Biodegradation of o-Benzyl-p-Chlorophenol

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The extent of biodegradation of o-benzyl-p-chlorophenol, marketed as a
germicide under the name Santophen® 1 (Monsanto Co.), in river water, sewage,
and activated sludge was determined. Biodegradation was assessed by use of a
colorimetric procedure for phenolic materials, carbon analysis, and CO₂ evolu-
tion. In unacclimated river water, 0.1 mg of Santophen 1 per liter was degraded
within 6 days. In sewage, 0.5 and 1.0 mg/liter levels of Santophen 1 were degraded
in 1 day. Acclimated activated sludge achieved 80% biodegradation of 1.0
mg/liter Santophen 1 in 8 h and 100% in 24 h. When effluent from a semicon-
tinuous activated sludge unit, acclimated to 20 mg of Santophen 1 per liter
was used as the inoculum for the CO₂ evolution procedure, 60% of the total
theoretical CO₂ was evolved from Santophen 1. Based on the results of these
studies, indicating Santophen 1 to be readily biodegraded in at least four
biological systems, the continued use of present levels of Santophen 1 should
present no significant environmental problems.

Santophen® 1, o-benzyl-p-chlorophenol, has been used as an antimicrobial agent in liquid
disinfectant compositions for several years. At present this is almost the sole use; its primary
route of disposal is, therefore, through domestic sewage into natural waters either with or with-
out waste treatment. Based on the quantity of Santophen 1 manufactured, probable levels of
Santophen 1 in sewage would be of the order of 0.05 to 0.20 mg/liter if no biodegradation oc-
curred. Assuming direct discharge into natural waters without sewage treatment, these levels
would decrease to the low part-per-billion range by simple dilution. A small percentage of San-
tophen 1 is also used to prevent microbial growth in such places as cooling towers, air
scrubbers, and cooling ponds. As a result, possible intermittent release directly to natural wa-
ters may occur. Due to the increasing numbers of various minor chemicals in wastewater, it is
becoming necessary to establish the possible environmental impact of such chemicals. Ac-
ccordingly, studies directed towards establishing the extent of biodegradation of Santophen 1 in
natural systems were undertaken.

Previous studies on the biodegradation of antimicrobial agents have been confined pri-
marily to the cationic compounds. Barden and Isaac (3) and Pitter (5) reported the primary
biodegradation (methylene blue analysis) of cetyl pyridinium bromide to occur in activated
sludge. Cetyl trimethylammonium bromide was also found to undergo primary biodegradation
(5). Oxygen uptake data from biochemical oxygen demand (BOD) and Warburg analysis for
these two compounds has also been presented (2, 3, 5, 7). The Warburg data of Barden and
Isaac (3) would indicate cetyl pyridinium bromide to be oxidized at a more rapid rate than
cetyl trimethylammonium bromide. Gawel and Huddleston (paper presented at Conf. Amer.
Oil Chem. Soc., 23-26 April 1973, Los Angeles) examined the biodegradation of two aliphatic
and two aromatic quaternary ammonium anti-
microbials (10 mg/liter) in an organic salts-
yeast extract medium which had received in-
ocula from soil and raw sewage. More than 90%
biodegradation, as measured by colorimetry and in one case ultraviolet analysis, was re-
ported for all four compounds within 48 h in
unacclimated systems. The aliphatic com-
pounds were degraded more rapidly than the aromatic ones. In another study the biodegra-
dation of 14C-labeled Hyamine 3500, n-alkyldi-
methylbenzyl ammonium chloride, in river
water (0.01 to 1.0 mg/liter) and in 24-h semi-
continuous activated sludge units (1.0 to 10.0 mg/
liter) indicated substantial ultimate biodegra-
dation of this product (4). Biodegradation of
other cationic compounds has been summarized
by Swisher (10).

In the studies reported here, Santophen 1 biodegradation was examined in systems closely
approximating those which would be encoun-

394
tered by this compound in nature, i.e., river water, domestic sewage, and activated sludge at levels of Santophen 1 which might be expected in these environments and with techniques permitting assessment of the degree of ultimate biodegradation.

**MATERIALS AND METHODS**

**Assay for phenolics.** For determination of Santophen 1, the standard 4-aminopyrine (AAP) procedure (1) was modified as follows: (i) 50 ml of sample containing 50 mg or less of Santophen 1; (ii) 1 ml of 5% NH₄Cl; (iii) 1 ml of 2% AAP; (iv) 1 ml of 8% K₃Fe(CN)₆; (v) 20 ml of CHCl₃; (vi) filter through glass wool into optical cell (5-cm path). Absorbance, read at 460 nm, was increased about 0.1 by the presence of 5 µg of Santophen 1 in the 50-ml sample (0.1 mg/liter). Thus, the procedure, in principle, permits detection of about 0.01 mg of Santophen 1 per liter, but because of variations in controls and natural interferences, the net sensitivity is somewhat decreased. All phenolics were calculated in terms of Santophen 1, whether or not it may actually have been present.

**River water.** River water was obtained from the Meramec River, and 1-liter quantities were added to Mason jars (2 qt., approximately 1.9 liters). The waters were spiked with 0.1 mg of Santophen 1 per liter or, for comparative purposes, 5.0 mg of C₄₀ linear alkylate sulfonate (LAS) (Soap and Detergent Ass., reference sample no. 2, Nov. 1964), stored in the dark at room temperature, and analyzed from time to time. The methylene blue procedure (Hellige modification [11]) was used to determine LAS primary biodegradation.

**Domestic sewage and activated sludge.** Natural, domestic sewage was obtained from a local treatment plant. To reduce its apparent phenolic content (as much as 1 mg/liter), 500 ml of sewage was added to a 1-liter flask and shaken on a rotary shaker for 4 days. At this time 0.5 mg of Santophen 1 per liter was added and the samples were assayed periodically by the AAP method. Activated sludge studies were carried out by using the Soap and Detergent Ass. 24-h, semicontinuous procedure (8) and modified feed (9) (300 mg of glucose + 200 mg of nutrient broth per liter) and glass vessels. Activated sludge was gradually acclimated by incremental feeding of Santophen 1. Biodegradation was assessed by measuring disappearance of dissolved organic carbon (DOC) (centrifuged sample) with a Beckman carbon analyzer and of phenolics by the AAP method. It was found that upon addition of 0.5 mg of Santophen 1 per liter to activated sludge mixed liquor (about 2,500 mg of suspended solids per liter), only 0.07 mg/liter was found in the liquid phase, the remaining 0.43 mg/liter presumably having been adsorbed onto the solids. Therefore, the entire mixed liquor was sampled for AAP analysis, rather than just the liquid phase. A sample (20 ml) of mixed liquor was acidified with 0.4 ml of concentrated HCl and extracted twice with 25-ml portions of chloroform. The extracts were combined and the chloroform was evaporated. Before the chloroform was completely gone, 5 ml of distilled water was added, and another 45 ml was added after, to avoid loss of phenolics by evaporation or by failure of a dried residue to redissolve. The AAP procedure was then performed in the usual manner. This extraction procedure permitted 75% recovery at the 1 mg/liter level of Santophen 1.

**CO₂ evolution.** CO₂ evolution studies were performed with the apparatus and procedure reported by Thompson and Duthie (12). An inoculum flask, containing 50 mg of yeast extract per liter in 500 ml of BOD dilution water (Ref. 1, p. 416-417), received 20 mg of Santophen 1 per liter and 50 ml of effluent liquor from the semicontinuous activated sludge unit that was degrading Santophen 1 at the 20 mg/liter level. After 2 weeks the flask contents were added to 5,500 ml of BOD medium containing 20 mg/liter of Santophen 1 in the CO₂ evolution bottle. Evolved CO₂ swept out by bubbling 50 ml/min of CO₂-free air through the medium, was trapped in 0.05 N Ba(OH)₂ and quantitated by titration with 0.1 N HCl. CO₂ values from a control bottle, receiving a similar inoculum and no test compound, were subtracted from the gross CO₂ values from the Santophen 1 unit.

**RESULTS AND DISCUSSION**

Figure 1 depicts the biodegradation of LAS and Santophen 1 in river water. From the curve at the left it can be seen that 5 mg of LAS per liter had undergone essentially complete primary biodegradation within 8 days. The apparent natural phenolic content of this particular river water was 0.04 mg/liter and was stable for the first week. The apparent decrease at days 8 and 10 may be an analytical artifact. The river water receiving 0.1 mg of Santophen 1 per liter, (Fig. 1, right) showed the full amount present at 2 days, but decreased to the level of the control unit after 6 days. At eight days another 0.1 mg of Santophen 1 per liter was added and this had substantially disappeared by the next day. Another 0.1 mg/liter was added at 10 days and had completely degraded by the time the waters were next sampled at 13 days.

The biodegradation of Santophen 1 in natural sewage is shown in Fig. 2. The sewage used had an apparent natural phenolic content of 0.95 mg/liter, which decreased to below 0.1 mg/liter after 4 days of incubation. At this time 0.5 mg of Santophen 1 per liter was added and was substantially gone after 1 day on the shaker. On day 6 the sewage was respiked with 1.0 mg of Santophen 1 per liter and this was degraded by the next day. A parallel shake flask containing 0.50 mg of Santophen 1 per liter in deionized water was shaken along with the experimental flask during days 4 to 8. Daily analysis by the AAP method yielded values of 0.45, 0.50, 0.59, 0.58, and 0.56 mg/liter, indicating both the stability of Santophen 1 in a nonbiological system.
and the relative reproducibility of the AAP procedure.

The behavior of a semicontinuous activated sludge unit receiving Santophen 1 over a 14-week period is shown in Fig. 3. Santophen 1 levels were gradually raised from 0.5 to 20 mg/liter over the first 60 days. The net DOC, an indication of the apparent residual Santophen 1, was obtained by subtracting the total DOC from a control unit receiving no Santophen 1 from the total DOC of the activated sludge unit receiving Santophen 1. The results indicate no significant upset in the performance of the unit and the DOC removal from Santophen 1 (20 mg/liter) averaged over 95%. Much of the Santophen 1 could have been adsorbed to solids, but results given below indicated that it was degraded by the system. During the 5 weeks of steady-state operation with 20 mg of Santophen 1 per liter in the daily feed, the control effluents averaged 6.9 ± 0.4 mg of DOC per liter and the Santophen 1 effluents averaged 0.5 ± 0.3 above the control. With the DOC of the control influent at 200 mg/liter and the Santophen 1 at 12.5, these values correspond to 96.5% DOC removal for the glucose-nutrient broth and 96% for the Santophen 1.

Figure 4 shows the results when 2.0 mg of Santophen 1 was added to 1,500 ml of mixed liquor (1.33 mg/liter) on day 15 of the semicontinuous activated sludge run. AAP analysis of the mixed liquor showed 0.04 mg/liter immediately before feeding and 1.0 mg/liter immediately after feeding (75% recovery). This amount was still present 15 min later, was 30% gone at 2.5 h, 80% gone at 8 h, and completely gone by 24 h. AAP analysis indicated the apparent phenolic content of a control sludge to range from 0.02 to 0.07 mg/liter throughout the experiment.

The CO₂ evolution procedure was used to further substantiate that the extensive removal of Santophen 1 from the activated sludge system indicated above was due to biodegradation. Formation of substantial amounts of CO₂ from the Santophen 1 could hardly result from any mechanism other than biodegradation. The clear effluent from the semicontinuous activated sludge unit at day 60 was used as seed to develop the inoculum. Figure 5 demonstrates the net CO₂ evolution curve for Santophen 1 and, for comparative purposes, typical curves from trisodium citrate and C₁₂ LAS (Soap and Detergent Ass. no. 2 Nov. 1964). The Santophen 1 curve shows a 1-week lag before initiation of CO₂ evolution followed by a relatively rapid rate of CO₂ production until about 60% of theoretical was achieved at 27 days. CO₂ evolution from both C₁₂ LAS and trisodium citrate proceeded with less of a lag period and also achieved approximately 60% at termination.
Acidification of the medium at the end of the run showed that an additional 30% of CO₂ had been formed in the case of the citrate, but had been retained in the medium by the three sodiums of the citrate. The Santophen 1 and LAS cultures did not show any significant additional amount of CO₂ upon acidification.

The 7-day lag in the Santophen 1 system very probably resulted from the microbiocidal action of the Santophen 1 at its relatively high concentration of 20 mg/liter in the simple minimal salts medium containing only a minimal inoculum. This situation differs from the activated sludge system, in which the biomass is some 1,000 times greater. It differs likewise from the calculated environmental situation wherein the Santophen 1 concentration is only about 0.001 as high. Successful biodegradation of a microbiocidal agent obviously must be dependent upon the agent to biomass ratio, and the fact that the 1,000-fold increase in ratio in the CO₂ test resulted in only a 7-day lag suggests that a considerable margin of safety exists.

The net CO₂ evolved in the Santophen 1 system must have come predominantly from the Santophen 1 itself since the only other carbon source was the inoculum. The endoge-
nous CO₂ from the inoculum alone in the control run is less than a third of the net from the Santophen 1 (Table 1). This endogenous CO₂ represents substantially all of the organic carbon present in the inoculum as indicated both by calculation and by organic carbon analysis (Table 1). The organic carbon analyses should be regarded as only semiquantitative since our procedure was near its limit of sensitivity at these low carbon levels, particularly so far as absolute rather than relative values are concerned.

Because of the variety of pathways by which microorganisms assimilate and use various sorts of organic carbon sources, quantitative interpretations of the CO₂ evolution figures cannot be offered. At one extreme, the carbon in all three materials may have been completely assimilated, but with a higher proportion converted to protoplasm in the case of the Santophen 1 and LAS and a higher proportion utilized for energy in the case of the citrate. At the other extreme, the lower CO₂ evolution from the Santophen 1 and the LAS may be the result of incomplete biodegradation of these two materials under the relatively mild conditions of the tests. In any case, CO₂ formation to the extent of 60% of theoretical indicates that biodegradation has been very extensive.

The data from these studies show Santophen 1 to be readily biodegraded in four different biological systems at concentrations ranging from 0.1 to 20 mg/liter. In those instances where comparisons were made, Santophen 1 degraded at least as readily as LAS. Based on these laboratory results we expect that Santophen 1 biodegrades in natural systems as well, and thus should introduce no significant environmental problems.

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**TABLE 1. Biodegradation in the CO₂ evolution system (6 liters)**

| Measurement                              | Amt degraded | Control | Santophen 1 | Citrate | C₄ LAS |
|-----------------------------------------|-------------|---------|-------------|---------|--------|
| Test compound (mg)                      |             | 120     | 120         | 120     |        |
| Test compound (mmol)                    |             | 0.550   | 0.571       | 0.345   |        |
| Atoms of C per mol                      |             | 13      | 6           | 18      |        |
| Theoretical CO₂ (mmol)                  | 1.55        | 7.15    | 3.43        | 6.22    |        |
| Total CO₂ evolved (mmol)                | 1.55        | 5.94    | 4.72        | 5.95    |        |
| Per cent of theoretical                 |             | 4.39    | 3.17        | 3.93*   |        |
| Calculated OC, initial (mg)             | 11*         | 86.0*   | 41.2*       | 74.7*   |        |
| Calculated OC, initial (mg/liter)       | 2*          | 14.3*   | 6.9*        | 12.5*   |        |
| Measured OC, initial (mg/liter)         | 2.5         | 13      | 8           | 11*     |        |
| Measured OC, final (mg/liter)           | 2.5         | 3       | 2.5         | 4*      |        |

* Run in another set, with different control.
* Seed culture started with: yeast extract, 25 mg (organic carbon ~10 mg); Santophen 1, 10 mg (organic carbon ~7 mg); raw sewage, 60 ml (organic carbon ~5 mg). Organic carbon content of initial seed culture was 22 mg; 11 mg at time of inoculation assuming 50% assimilation during incubation.
* Exclusive of inoculum.