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# Metabolism of Niclosamide in Sediment and Water Systems

PHILLIP W. GRAEBING AND J. S. CHIB*

Pittsburgh Environmental Research Laboratory, Inc., Pittsburgh, Pennsylvania 15238

TERRANCE D. HUBERT AND WILLIAM H. GINGERICH

Upper Midwest Environmental Sciences Center, U.S. Geological Survey, La Crosse, Wisconsin 54603

A series of experiments analyzed the kinetics and mechanisms of [$^{14}$C]niclosamide degradation. The aerobic aquatic metabolism of [$^{14}$C]niclosamide was studied in nonsterile river water/sediment mixtures. Test systems, maintained under aerobic conditions, were treated with niclosamide and incubated in the dark at 25.0 ± 1.0 °C for 30 days. Half-lives of 4.9 and 5.4 days were calculated for the chlorosalicylic acid- and chloronitroaniline-labeled test systems, respectively. From 0 to 21 days after treatment (DAT), the only metabolism product observed in either test system was aminoniclosamide. At the final sampling interval, five peaks were resolved from the chlorosalicylic acid label, and three peaks were resolved from the chloronitroaniline label test substance. By 30 DAT, sediment-bound residues represented ~70% of the observed radioactivity. For the anaerobic aquatic metabolism of [$^{14}$C]niclosamide, test systems were incubated under anaerobic conditions for 365 days. Half-lives of 0.65 day for the chlorosalicylic acid label and 2.79 days for the chloronitroaniline label were calculated. From 0 to 3 DAT, niclosamide was first transformed into aminoniclosamide. Aminoniclosamide is readily formed, as it was observed in the chlorosalicylic acid label 0 DAT sampling. Several minor metabolites were observed in the water and sediment extracts. None of these metabolites were formed to a significant amount until the parent niclosamide dissipated below the detection limit. Two of the byproducts from these metabolism studies are polar unknowns eluting at 3 and 5 min by HPLC, similar to the unknowns observed in aqueous photolysis studies.

**KEYWORDS: Niclosamide; lampricide; half-life; aerobic soil metabolism; anaerobic soil metabolism**

## INTRODUCTION

Understanding the fate and metabolism of pesticides is very important in the decision to approve their use. The behavior of pesticides in the environment is of great importance, because the disappearance, persistence, and transformation of a chemical, along with its efficacy, determines its usefulness and effects. Interest in the fate of pesticides in the environment arises from concerns of the possibilities of environmental contamination. Not only must the efficacy of the compound be proven, but the kinetics of its degradation mechanism must be characterized to ensure public safety and environmental protection from potentially harmful degradates. Therefore, it is essential that laboratory studies be conducted under accurate conditions to understand what will happen to the chemical in the environment. Degradation is a major concern in the environment, where the compound or its degradates may persist and become available to waterways and nontarget organisms (*1*, *2*). Hydrolysis, microbial degradation, oxidation, and reactions with sediment and surface water components are some of the mechanisms by which pesticides degrade.

Niclosamide, 5-chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide, is the active ingredient of Bayluscide, a chemical used to control sea lamprey (*Petromyzon marinus*) in the Great Lakes region of the United States, where native fish populations have been devastated (*3*). Sea lamprey first gained access to Lake Erie upon completion of the Welland Canal around Niagara Falls in 1829, but they were not reported in Lake Erie until 1921. Thereafter, the infestation quickly spread. Sea lampreys were found in Lake Huron in 1932, in Lake Michigan in 1936, and in Lake Superior in 1946. Adult sea lampreys feed by attaching onto other fish with their suctorial mouths and extracting blood and other body fluids from the fish. A single sea lamprey is capable of killing as much as 18 kg of fish during the 12−20 months of its adult life. The devastation was best documented in Lake Superior, where from 1930 to 1952 lake trout production was consistent at 1.8 million kg. In the following decade, however, production decreased by 90%, whereas the number of sea lampreys caught in a fixed number of assessment weirs rose from 1000 to 70000 (*4*). Salicylanilides as a class of chemicals had been found to be effective as molluscicides, with niclosamide possessing the optimal properties (*5*). Niclosamide residues in fish begin to decline 12−18 h

* Author to whom correspondence should be addressed [telephone (412) 826-5161; fax (412) 826-3946].

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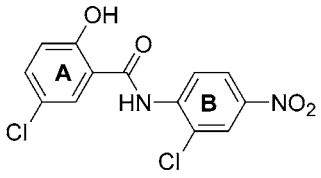
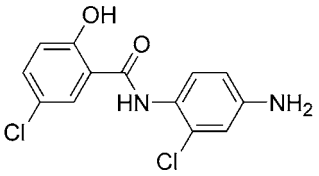
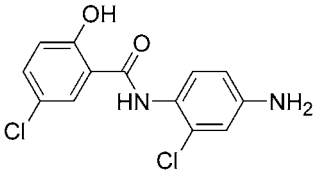



Label A: [$^{14}$C-Chlorosalicylic acid URL]-Niclosamide
Specific Activity 7.35 mCi/mmol

Label B: [$^{14}$C-Chloronitroaniline URL]-Niclosamide
Specific Activity 6.45 mCi/mmol




Aminoniclosamide




Hydroxyniclosamide

**Figure 1.** Structures of test and reference substances.

after exposure as the compound is transformed to niclosamide glucuronide and eliminated (*6*). The efficacy of niclosamide appears to be similar to that of 3-trifluoromethyl-4-nitrophenol (TFM), the primary chemical used for sea lamprey control in the Great Lakes. TFM takes advantage of the lower glucuronyltransferase activity in lampreys, so that more of the compound is accumulated (*7*). The environmental fate of niclosamide has been studied previously. It is stable to hydrolysis in pH 5, 7, and 9 buffers for over 4 months (*8*), but photolysis of the 70% wettable powder formulation in aqueous solutions produces a significant decrease in biological activity within 24 h (*9*−*11*). Photolysis causes 51% degradation of the parent compound after 7 days of exposure in pH 6.9 buffer (*12*). Degradation in river and pond sediment/water systems yielded half-lives of 1.1−3.9 days (*13*). The purpose of this study was to determine the rates of degradation and half-lives of niclosamide in sediment/water environments and to identify and quantify niclosamide and its metabolites, thereby expanding on the previous studies.

## MATERIALS AND METHODS

**Test Substances and Reference Substances.** [$^{14}$C]Niclosamide test substances were synthesized by DuPont/New England Nuclear (Boston, MA). Two test substances were used in these studies, differing by the position of the $^{14}$C-labeled aromatic ring (**Figure 1**). Prior to use in the study, HPLC was used to verify the radiochemical purity of both label A (chlorosalicylic acid label, 99.0%) and label B (chloronitroaniline label, 98.1%) test substances. The solubility of niclosamide in water is 5−8 mg/L (*14*).

Non-radiolabeled reference substances of aminoniclosamide (2′,5-dichloro-4′-aminosalicylanilide) and hydroxyniclosamide (2′,5-dichloro-4′-hydroxysalicylanilide) were synthesized by Derse and Schroeder Associates., Ltd. (Madison, WI). Additional reference substances were obtained from Aldrich Chemical Co., St. Louis, MO (**Table 1**). All reference substances arrived with a certificate of analysis confirming their identities and purities and were used without further purification. Stock solutions of reference substances were prepared in acetonitrile or methanol and stored refrigerated or at room temperature. Dosing solutions were prepared in methanol. Under these preparation and storage conditions, the reference substance stock solutions were found to be stable over the course of the study.

**Table 1.** Other Reference Standards

| | |
|---|---|
| 5-chlorosalicylic acid | 3-chlorobenzoic acid |
| 2,3-dihydroxybenzoic acid | salicylic acid |
| 2,4-dihydroxybenzoic acid | 1,2,4-benzenetriol |
| 2,5-dihydroxybenzoic acid | 4-chlorophenol |
| 2,4-dihydroxybenzoic acid methyl ester | 2-aminophenol |
| 2,5-dihydroxybenzoic acid methyl ester | 4-aminophenol |
| 2,6-dihydroxybenzoic acid methyl ester | 4-aminoresorcinol |
| 3-hydroxybenzoic acid | 2-chloroaniline |
| 2-chloro-4-nitroaniline | 4-nitrocatechol |
| 2-chloro-4-nitrophenol | 4-nitroaniline |
| 2-amino-5-nitrophenol | chlorohydroquinone |
| 4-amino-3-chlorophenol | hydroquinone |

**Table 2.** Characteristics of Sediment and Water Used in the Aquatic Metabolism Studies

| | |
|---|---|
| Sediment | |
| organic matter | 1.0% |
| pH | 7.9 |
| sand | 94% |
| silt | 4% |
| clay | 2% |
| USDA textural class | sand |
| cation exchange capacity | 6.7 mequiv/100 g |
| bulk density | 1.47 g/cm$^3$ |
| Water | |
| pH | 8.11 |
| dissolved oxygen | 9.8 mg/L |
| conductance | 282 FS/cm |
| hardness | 174 mg of $CaCO_3$/L |
| total organic carbon | 3.5 mg/L |

**River Water and Sediment.** The test system matrix was obtained from a tributary of Lake Huron, typical of the niclosamide application area. Sediment from Black River, Presque Isle County, MI, and water from the Black River, Cheboygan County, MI, were provided by the U.S. Geological Survey. Sediment and water characterization was performed by Midwest Laboratories, Inc., Omaha, NE. The sediment and water technical information and characterization results are presented in **Table 2**.

For the aerobic aquatic metabolism study, the sediment and water were first combined at a ratio of one part sediment to three parts river water and preincubated at 25 °C to acclimate the microbial populations. Air was continuously purged into the water phase. Prior to initiation of the study and again at its conclusion, the microbial viability of the sediment/water mixture was verified by enumerating the colony-forming units after 24, 48, and 72 h of incubation on Standard Methods Agar and Center for Disease Control Anaerobe Blood Agar (Becton-Dickinson Microbiology Systems, Cockeysville, MD). The test systems were microbially viable throughout the study.

In preparation for the anaerobic aquatic metabolism study, a 1:3 sediment/water mixture was transferred into a 2 L bottle. Nitrogen was purged into the water for 30 min, and then the headspace was purged with nitrogen for an additional hour. The vessel was sealed, and the mixture was then preincubated for at least 2 weeks in a static nitrogen atmosphere at 25 °C. The microbial viability of the test system was verified by plating serial dilutions of the sediment/water mixture onto CDC Anaerobe Blood Agar plates for the enumeration of anaerobes. The plates were incubated anaerobically in a Becton-Dickinson GasPak container. After 144 h of plate incubation at 25 °C, the colonies were counted and the colony-forming units per gram of sediment/water slurry was calculated. The anaerobic test systems were microbially active throughout the study.

**Liquid Scintillation Counting (LSC).** Liquid scintillation analyses were conducted using a Packard Instruments (Meriden, CT) TriCarb model 1900TR liquid scintillation analyzer. Results are reported in

disintegrations per minute (dpm). Triplicate aliquots (100 or 200 μL) of the river water and sediment extracts were analyzed using 4 mL of Packard Ultima Gold scintillant. Trapping solutions were analyzed in triplicate 1 mL aliquots. The samples were counted for at least 2 min. All counts were automatically corrected for instrument background (~25 dpm) and efficiency (>96%).

**High-Performance Liquid Chromatography (HPLC).** A Waters (Milford, MA) HPLC system, configured with two model 501 pumps, a model 715 WISP autosampler, and Maxima 820 data acquisition software, was used for the primary method of analysis. The gradient method developed for the aqueous photolysis study (*15*) was used to analyze the samples from these metabolism studies. An Advanced Chromatography Technologies (Chadds Ford, PA) Ace 5 C18 4.6 × 250 mm column with a mobile phase of 20 mM potassium phosphate monobasic, pH 3.4 (A), and acetonitrile (B) achieved the separation using a gradient beginning with a 3 min hold at 10% solvent B, rising to 70% solvent B at 14 min and 80% solvent B at 15 min, and holding until 35 min. Peaks were detected with a Waters 484 tunable UV detector at 220 or 254 nm and a Packard FLO-ONE/Beta radioflow detector. The scintillant was Flo-Scint II (Packard), flowing at 3.0 mL/min. Those radiolabeled peaks that cochromatographed with reference substances were considered tentatively identified and selected for further analysis by TLC. The half-lives ($t_{1/2}$) of [$^{14}$C]niclosamide in the test systems were determined by performing linear regression analysis on the natural logarithm of the average ratio of test substance concentration ($C_t$) observed at each sampling interval $t$ to the time 0 concentration ($C_0$):

$$\ln(C_t/C_0) = kt \qquad t_{1/2} = \ln(0.5)/k$$

To more accurately quantify the degradate peaks, selected samples were also quantified by HPLC fractionation and LSC analysis. Fractionation was also used to isolate and collect unknowns for identification. Throughout the study, the HPLC column recoveries were ≥90%.

**Thin-Layer Chromatography (TLC).** TLC was utilized as a secondary analytical method to identify and confirm the observed degradates. Aliquots of test samples and reference substances were spotted on silica gel 60F (EM Science, Gibbstown, NJ) plates and developed in dichloromethane/ethyl acetate/acetic acid (80:20:2 v/v/v; solvent system 1), dichloromethane/methanol/10% $NH_4OH$ (80:20:3 v/v/v; solvent system 2), or chloroform/methanol/water/acetic acid (13:7:1:2 v/v/v/v; solvent system 3).

Following TLC development, the plates were dried, and the areas where the reference substances had migrated were visualized with a hand-held UV lamp at 254 nm or by staining with iodine vapor. The plate was then scanned for radioactivity using an AMBIS (San Diego, CA) radioanalytical imaging system. Those radiolabeled peaks that cochromatographed with reference substances by both HPLC and TLC were considered to be confirmed.

**Combustion Analysis.** Triplicate aliquots of extracted air-dried sediment were combusted using a Harvey (Hillsdale, NJ) model OX-500 biological oxidizer to determine the levels of bound radioactivity remaining in each sample. The liberated $^{14}CO_2$ was trapped directly into scintillant (Harvey C14 cocktail) and analyzed by LSC. The efficiency of the oxidizer was determined by fortifying the sediment matrix with a known level of [$^{14}$C]niclosamide standard. The average efficiency of the oxidizer was >95% during the course of the study.

**Sediment-Bound Radioactivity.** In the later sampling intervals of the metabolism studies, sediment-bound radioactivity following extraction was >50%. For this reason, the bound residues were further characterized by fractionation of the sediment organic matter. Aliquots (~2 g) of the extracted sediments were processed as follows: The sample was extracted overnight with 5 mL of 5% aqueous sodium hydroxide. The sample was centrifuged, and the supernatant, containing fulvic acid and humic acid, was decanted. The sample was extracted for 3 h with an additional 5 mL of 5% sodium hydroxide. Following centrifugation, the supernatants were pooled. The volume was determined, and triplicate aliquots of the NaOH extract were assayed by LSC. The pH of the supernatant was then adjusted to pH 1 with concentrated hydrochloric acid, resulting in the precipitation of the humic acid fraction. The sample was centrifuged, and the fulvic acid supernatant was decanted. The volume was determined, and triplicate aliquots were assayed by LSC. The humic acid precipitate was redissolved in 2 mL of 5% sodium hydroxide and assayed in triplicate aliquots by LSC. Aliquots of the remaining sediment sample were combusted to determine the humin fraction.

**Aerobic Aquatic Metabolism Rate Determination.** The test systems used for the aerobic aquatic study were 1 L bottles with screw caps equipped with inlet and outlet ports. Both test systems (labels A and B) contained 180 g of sediment and 540 mL of water (1:3 ratio). Tubing from the inlet port had enough length to extend into the water phase. The outlet port tubing was shorter and reached only the headspace. During incubation in the dark at 25 ± 1 °C, $CO_2$-free air was continuously purged into the water phase to maintain aerobic conditions. The outlet port of each test system was connected to an empty back-flow trap and then to two traps containing 1 N NaOH for volatile $^{14}CO_2$ collection.

One test system was dosed with chlorosalicylic acid-labeled niclosamide (label A, 30.2 μCi applied) and the other with chloronitroaniline-labeled niclosamide (label B, 26.6 μCi applied) test substance. The methanol cosolvent from dosing represented 0.35% of the water volume. The application rate was 2.48 μg/mL of water for both labels. Following dosing, both test systems were mixed thoroughly by shaking for 1 min. The test systems were allowed to settle for 15 min, and then 0 DAT samples were taken from both systems. Succeeding samples were taken at 1, 2, 3, 5, 7, 14, 21, and 30 DAT.

Prior to sampling, each test system was mixed thoroughly by shaking for ~1 min. The test systems were allowed to settle for 15 min, and then ~25 mL of sediment/water sample was taken by pipet from both systems. Samples were transferred into 40 mL vials, which were then centrifuged at 2000 rpm for 8−10 min. Following centrifugation, the water phase was decanted, and the volume was measured. Aliquots of the water phases were analyzed directly in triplicate by LSC and in duplicate by HPLC. At each sampling interval, the NaOH trapping solutions were removed and assayed by LSC. Fresh trapping solutions were added, and incubation was resumed with continuous air flow. Each sediment pellet was extracted three times with 10 mL of methanol/water (4:1 v/v) followed by three extractions with 10 mL of acetonitrile/1 N phosphoric acid (9:1 v/v). This extraction procedure was validated prior to study initiation. The extracts were analyzed by LSC in triplicate. Aliquots of extracts containing high enough levels of radioactivity were analyzed directly in duplicate by HPLC. Aliquots of extracts containing low levels of radioactivity were concentrated 5−10-fold by nitrogen evaporation before duplicate aliquots were analyzed by HPLC. The extracted sediment was air-dried overnight, and aliquots of each extracted sediment were combusted to determine the levels of remaining bound radioactivity. At selected sampling intervals, the dissolved oxygen in each test system was measured using a Yellow Springs Instruments (Yellow Springs, OH) model 5300 biological oxygen monitor and a model 5331 standard oxygen probe. The instrument was calibrated to 100% dissolved oxygen using air-saturated reagent water. Dissolved oxygen readings of >75% saturation were observed in every sample that was taken, indicating that aerobic conditions were maintained in the test systems.

**Anaerobic Aquatic Metabolism Rate Determination.** Label A niclosamide and label B niclosamide were studied independently of each other in two test systems consisting of 22 100 mL bottles with screw caps with inlet and outlet ports connected in series. Each test system bottle contained 10 g of sediment and 30 mL of water. Tubing from the ports extended into only the headspace of the bottles. Sediment and water were dispensed from the batch preincubated mixture and further incubated in a static nitrogen atmosphere in the individual sample bottles. At the end of the series an untreated sediment and water sample containing an anaerobic indicator strip (Becton-Dickinson) was added to monitor the system anaerobicity. The outlet port from the series was connected to a trap containing 100 mL of 1 N NaOH for volatile $^{14}CO_2$ collection.

The application rates were 1.6 μCi/bottle, or 2.56 μg/mL for label A and 2.66 μg/mL for label B, calculated on the basis of 30 mL of water. Following dosing, each sample bottle was swirled to mix.

**Table 3.** Summary of the Percent Distribution of Total Radioactivity over Time from the Aerobic Aquatic Metabolism Study[a]

| DAT[b] | label | water phase | sediment extracts | sediment bound | volatiles | total |
|---|---|---|---|---|---|---|
| 0 | A | 1.3 | 3.5 | 0.1 | na[c] | 4.9 |
| | B | 1.1 | 3.4 | 0.0 | | 4.4 |
| 1 | A | 1.8 | 2.2 | 0.2 | 0.0 | 4.2 |
| | B | 1.8 | 3.9 | 0.3 | 0.0 | 6.0 |
| 2 | A | 1.2 | 4.5 | 1.0 | 0.0 | 6.8 |
| | B | 1.7 | 3.1 | 0.6 | 0.0 | 5.3 |
| 3 | A | 1.4 | 3.1 | 0.7 | 0.0 | 5.3 |
| | B | 0.8 | 5.0 | 2.2 | 0.0 | 8.1 |
| 5 | A | 0.7 | 3.2 | 1.4 | 0.0 | 5.3 |
| | B | 0.9 | 4.5 | 4.3 | 0.0 | 9.7 |
| 7 | A | 0.8 | 0.5 | 0.3 | 0.0 | 1.7 |
| | B | 0.6 | 3.9 | 4.1 | 0.0 | 8.6 |
| 14 | A | 0.6 | 3.2 | 5.2 | 0.1 | 8.9 |
| | B | 0.5 | 1.2 | 2.2 | 0.0 | 4.0 |
| 21 | A | 0.7 | 1.7 | 2.8 | 0.1 | 5.2 |
| | B | 0.6 | 1.3 | 3.1 | 0.1 | 5.1 |
| 30 | A | 0.4 | 1.5 | 5.4 | 0.4 | 7.7 |
| | B | 0.3 | 1.5 | 5.9 | 0.1 | 7.8 |
| test system | A | 4.7 | na | 38.5[a] | na | 43.4 |
| | B | 3.4 | | 48.1 | | 51.6 |
| total | A | 13.6 | 23.4 | 55.6 | 0.6 | 93.3 |
| | B | 11.8 | 27.8 | 70.9 | 0.2 | 111 |

[a] Percentage of total dpm applied to the test system. [b] Days after treatment. [c] Not applicable. [d] Total of extractable and nonextractable radioactivity.

Nitrogen flow was continuous through the test systems during dosing. Sampling occurred at 0, 1, 3, 7, 14, 30, 60, 120, 180, 270, and 365 DAT.

Prior to sampling, the test system was purged with nitrogen into the sodium hydroxide trap for 20 min−3 h, longer for the later sampling times. The test system was then clamped off. The final two sample bottles in the series were removed from both systems. The redox potentials of the samples were measured immediately using an Orion Research, Inc. (Beverly, MA), model 96-78-00 redox electrode and a Fisher (Pittsburgh, PA) model 805 MP pH meter. Redox potential readings of <350 mV were observed in every sample that was taken, indicating that anaerobic conditions were maintained in the test systems (*13*). Samples were then centrifuged at 2000 rpm for ∼4−10 min. Following centrifugation, the water phase was decanted, and the volume was measured. Aliquots of the water phases were then analyzed directly in triplicate by LSC. Aliquots were also analyzed directly by HPLC if radioactivity levels were sufficiently high. The sediment pellets and the NaOH trapping solution were analyzed as described for the aerobic aquatic metabolism samples. In the later sampling intervals, sediment-bound radioactivity following extraction was ∼75% of the applied radioactivity, so the bound residues were further characterized by fractionation of the sediment organic matter.

## RESULTS

**Aerobic Aquatic Metabolism.** The distribution of radioactivity between the river water and sediment of the aerobic aquatic metabolism study is presented in **Table 3**. Overall material balances of 93.3% in the label A system and 111% in the label B system were observed. At 0 DAT ∼75% of the recovered radioactivity was extractable from the sediment phase, and there was very little bound material. By 14 DAT, however, bound residues were ∼55% of the recovered radioactivity. At 30 DAT, bound residues represented ∼70% of the recovered radioactivity. Volatile radioactivity formation was insignificant, at <1% of applied radioactivity.

**Table 4.** Percent Distribution of Aerobic Aquatic Metabolites of [$^{14}$C]Niclosamide

| DAT[a] | label | unk 1, 3 min[b] | unk 2, 21 min | unk 3, 22 min | unk 4, 23 min | unk 5, 23.8 min | amino-niclosamide, 24.5 min | niclos-amide, 27 min |
|---|---|---|---|---|---|---|---|---|
| 0 | A | | | | | | | 99.3 |
| | B | | | | | | | 99.4 |
| 1 | A | | | | | | 7.4 | 89.6 |
| | B | | | | | | 6.0 | 90.5 |
| 2 | A | | | | | | 14.7 | 73.5 |
| | B | | | | | | 15.1 | 76.5 |
| 3 | A | | | | | | 23.2 | 65.6 |
| | B | | | | | | 15.6 | 63.4 |
| 5 | A | | | | | | 29.7 | 49.4 |
| | B | | | | | | 14.0 | 50.2 |
| 7 | A | | | | | | 36.3 | 49.0 |
| | B | | | | | | 20.6 | 40.3 |
| 14 | A | | | | | | 44.7 | 5.0 |
| | B | | | | | | 38.7 | 13.8 |
| 21 | A | | | | 4.1 | | 46.3 | 2.9 |
| | B | | | | 0.0 | | 37.3 | 5.4 |
| 30 | A | 0.6 | 4.3 | 0.8 | 0.6 | 6.9 | 15.4 | 2.6 |
| | B | 0.9 | 0.0 | 3.2 | 0.0 | 9.7 | 13.9 | 2.4 |

[a] Days after treatment. [b] HPLC retention time.

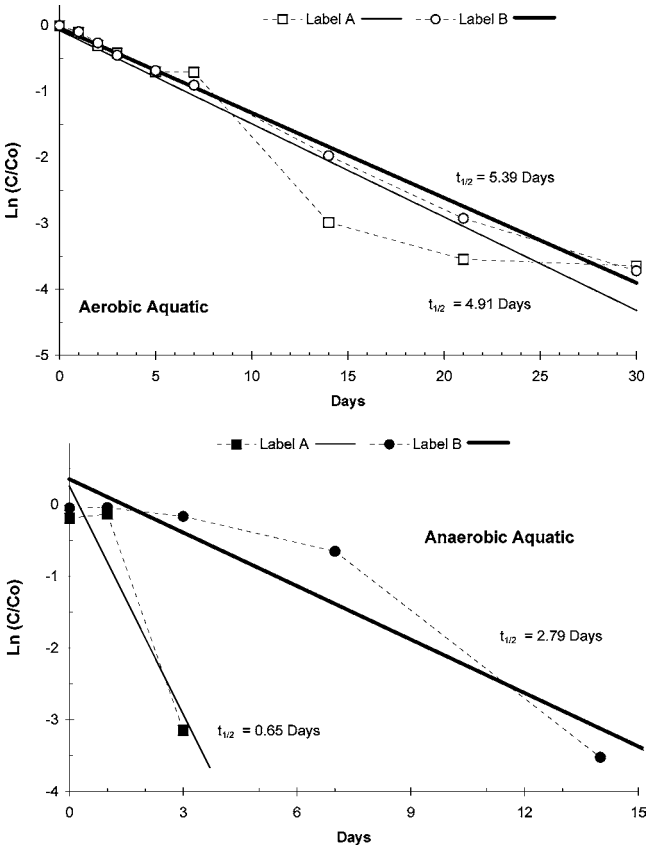



**Figure 2.** Decline of [$^{14}$C]niclosamide concentrations in the aerobic aquatic (top) and anaerobic aquatic (bottom) metabolism studies.

The concentrations of [$^{14}$C]niclosamide and its degradates are summarized for both test systems in **Table 4** as percent of recovered radioactivity. In the both test systems, [$^{14}$C]niclosamide represented 99% of the recovered dpm at 0 DAT and then declined to 2.5% by 30 DAT. The values in **Table 4** were used to perform linear regression analyses to determine the half-life of niclosamide. The results are presented in **Figure 2**. Half-lives of 4.9 days ($k = 5.89 \times 10^{-3}$ h$^{-1}$) for label A and 5.4 days ($k = 5.36 \times 10^{-3}$ h$^{-1}$) for label B were calculated. The

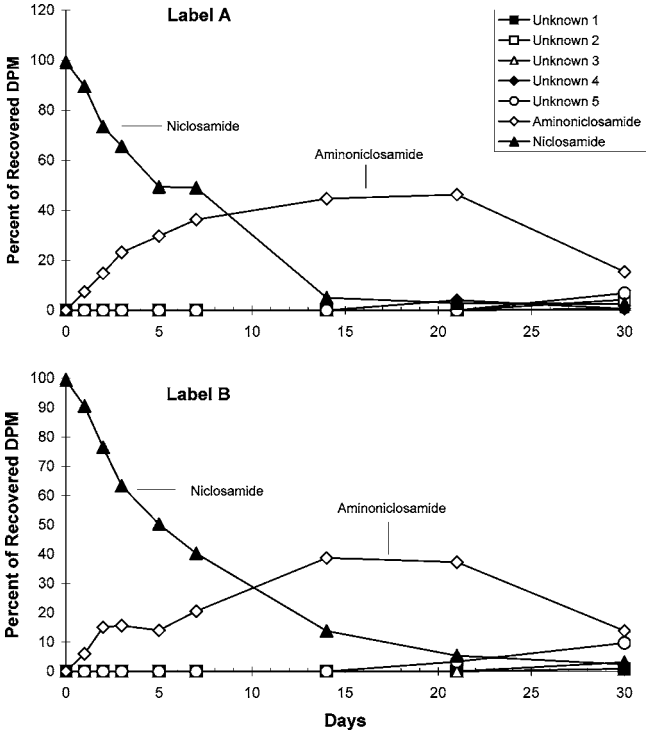



**Figure 3.** Formation and decline of aerobic aquatic metabolites.

coefficients of determination for the regressions were 0.993 and 0.909, respectively, thus showing good correlation to first-order degradations.

From 0 to 21 DAT, the only transformation product observed was aminoniclosamide. This metabolite was identified by HPLC and TLC cochromatography. Aminoniclosamide, first reported as a degradate in aquatic systems by Muir and Yarechewski (*13*), increased to a maximum of 38−46% of the recovered radioactivity at ∼21 DAT and then declined to 14−15% at 30 DAT. However, in the final sampling, the metabolite pattern was more complex. Along with aminoniclosamide, both labels shared three common metabolites. In the HPLC chromatograms from both test systems, the polar unknown 1 at 3 min represented a total of ∼0.6−0.9% of the radioactivity (0.02 $\mu$g/mL). This material is very similar in nature to polar degradates observed in the aqueous photolysis of niclosamide (*15*). In the photolysis of niclosamide in pH 9 buffer, two radioactive peaks eluting by HPLC at about 3 and 5 min were found to be composed mostly of carbon dioxide (identified using $Na_2{}^{14}CO_3$) plus five or six aliphatic acids, formed from the opening of the aromatic rings. Two other metabolites were common to both labels of the aerobic aquatic study. Unknown 3 at 22 min made up 0.8% of the radioactivity (0.02 $\mu$g/mL) in label A and 3.2% of the radioactivity (0.08 $\mu$g/mL) in label B. Unknown 5 at 23.8 min contained 7−10% of the radioactivity (0.17−0.24 $\mu$g/mL). Unknown 5 was the most significant unknown and eluted slightly before aminoniclosamide. This suggested that this unknown was a further transformation of aminoniclosamide. Two unknowns were unique to the label A test system. Unknown 2 at 21 min represented 4.3% of the radioactivity (0.11 $\mu$g/mL), and unknown 4 at 23 min represented 0.6% of the radioactivity (0.02 $\mu$g/mL). The formation and decline of the metabolites are graphically presented in **Figure 3**.

In the later sampling intervals (14, 22, and 30 DAT), the bound residues were characterized by fractionation of the sediment organic matter. The results from both test systems are presented in **Table 5**. In the 30 DAT sediment samples, 12% of the bound residues was distributed in the fulvic acid fraction, 24% in the humic acid fraction, and 63% remained in the humin fraction.

**Table 5.** Percent Distribution of Sediment-Bound Radioactivity in Label A and Label B Aquatic Metabolism Studies

| DAT[a] | label | fulvic acid fraction | humic acid fraction | humin fraction |
|---|---|---|---|---|
| | | Aerobic Aquatic Sediment | | |
| 14 | A | 10.0 | 26.9 | 63.1 |
| | B | 18.0 | 41.3 | 40.7 |
| 21 | A | 14.5 | 33.2 | 52.3 |
| | B | 10.3 | 30.2 | 59.5 |
| 30 | A | 15.4 | 20.7 | 63.9 |
| | B | 9.9 | 27.1 | 63.0 |
| | | Anaerobic Aquatic Sediment | | |
| 120 | A | 17.4 | 28.2 | 54.5 |
| | B | 18.1 | 19.2 | 62.8 |
| 181 | A | 20.4 | 18.9 | 60.8 |
| | B | 16.2 | 20.5 | 63.4 |
| 365 | A | 24.2 | 24.1 | 51.7 |
| | B | 12.6 | 24.0 | 63.5 |

[a] Days after treatment.

**Table 6.** Distribution of Radioactivity over Time in the Anaerobic Aquatic Metabolism Study

| DAT[a] | label | water phase | extract 1[b] | extract 2[c] | bound | volatiles | total |
|---|---|---|---|---|---|---|---|
| 0 | A | 61.3 | 35.1 | 3.4 | 0.8 | na[d] | 101 |
| | B | 65.1 | 29.9 | 0.8 | 0.2 | | 96.0 |
| 1 | A | 64.8 | 30.8 | 2.3 | 1.0 | 0.01 | 98.9 |
| | B | 64.5 | 31.3 | 1.6 | 0.7 | 0.0 | 98.1 |
| 3 | A | 75.9 | 15.4 | 3.8 | 3.0 | 0.03 | 98.3 |
| | B | 46.6 | 44.9 | 4.3 | 2.2 | 0.0 | 98.1 |
| 7 | A | 54.1 | 26.4 | 7.0 | 8.0 | 0.07 | 95.5 |
| | B | 40.4 | 44.6 | 7.5 | 5.9 | 0.0 | 98.4 |
| 14 | A | 41.0 | 32.1 | 10.4 | 9.5 | 0.1 | 93.1 |
| | B | 42.1 | 31.1 | 10.7 | 11.4 | 0.0 | 95.4 |
| 30 | A | 29.4 | 36.8 | 13.7 | 13.7 | 0.2 | 93.7 |
| | B | 38.7 | 28.6 | 12.9 | 14.0 | 0.01 | 94.2 |
| 62 | A | 12.8 | 14.1 | 16.5 | 44.4 | 0.3 | 87.9 |
| | B | 22.2 | 26.3 | 19.9 | 27.7 | 0.1 | 96.2 |
| 120 | A | 3.9 | 4.7 | 10.5 | 71.5 | 0.4 | 91.0 |
| | B | 6.8 | 5.6 | 13.6 | 69.5 | 0.2 | 95.6 |
| 181 | A | 4.5 | 2.7 | 6.5 | 77.1 | 0.6 | 91.3 |
| | B | 7.5 | 3.3 | 13.0 | 76.5 | 0.5 | 101 |
| 272 | A | 4.6 | 1.6 | 16.7 | 70.1 | 1.3 | 94.2 |
| | B | 4.1 | 0.4 | 11.2 | 78.3 | 0.6 | 94.7 |
| 365 | A | 3.0 | 4.3 | 8.0 | 77.3 | 1.4 | 94.0 |
| | B | 2.4 | 2.1 | 7.8 | 77.5 | 0.7 | 90.4 |
| | | | overall material balance | | | | 94.4<br>96.2 |

[a] Days after treatment. [b] Methanol/water (4:1; v/v), includes sample container rinse after DAT 120. [c] Acetonitrile/1 N $H_3PO_4$ (9:1; v/v). [d] Not applicable.

**Anaerobic Aquatic Metabolism.** The distribution of radioactivity in the anaerobic systems is presented in **Table 6**. An overall material balance of >94% was observed throughout the study. The amount of applied radioactivity in the water steadily decreased from 3 DAT and leveled off at 2−7% from 120 to 365 DAT. By 62 DAT, the levels of bound radioactivity had increased significantly and remained constant at ∼70−75% in succeeding samplings. The volatile radioactivity data, calculated on a per sample basis and accumulated throughout the study, were insignificant, representing ∼1% of applied.

**Table 7.** Percent Distribution of Metabolites of [$^{14}$C]Niclosamide in the Anaerobic Aquatic Metabolism Study

| DAT[a] | label | unk 4, 3 min | unk 6, 5 min | unk 2, 21 min | unk 3, 23 min | unk 1, 24 min | amino-niclosamide, 24.5 min | unk 5, 25 min | niclos-amide, 27 min |
|---|---|---|---|---|---|---|---|---|---|
| 0 | A | | | | | | 4.6 | | 82.7 |
| | B | | | | | | 0.0 | | 95.0 |
| 1 | A | | | | | | 10.5 | | 87.4 |
| | B | | | | | | 0.0 | | 95.8 |
| 3 | A | 0.0 | | | | | 90.9 | | 4.3 |
| | B | 0.4 | | | | | 10.9 | | 84.6 |
| 7 | A | | | | | 16.8 | 70.7 | | 0.0 |
| | B | | | | | 0.0 | 40.4 | | 52.0 |
| 14 | A | | | | | 9.5 | 74.0 | | 0.0 |
| | B | | | | | 1.8 | 79.3 | | 3.0 |
| 30 | A | | | 0.0 | | 10.3 | 69.6 | | |
| | B | | | 1.6 | | 1.5 | 77.1 | | |
| 60 | A | | | | 0.0 | 3.8 | 39.5 | | |
| | B | | | | 1.5 | 6.4 | 60.5 | | |
| 120 | A | 0.0 | | 1.9 | 2.1 | 0.0 | 15.1 | | |
| | B | 2.1 | | 6.9 | 5.9 | 2.7 | 7.7 | | |
| 180 | A | 4.5 | | 0.0 | | 0.0 | | 9.2 | |
| | B | 10.2 | | 1.3 | | 0.0 | | 9.0 | |
| 270 | A | 1.5 | 0.0 | 3.1 | 8.3 | 0.0 | | 8.4 | |
| | B | 3.2 | 0.6 | 0.4 | 1.6 | 1.6 | | 8.0 | |
| 365 | A | 0.4 | 0.0 | 1.6 | 1.4 | 0.0 | | 7.6 | |
| | B | 4.5 | 0.5 | 0.0 | 2.2 | 0.6 | | 2.5 | |

[a] Days after treatment.

The distribution of metabolites is presented in **Table 7**. HPLC analyses revealed that the degradation patterns of the two labeled test substances were very similar. [$^{14}$C]Niclosamide represented 95% of the applied dpm at 0 DAT and was not detected in the label A test system by 7 DAT and by 30 DAT in the label B system. Aminoniclosamide was the major anaerobic metabolite through 4 months post-treatment, identified by HPLC and TLC cochromatography. More aminoniclosamide was formed in the anaerobic test system than in the aerobic test system (**Tables 4** and **7**). Its formation is related to low redox potential (*13*). All other metabolites were formed only after the parent niclosamide had transformed into aminoniclosamide. These metabolites are therefore derivatives of aminoniclosamide. Aminoniclosamide was not detected after 120 DAT.

The values in **Table 7** were used to perform linear regression analyses to determine the half-life of niclosamide. These results are presented in **Figure 2**. Half-lives of 0.65 day ($k = 1.0598$ day$^{-1}$) and 2.8 days ($k = 0.24818$ day$^{-1}$) were calculated for the label A and label B niclosamide, respectively. The disappearance of niclosamide showed good agreement with a first-order degradation, as the coefficients of determination for the regressions were 0.883 and 0.897.

**Figure 4** is a graphical representation of the formation and decline of aminoniclosamide in the test systems. The formation and decline of the other metabolites are graphically presented in **Figure 5**.

Only after the transformation of niclosamide to aminoniclosamide were several minor metabolites observed in the water and sediment extracts. Most notable of these metabolites are the very polar unknowns 4 and 6, eluting at 3 and 5 min by HPLC which, as in the aerobic aquatic metabolism study, are similar to the unknowns observed and identified in the aqueous photolysis study (*15*) as carbon dioxide and short-chain aliphatic acids. Unknown 4 reached a maximum level of 5% of the applied dpm in the label A study and ~10% in the label B study,

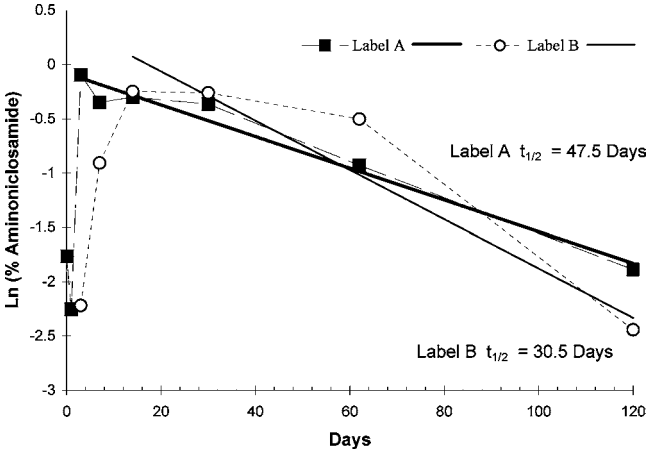



**Figure 4.** Formation and decline of aminoniclosamide concentrations in the anaerobic aquatic metabolism study.

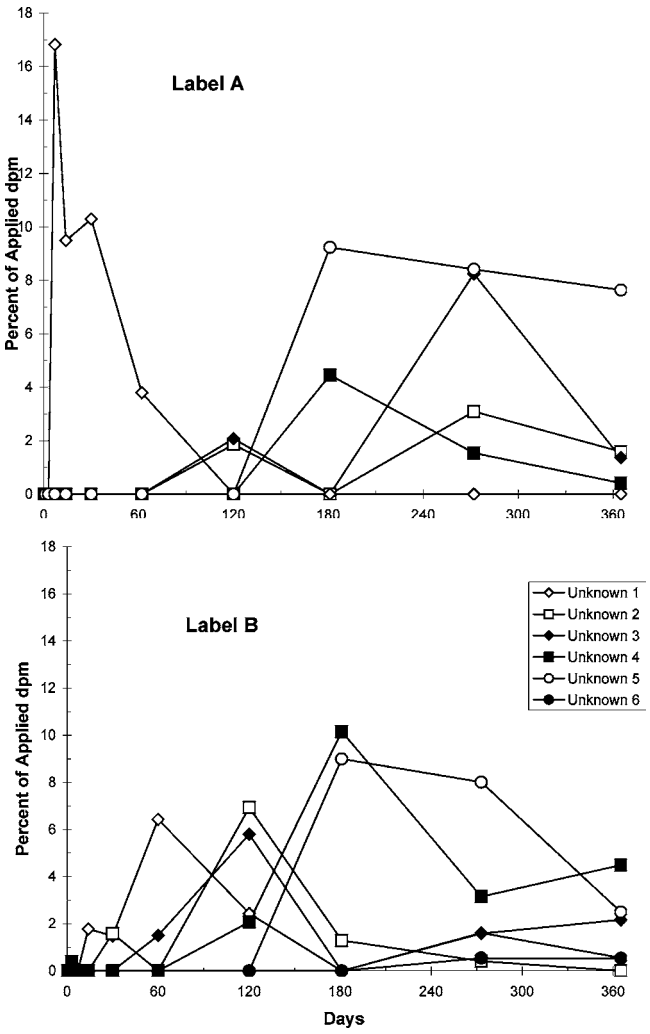



**Figure 5.** Formation and decline of anaerobic aquatic metabolites.

both at 180 days. By 365 DAT it had declined below 1% in the label A samples and 5% in the label B samples. At 270 DAT a metabolite unique to the label B system, unknown 6, was observed at 5 min. This was the only metabolite unique to a specific test system.

The initial characterization of the metabolites was accomplished using radioflow HPLC cochromatography with available standards. By using a systematic approach, each of the reference standards listed in **Table 1** was ruled out as a

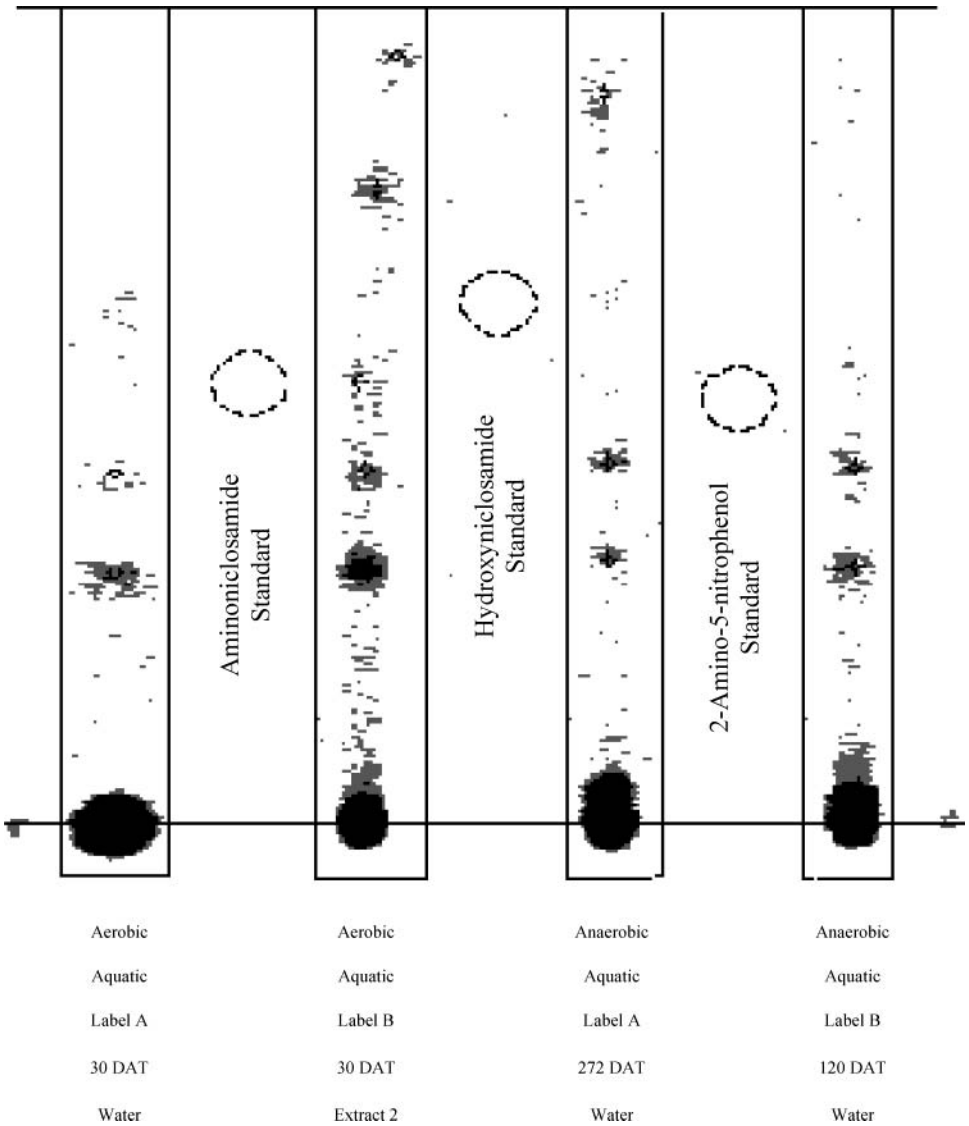



**Figure 6.** Comparison of aerobic aquatic metabolites with anaerobic aquatic metabolites using the primary TLC method. Nonlabeled standards are indicated by broken circles.

match for unknowns 1, 2, 3, and 5 formed in the study. None provided matches for these unknowns from either label. For the label A samples, TLC analysis did not show correspondence of 3-hydroxybenzoic acid or 4-chlorophenol with any radioactive areas. Analysis of 2,5-dihydroxybenzoic acid by HPLC did not match any radioactive peaks. From the label B study, 4-aminoresorcinol, 2-chloroaniline, 4-chlorophenol, 4-nitroaniline, 4-aminophenol, and 2-aminophenol were cochromatographed with extracts by HPLC and/or TLC with no match with radioactive peaks. Similar HPLC and TLC degradation patterns are observed from the two labeled test substances. It is obvious from these patterns that the anaerobic metabolism of niclosamide results first in the formation of aminoniclosamide. The aminoniclosamide is then further metabolized into several compounds.

A side-by-side comparison was done of aerobic aquatic metabolism samples with anaerobic aquatic metabolism samples. **Figure 6** shows the TLC plate run using the primary dichloromethane/ethyl acetate/acetic acid 80:20:2 method. From this scan, it can be concluded that there are no metabolites formed in the anaerobic aquatic study that are not formed in the aerobic aquatic study. **Figure 7** is the TLC scan of the same samples run using the identification method chloroformm/methanol/water/acetic acid 13:7:1:2. The radioactive areas at $R_f$ 0.33 are unique to the anaerobic aquatic study samples. Both plates also confirm that the metabolites from the two labeled test substances are similar.

In the later sampling intervals (120−365 DAT), sediment-bound radioactivity following extraction exceeded 70% of the applied radioactivity. Therefore, the bound residues were further characterized by fractionation of the sediment organic matter. The results are presented in **Table 5**. Most of the bound radioactivity was contained in the humin fraction of the sediments.

## DISCUSSION

Niclosamide degraded rapidly in aquatic systems. In the aerobic aquatic metabolism, similar HPLC and TLC metabolite patterns were produced by the label A and label B test substances. From 0 to 21 DAT, the only transformation product observed in either test system was aminoniclosamide. More metabolites were observed to have formed by 30 DAT, with unknowns 2−5 exhibiting a nonpolar nature. Unknowns 3 and 5, being observed as metabolites arising from both labeled test substances, suggest that the benzoic acid-aniline bond linking

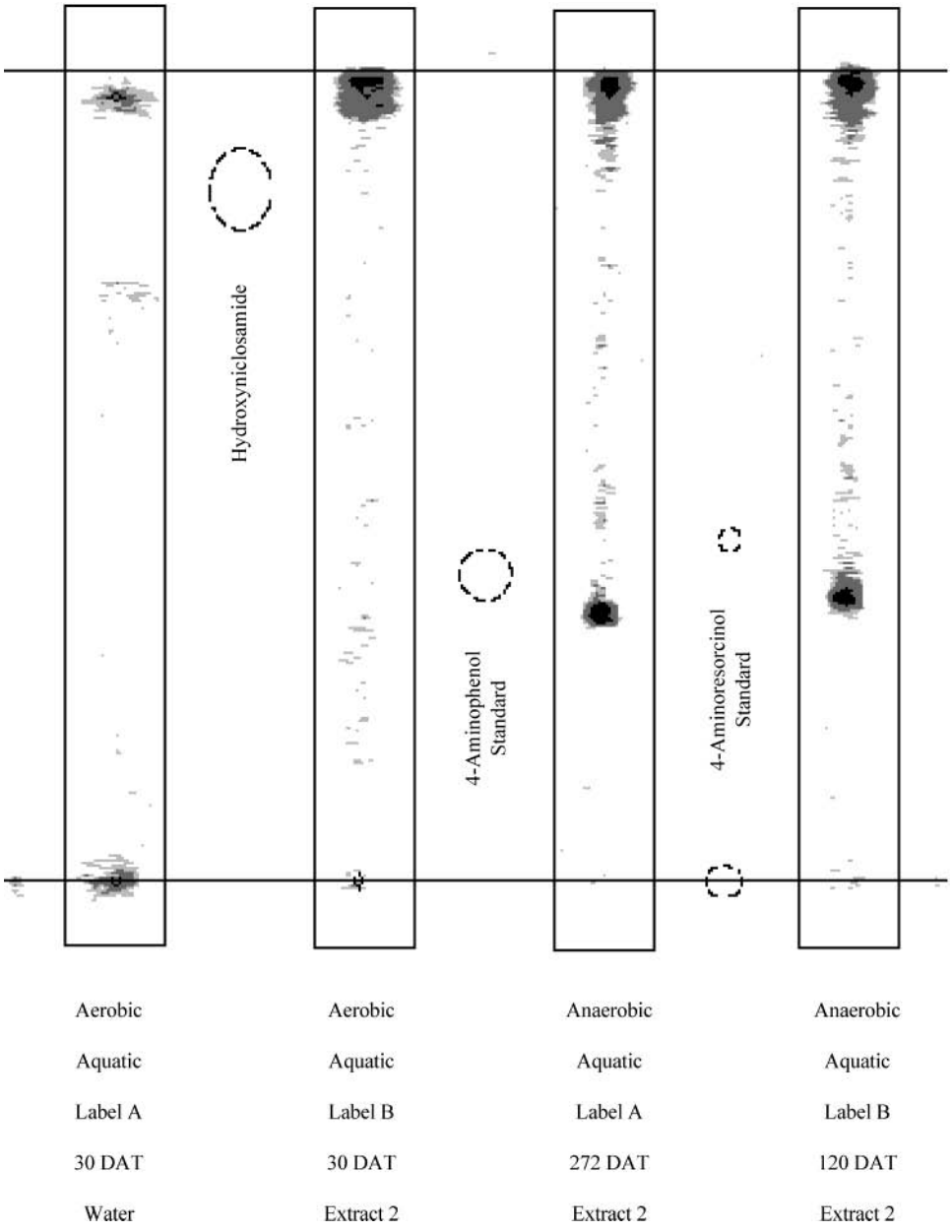



**Figure 7.** Comparison of aerobic aquatic metabolites with anaerobic aquatic metabolites using the secondary TLC method. Nonlabeled standards are indicated by broken circles.

the two ring structures in the molecule is intact. The proximity of their retention times to aminoniclosamide also suggested that these unknowns are a further transformation of aminoniclosamide, perhaps by hydroxylation. Hydroxyniclosamide reference standard, however did not coelute with radioactivity in the samples. None of the new peaks observed at 30 DAT matched the available reference substances. From these patterns of formation and decline, the aerobic aquatic metabolism of niclosamide initially produces aminoniclosamide, which itself is subsequently metabolized to form several compounds.

Niclosamide was metabolized even more rapidly under anaerobic aquatic conditions. The major transformation product observed in either test system was again aminoniclosamide. In the label A test system, aminoniclosamide was detected in the 0 DAT water sample and reached a maximum by 3 DAT. Other metabolites were formed by conversion of the aminoniclosamide. In fact, the other metabolites were not formed in appreciable quantities until the niclosamide had metabolized to aminoniclosamide. Therefore, they are metabolites of aminoniclosamide. The label B study produced one polar compound that was unique to the chloronitroaniline label. Otherwise, similar HPLC and TLC degradation patterns are observed from the two labeled test substances. Like the aerobic aquatic metabolism, anaerobic metabolism leads directly to the formation of aminoniclosamide. Several compounds are then formed from the continued metabolism of aminoniclosamide.

The polar materials formed in both aquatic studies are very similar in nature to polar degradates observed in the aqueous photolysis of niclosamide (*15*). Niclosamide was found to be completely photolyzed in pH 9 buffer into carbon dioxide and five or six aliphatic acids. This suggests that the aromatic ring can also be opened under typical environmental conditions.

The rate of degradation of aminoniclosamide was much slower than that of niclosamide in the aquatic studies. Once formed, aminoniclosamide did not metabolize appreciably over the course of the 30 day aerobic aquatic study. However, estimated half-lives were able to be calculated in the anaerobic aquatic metabolism study from the decline of aminoniclosamide

from its maximum observed concentrations at 3 DAT (label A) and 14 DAT (label B). For the label A aminoniclosamide a half-life of 47.5 days was calculated, compared to 30.5 days for label B. Muir and Yarechewski (*13*) reported half-lives of aminoniclosamide in static systems of 143 and 20 days for river and pond sediment, respectively. Although similar levels of aminoniclosamide were formed in the aerobic experiments of both the Muir and Yarechewski study (*13*) and the present study, our analyses found comparatively higher aminoniclosamide production in the anaerobic aquatic experiment (**Table 7**). Given the transformation of niclosamide to aminoniclosamide and the persistence of aminoniclosamide, particularly in the aerobic aquatic metabolism study, it would be interesting to conduct aquatic studies using aminoniclosamide as the test substance. It should be noted that the transformation of the nitro group of niclosamide to the amino group results in a very considerable loss of molluscicidal activity (*5*).

Muir and Yarechewski (*13*) observed 5-chlorosalicylic acid in sediment samples only after 40 h of water reflux. Their conclusion was that its presence in this extract was due to hydrolysis of aminoniclosamide in hot water. They also reported a less than complete material balance in static test systems, attributed to the lack of a $^{14}CO_2$ trapping system or poor combustion efficiency. In the current aquatic metabolism studies very few volatiles were formed. Volatile radioactivity formation was slightly greater under anaerobic than under aerobic conditions, but was still <1.5% after 365 days of incubation.

Transformation and sorption of niclosamide in the sediments were apparent by the increase in bound residues. The bound residues from both the aerobic aquatic and the anaerobic aquatic metabolism studies were composed mostly of humin.

## ABBREVIATIONS USED

DAT, days after treatment; dpm, disintegrations per minute; HPLC, high-performance liquid chromatography; LSC, liquid scintillation counting; TLC, thin-layer chromatography.

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