution of CO_2 from aniline at 250 μg ml^{-1} is shown in Fig. 2 and corresponds well to the aniline disappearance patterns shown in Fig. 1. Background CO_2 evolution from pond water and pond water with sewage sludge inoculum was 25 and 20% (data not shown) of the respective CO_2 evolutions with 250 μg of aniline added per ml. The curves in Fig. 2 are corrected for the respective background CO_2 evolutions and thus represent net CO_2 production from the utilization of aniline.

Table 1 provides a balance sheet for the fate of aniline in sewage sludge-inoculated pond water during a 1-week incubation period. The biodegradation of aniline itself was virtually complete by day 4. The last traces remaining appeared to be protected from biodegradation by some mechanism (e.g., by binding to humus) that was reversible by solvent extraction. CO_2 evolution continued beyond the 4-day peri-

TABLE 1. Balance sheet of aniline (250 μg ml^{-1}) transformations in sewage sludge-inoculated pond water

Day	μmol in pond water[a]						μmol of biomass incorporation[e]
	Aniline	Acetanilide	Biodegradation products of aniline[b]	Evaporated plus autoxidated aniline[c]	Bound aniline	CO_2[c,d]	
0	134						
1	43 ± 3.5	19	11	1	5	15 ± 0.5	40
2	26 ± 5.0	10	9	2	3	20 ± 0.3	63
3	18 ± 1.5	6	11	3	3	25 ± 0.3	73
4	5 ± 0.7	3	17	4	4	28 ± 0.4	69
5	6 ± 1.1	3	16	5	4	32 ± 0.4	69
6	6 ± 0.6	3	9	6	4	35 ± 0.7	71
7	5 ± 0.6	3	7	7	4	38 ± 1.2	70

[a] Values represent the average of triplicate measurements. Standard error is noted only for the most significant columns of data.
[b] Estimate based on GC peak areas of pooled and derivatized samples.
[c] Cumulative values.
[d] Micromoles of CO_2 were divided by six, the number of carbons in aniline.
[e] Not a direct measurement, calculated by difference.

od, although at a decreasing rate, reflecting the utilization of catabolic intermediates and biomass. The aniline carbon not accounted for by the actual analytical measurements was 30 to 52%. Most, if not all, of this is attributed to an incorporation of aniline carbon into the cell biomass and corresponds to average incorporation efficiencies.

In pond water without sewage sludge inoculum, the disappearance of aniline was slower (Fig. 1 and 2). Acetanilide and some other biodegradation intermediates were not detected, but otherwise, the balance sheet of this experiment (data not shown) was very similar to the data shown in Table 1. To ascertain that our inability to detect acetanilide was not due to the slower rate of aniline biodegradation, in one experiment (Table 2) we concentrated pond water biomass twofold. In this case, the time course of aniline biodegradation and CO_2 evolution were comparable with sewage sludge-inoculated pond water (Table 1), yet acetanilide was not detected. Biomass protein more than doubled due to aniline utilization during the first 5 days of the experiment. With no added aniline, the biomass declined.

In untreated pond water, biomass protein ranged between 90 and 117 μg ml^{-1}. Sewage sludge inoculum added 8 μg of biomass protein per ml. As evident from both aniline depletion (Fig. 1) and CO_2 evolution (Fig. 2), the microbial community of sewage sludge exhibited aniline biodegradation activity per microgram of biomass protein that was at least one order of magnitude higher than that in the microbial community of pond water.

Acetanilide, added at 25 or 250 μg ml^{-1}, was rapidly utilized in pond water (Table 3). Biomass yields on acetanilide were comparable with those obtained by using aniline as growth substrate (Table 2).

Intermediates and pathways of aniline degradation. The study of biodegradation pathways in environmental samples is complicated by low concentrations of intermediates and interference by unrelated metabolic products. Nevertheless, GC-MS evidence for several metabolic intermediates demonstrated the operation in pond water of several previously established aniline transformation pathways. The same metabolic intermediates were not detected in methylated solvent extracts of pond water with or without sewage sludge inoculum, unless they were supplemented with aniline.

The MS evidence for the most important metabolic products generated from aniline in sewage sludge-supplemented pond water is presented in Fig. 3. Evidence for acetanilide (Fig. 3A) was the molecular ion (M^+) at *m/e* 135 with major fragments at *m/e* 93, 66, 65, and 43. The fragment at *m/e* 93 represented a direct ketene loss by alpha-cleavage. The *m/e* pattern 121 (M^+), 122 (M + 1) and 123 (M + 2) in combination with fragments at *m/e* 93, 66, and 65 (data not shown in Fig. 3) was indicative of formanilide. The spectra of the two aniline acylation products described above were identical with the spectra of corresponding standards and with previously published spectra (15). Quantitatively, acetanilide was the dominant acylation product (see also Fig. 1); formanilide appeared only in traces on days 6 and 7.

Evidence for *N*-oxidation resulting in phenylhydroxylamine (Fig. 3B) was *m/e* 109 (M^+). A major fragment at *m/e* 92 represented loss of a hydroxyl group. The subsequent elimination of HCN resulted in the cyclopentadienyl ion at *m/e* 65 (10). No further oxidation of the nitrogen was

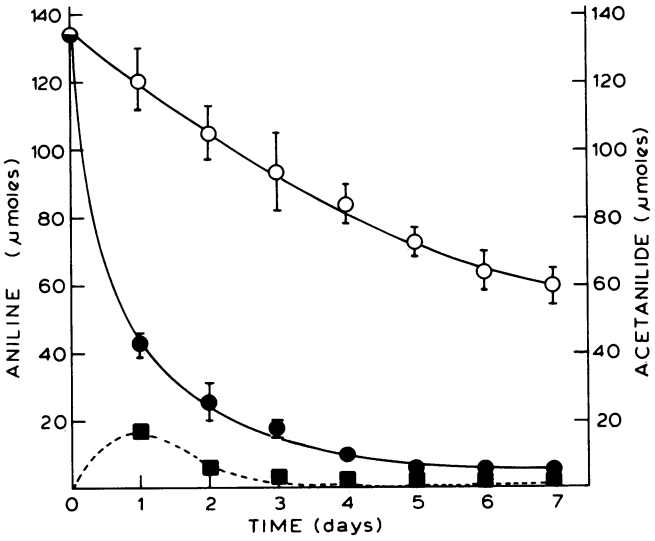

FIG. 1. Time course of aniline biodegradation in pond water (○) and pond water with sewage sludge inoculum (●). Coincident with the initial rapid rate of aniline biodegradation in pond water with sewage sludge inoculum was the transient appearance of acetanilide (■). In cases in which no error bars are depicted, the standard deviation was smaller than the symbol size.

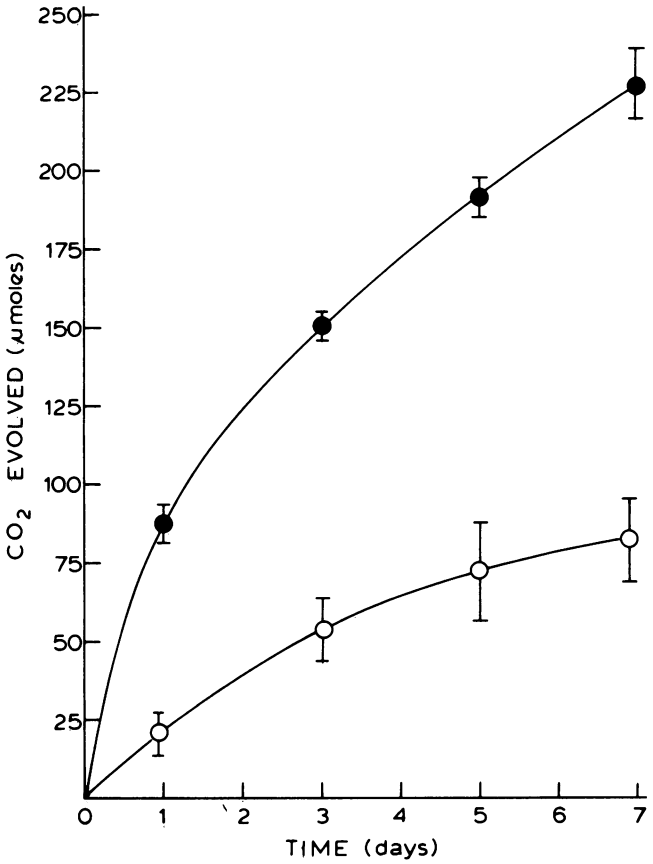

FIG. 2. Evolution of CO_2 from aniline in pond water (○) and pond water with sewage sludge inoculum (●). The totals of 28% (227 μmol) and 11% (88 μmol) aniline carbon were evolved as CO_2 during the 1-week incubation period.

observed, but the reactive phenylhydroxylamine was responsible for the formation of several condensation products. High *m/e* values and fragmentation patterns (data not shown in Fig. 3) indicative of phenoxazine and anilinoazobenzene structures were obtained (9, 14, 28). Due to the unavailability of authentic standards, previously published detailed spectra, or both, positive identification of the condensation products described above was not attempted.

In the case of *cis,cis*-muconic acid (methylated, Fig. 3C),

TABLE 2. Balance sheet of aniline (250 μg ml^{-1}) transformation in pond water with doubled biomass

Day	μmol in pond water[a]				Biomass protein (μg ml^{-1})
	Aniline	Biodegradation products of aniline[b]	Evaporated plus autoxidated aniline[c]	CO_2[c,d]	
0	134				235
1	72 ± 8.5	5	1	8 ± 0.7	340
2	54 ± 1.5	7	2	11 ± 5.9	450
3	38 ± 7.5	10	3	16 ± 1.7	480
4	24 ± 1.4	9	4	19 ± 1.2	495
5	23 ± 1.5	6	5	24 ± 0.2	555
7	21 ± 1.5	3	7	31 ± 0.2	515
7	None added			10	160

[a] Values represent the average of triplicate measurements. Standard error is noted only for the most significant columns of data.
[b] Estimate based on GC peak areas of pooled and derivatized samples.
[c] Cumulative values.
[d] Micromoles of CO_2 were divided by six, the number of carbons in aniline.

TABLE 3. Utilization of acetanilide in pond water with doubled biomass

Day	Acetanilide (μg/ml)	Protein (μg/ml)
Expt 1		
0	25.0	165
0.5	1.2	201
1	0.5	215
2	0.2	218
3	Not detected	200
Expt 2		
0	250.0	180
1	36.5	325
3	19.5	445

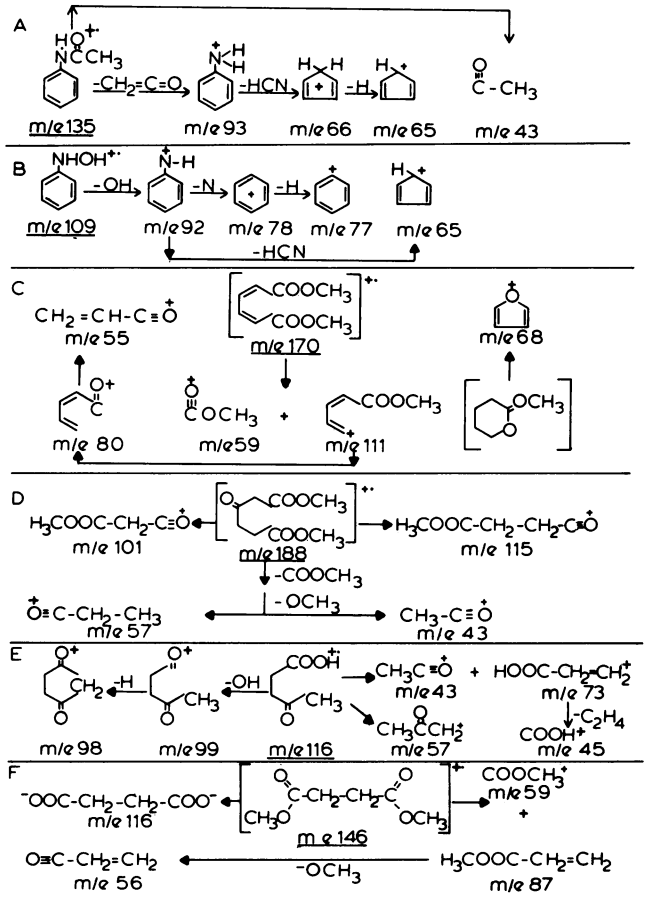

FIG. 3. Fragmentation pathways for key intermediates of aniline biodegradation in pond water with sewage sludge inoculum. The ions in brackets were postulated but not actually observed. Underlining indicates the molecular ion. (A) Acetanilide; (B) phenylhydroxylamine; (C) muconic acid methyl ester; (D) beta-ketoadipic acid methyl ester; (E) levulinic acid; (F) succinic acid methyl ester. The same intermediates were also detected in pond water without sewage sludge, with the exception of (A) and (B).

the molecular ion (*m/e* 170) itself was not observed, but fragments representing the loss of $COOCH_3$ (*m/e* 111 + *m/e* 59) followed by the loss of OCH_3 from *m/e* 111 yielding *m/e* 80 were evident. A further loss of $COCH_3$ from *m/e* 111 after rearrangement and cyclization resulted in *m/e* 68 (furan ion) plus other minor fragments (data not shown). A major component of the spectrum was *m/e* 55 derived from *m/e* 80 by loss of ethylene (10, 12, 15, 18). The fragments described above provided strong evidence for the presence of *cis,cis*-muconic acid as a breakdown product of aniline.

For beta-ketoadipic acid (methylated, Fig. 3D) the molecular ion (*m/e* 188) was not observed. However, *m/e* 101 and *m/e* 115, due to alpha-cleavage of the molecular ion, strongly suggested its presence among the catabolic products of aniline (10, 12, 23, 31). Loss of $COOCH_3$ and OCH_3 from the postulated molecular ion followed by alpha-cleavage resulted in fragments *m/e* 57 and 43, respectively. The strong *m/e* 43 signal in the obtained spectrum is characteristic of a keto group and, in conjunction with the other detected fragments, provides strong evidence for the presence of beta-ketoadipic acid (10, 12, 23, 31).

Levulinic acid (Fig. 3E), indicated by *m/e* 116 (M^+) 117 (M + 1) and 118 (M + 2), was fragmented to *m/e* 99, 98, 73, 57, 45, and 43. The ions *m/e* 43 And 45 were due to alpha cleavage of the carbonyl and carboxyl groups, respectively. The *m/e* 73 and 57 peaks were due to gamma- and beta-cleavage of the carboxyl and keto groups, respectively. The loss of an OH radical plus a hydrogen and subsequent cyclization resulted in *m/e* 98 (23). Comparison of the data described above with published spectra (15, 31) confirmed our identification. Levulinic acid was not methylated before MS and was introduced into the instrument by direct probe inlet.

No molecular ion (*m/e* 146) was observed for succinic acid dimethyl ester (Fig. 3F), but due to the loss of two methyl groups, *m/e* 116 was present. alpha-Cleavage yielded the fragments *m/e* 87 and 59, and loss of OCH_3 yielded *m/e* 56. This spectrum correlated well with previously published spectra of methylated succinic acid (13, 15).

Without a sewage sludge inoculum, acetanilide, formanilide, and phenylhydroxylamine were not detected, even when pond water biomass was doubled to obtain metabolic rates similar to the ones observed with sewage sludge inoculum. However, metabolites shown in Fig. 3C, D, E, and F were formed also in the absence of a sewage sludge inoculum (data not shown).

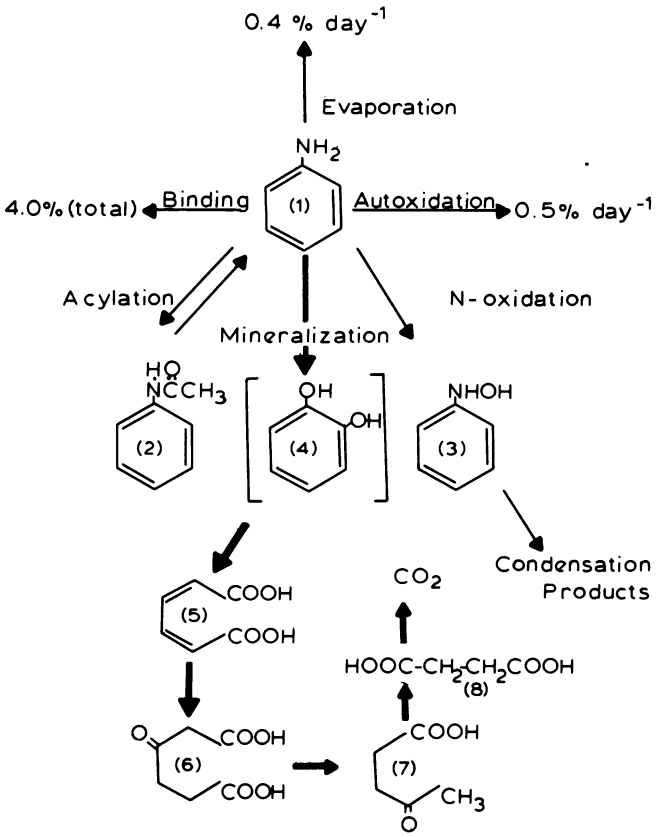

FIG. 4. Suggested mechanisms and pathways of aniline elimination from pond water with sewage sludge inoculum. Names of the illustrated intermediates are as follows: (1) aniline; (2) acetanilide; (3) phenylhydroxylamine; (4) catechol; (5) *cis,cis*-muconic acid; (6) beta-ketoadipic acid; (7) levulinic acid; (8) succinic acid. Evidence for the same catabolic pathway was obtained also in uninoculated pond water, but acylation and *N*-oxidation products were not detected in the latter system.

DISCUSSION

Significance of various processes in aniline removal. From natural waters, aniline is eliminated by various mechanisms (Fig. 4). Evaporation and autoxidation in the absence of light are slow processes and contribute little to the removal of aniline from natural waters. Binding to humic substances is a rapid process, and its significance to total aniline removal varies with the amount of dissolved or suspended humic substances in the water. Removal of aniline by this mechanism was undetectable in pond water alone but amounted to ca. 4% when sewage sludge was added.

Photodegradation of aniline in surface waters is a slow process unless catalyzed by humic substances (36). Since in humus-rich waters light penetration is rather limited, we roughly estimate that the contribution of photodegradation to aniline removal from surface waters is at least an order of magnitude lower than biodegradation.

Removal of aniline by microbial processes, resulting ultimately in CO_2 and microbial biomass, was by far the most efficient aniline removal mechanism from those measured and is indicated in Fig. 4 with heavy arrows. Aniline degradation did not seem to require an induction period, or the induction was too rapid to be detected in our test. It is very unlikely that either the pond water or the domestic sewage sludge had any previous exposure to aniline.

Pathways of aniline biodegradation. As summarized above, a considerable amount of information is available about aniline transformation pathways in pure culture experiments and in soil, but without direct measurements, it was difficult to predict which of these would predominate in natural waters. The identification of a number of key metabolic intermediates in conjunction with the previously discussed background literature allows us to propose the pathways illustrated in Fig. 4.

The principal biodegradation pathway for aniline in pond water with or without sewage sludge inoculum appears to involve dioxygenase attack (2), resulting in oxidative deamination to catechol. Catechol itself was not detected, but this is a notoriously difficult intermediate to isolate (35). Detection of the muconic, beta-ketoadipic, levulinic, and succinic acids clearly delineates the pathway that leads through the tricarboxylic acid cycle to the ultimate release of aniline carbon as CO_2.

Formation of acetanilide, formanilide, phenylhydroxylamine, and some unidentified condensation products was detected only in pond water with sewage sludge inoculum. Microorganisms that are responsible for these minor pathways were apparently not sufficiently represented in the microbial community of pond water but were active in sewage sludge. Acetanilide and formanilide are unusual biodegradation intermediates, as they are products of acylation rather than of a catabolic reaction. Acetanilide and formanilide analogs of corresponding anilines have been reported by several authors (20, 21, 30, 32, 35) and may be considered products of fortuitous or detoxification reactions. These reactions are clearly reversible and do not interfere with the utimate mineralization of aniline. Phenylhydroxylamines were identified as the active intermediates of *N*-oxidation reactions (7) that lead to condensation products of considerable persistence (5). This type of transformation was detected only in the presence of sewage sludge inoculum, and as estimated from the GC peak areas, not more than 3% of the added aniline was transformed in this manner.

Although the minor pathways of aniline biodegradation described above were detected only in the presence of sewage sludge, the principal biodegradation pathway (Fig. 4, heavy arrows) leading to aniline mineralization was found to be identical for both the pond water and the sewage sludge microbial communities. Considering that the pond water community had a large photoautotrophic component, consisting mainly of chlorophycophyta, whereas the sewage sludge consisted principally of heterotrophic bacteria, it is not surprising that per microgram of biomass protein, the latter community exhibited a much higher aniline biodegradation activity than the former.

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