Biodegradation of 2,4-dichlorophenoxyacetic acid using an acidogenic anaerobic sequencing batch reactor

H. Chin, P. Elefsiniotis, and N. Singhal

Abstract: A bench-scale study was carried out to investigate the potential to biologically treat 2,4-dichlorophenoxyacetic acid (2,4-D) contaminated wastewater in an anaerobic sequencing batch reactor (ASBR), operated in the acid-phase digestion mode. The effects of 2,4-D feed concentration (20 to 200 mg L\(^{-1}\)) and temperature (ambient and 33 °C) on biodegradation were investigated at a hydraulic retention time of 48 h and a solids retention time of 10 d, using glucose as a supplemental substrate. Following a long acclimation period of about 100 d, complete 2,4-D degradation was observed at feed concentrations of 20 and 100 mg L\(^{-1}\). However, at a 2,4-D concentration of 200 mg L\(^{-1}\), only 65% removal was achieved. Overall, operation at an ambient temperature resulted in a slightly better performance than that at 33 °C. An adaptation period of approximately a week was required any time the 2,4-D concentration was increased, indicating a sensitive behavior towards shock loadings. On the other hand, glucose was completely and readily degraded throughout the study. A sequential utilization pattern of glucose and 2,4-D was also observed, with degradation of both substrates following first-order kinetics. Moreover, volatile fatty acids (VFAs) were the main products of acidogenesis, accounting for 65% of the effluent soluble chemical oxygen demand (COD), with acetic acid being by far the most predominant VFA detected.

Key words: anaerobic sequencing batch reactor, acidogenesis, kinetics, 2,4-D, glucose, volatile fatty acids.

Introduction

Herbicide use in pastoral agriculture has been dominated by phenoxy hormone products for the control of broadleaf weeds in pastures. 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most widely applied phenoxy herbicides in many parts of the world (Wilson et al. 1997b; Botrè et al. 2000). In New Zealand, for instance, 68% of all pesticides used belong to the phenoxy herbicide class (Holland and Anis 1999). Consequently, 2,4-D has been frequently detected in surface and ground waters not only in New Zealand (Close 1993) but also in North America and Europe (Fielding et al. 1992; Mangat and Elefsiniotis


P. Elefsiniotis\(^1\) and N. Singhal. University of Auckland, Department of Civil and Environmental Engineering, Private Bag 92019, Auckland, New Zealand.

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\(^1\)Corresponding author (e-mail: t.elefsiniotis@auckland.ac.nz).
Extensive use of pesticides has a potential adverse impact on human health and the environment. 2,4-D is toxic to plants, microflora, and human beings (Papanastasiou and Maier 1982; McTernan and Pereira 1991; Poggi-Varaldo et al. 1999). Various regulatory authorities worldwide have introduced control measures to address pesticide application (Manitoba Agriculture 1997; Ministry of Agriculture and Fisheries 2001). Contamination of the environment, however, due to pesticide discharges from manufacturing plants (i.e., untreated or partially treated effluents), storage sites, accidental spills, surface runoff, and inefficient application technologies still remains a particular concern.

In recent years, biological treatment methods have become more common in the treatment of pesticide-contaminated wastewaters. Although a plethora of studies exists on 2,4-D biodegradation, they have mainly explored the behavior of mixed and (or) pure batch cultures under aerobic conditions, with a focus on biochemical or genetic aspects (Saleh et al. 1980; Hill et al. 1986; Shaler and Klecka 1986; Smith et al. 1994). There is limited information, however, on the feasibility of biodegrading concentrated 2,4-D-contaminated wastewaters in continuously operating bioreactors. Activated sludge systems have been used to treat 2,4-D and related compounds with mixed results (Hill et al. 1986; Orhon et al. 1989; Ettala et al. 1992; Meri et al. 2003). Although other technologies investigated such as anaerobic fluidized beds (Wilson et al. 1997a) and membrane bioreactors (Buenrostro-Zagal et al. 2000) have been promising from a pesticide removal perspective, operational complexity and associated costs can be negative factors. Therefore, removal of concentrated 2,4-D from wastewaters prior to discharge into aquatic systems or municipal treatment plants remains a challenge for many industrial treatment systems.

Industrial wastewater can contain up to 500 mg L\(^{-1}\) of 2,4-D, and there may be a great variability in the 2,4-D loading (Tyler and Finn 1974; Mangat 1997; Buenrostro-Zagal et al. 2000). Fluctuations in wastewater quantity, quality, and temperature normally experienced in practice may render continuous-flow, multiple-tank processes less effective. Sequencing batch reactors (SBRs) can be an attractive alternative, mainly because of their simple and flexible operation and cost effectiveness, especially for small-scale operations (Irvine and Ketchum 1989). Mangat and Eflesiniotis (1999) have reported that a removal efficiency of over 95% was obtained in an aerobic SBR treating up to 300 mg L\(^{-1}\) of 2,4-D. Sequencing batch reactors are versatile enough to handle aerobic, anaerobic, and anoxic conditions simply by varying operating strategies. Anaerobic sequencing batch reactors (ASBR) have been a relatively recent addition to wastewater treatment practice (Bagley and Brodkorb 1999; Chin et al. 2002). Anaerobic sequencing batch reactor technology has been successfully employed in removing organic compounds and (or) nutrients from wastewaters (Dockhorn et al. 2001, Fongsatitkul et al. 2004).

This research investigated the treatability of 2,4-D-contaminated wastewater in a continuously operated acidogenic ASBR. To explore the biodegradation behavior of the system, the effects of variation in 2,4-D feed concentration and temperature were studied in detail, in the presence of glucose serving as a supplemental substrate.

**Materials and methods**

**Reactor setup and operation**

The bioreactor used was made of 10-mm-thick Plexiglas® with an internal diameter of 0.10 m, a total volume of 3 L, and an operating liquid volume of 2 L. Sealing was achieved by a bolted O-ring-mounted Plexiglas® stopper. Five ports were installed for feeding, decanting, sludge sampling and wasting, biogas collection, and nitrogen gas addition. Nitrogen gas was introduced in the headspace during feeding to purge the system from any remaining oxygen. Magnetic stirrers provided mixing, while peristaltic pumps carried out feeding and decanting. The operation was controlled by a series of timers.

The 24-h operating cycle consisted of the following periods: (a) filling, 15 min; (b) reaction, 22 h 55 min; (c) settling, 30 min; and (d) decanting, 20 min. At the end of each cycle, 1 L of the supernatant was decanted, followed by feeding of an equal amount of synthetic wastewater. A control wastage spigot was installed to allow control of solids retention time (SRT). The system operated at a nominal SRT of 10 d and a hydraulic retention time (HRT) of 48 h. Both values selected for the operational parameters were based on previous 2,4-D degradation studies (Ndon and Dague 1997; Mangat and Eflesiniotis 1999).

During the temperature-controlled run, a heating coil and a glass-fiber insulation jacket were employed. The temperature was controlled by a Variac voltmeter attached to the heating coils that were wrapped around the base of the reactor. Furthermore, throughout the experiment, the reactor pH was maintained between 4.5 and 5.0 to encourage the growth of acido-genic microorganisms (Maharaj and Eflesiniotis 2001). When necessary, the pH was controlled by the addition of an aqueous solution either of 0.05 N sulfuric acid or 0.05 N sodium hydroxide, respectively.

**Biomass and feed characteristics**

The ASBR was seeded with anaerobic sludge from the digesters of a local facility (North Shore Wastewater Treatment Plant, Auckland, New Zealand). The initial concentration of mixed liquor volatile suspended solids (MLVSS) was approximately 10 000 mg L\(^{-1}\). The synthetic wastewater used was prepared by modifying a recipe available in the literature (Bhattacharya et al. 1995). To ensure that a sufficient amount of carbon was available, glucose was added as a supplemental substrate at a concentration of 1000 mg L\(^{-1}\) (expressed as chemical oxygen demand (COD)), throughout the study, except for a 5-d period during one of the runs to observe the 2,4-D degradation pattern in the absence of glucose. Tap water was used to provide additional micronutrients. Further details can be found in Chin (2002). During acclimation, the reactor was kept at ambient temperature (i.e., 20 to 25 °C) and fed daily with the synthetic wastewater containing 20 mg L\(^{-1}\) of 2,4-D (run 1). After complete...
Analytical methods

The influent, reactor content, and effluent were regularly analyzed (several times a week), except during the track studies when analysis was normally done on an hourly basis. The parameters determined included chloride ion, glucose, volatile fatty acids (VFAs), COD, total suspended solids (TSS), and volatile suspended solids (VSS). All samples, except those used for solids determination, were centrifuged at 3200 rpm (or 4000 rpm) for 8 min, filtered through a 25-mm-diameter 0.2-m filter (Phenomenex AFO-0501), and acidified to a pH of 2 with phosphoric acid. Samples analyzed for TSS and VSS were filtered using 55-mm-diameter glass microfiber filters (GF/C 1822 055).

Determination of 2,4-D involved measuring the amount of chloride ion in the sample. This was based on the observation that the amount of chloride ion present can be directly correlated to the amount of 2,4-D that has been degraded (Singhal and Roy 1988). Chloride ion determination was carried out by a Dionex DX-120 ion chromatograph with an AS9-HC column. Glucose was analyzed using the colorimetric assay method developed by Dische (1962). The reagent used was 2.2% w/v anthrone dissolved in concentrated H₂SO₄. Glucose readings were taken by a Hach spectrophotometer (model DR/2000). Volatile fatty acids (i.e., acetic, propionic, butyric, isobutyric, and valeric) were analyzed by gas chromatography (GC) using a Hewlett-Packard HP 6890 GC equipped with an EC1000 column and a flame ionization detector (FID). Chemical oxygen demand was determined using the closed reflux method outlined in section 5220D, and TSS and VSS by the methods outlined in sections 2540D, 2540 E, and 2540 F in APHA-AWWA-WEF (1998). The pH meter used was a Metrohm 704 meter and was calibrated weekly with HACH standard buffer solutions. Temperature measurements were conducted using a standard thermometer. Details for all analytical tests can be found elsewhere (Chin 2002).

Table 1. Summary of operating characteristics for all runs.

<table>
<thead>
<tr>
<th>Run</th>
<th>Feed 2,4-D conc. (mg L⁻¹)</th>
<th>Temperature (°C)</th>
<th>Mean MLVSS conc. (mg L⁻¹)</th>
<th>Days of operation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>22 ± 2</td>
<td>2450 ± 440</td>
<td>129</td>
</tr>
<tr>
<td>2A</td>
<td>100</td>
<td>22 ± 2</td>
<td>2630 ± 110</td>
<td>33</td>
</tr>
<tr>
<td>2B</td>
<td>100</td>
<td>33 ± 1</td>
<td>2480 ± 225</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>23 ± 2</td>
<td>2350 ± 60</td>
<td>26</td>
</tr>
</tbody>
</table>

*Excluding acclimation period.

2,4-D degradation was achieved, the concentration of 2,4-D was increased to 100 mg L⁻¹, which was held constant for both parts of run 2 (i.e., operated either at an ambient or a 33 °C temperature). Finally, during run 3, the 2,4-D concentration was raised to 200 mg L⁻¹. A summary of the experimental conditions is depicted in Table 1.

Results and discussion

Acclimation period

The dual purpose of acclimation is to increase the starting population of 2,4-D degraders and to induce enzyme-producing genes in other species. Biodegradation is possible only when an adequate amount of enzymes that are capable of metabolizing 2,4-D is produced. During the first 100 d of operation, 2,4-D degradation was practically negligible. On the other hand, glucose, serving as a supplemental substrate, was readily and completely consumed within the first 3 h of the reaction period (discussed later), indicating that a 2,4-D concentration of 20 mg L⁻¹ had no direct inhibitory effect on the biomass. However, after the first sign of noticeable 2,4-D degradation appeared on day 101, its percent removal increased rapidly until complete degradation was achieved within a few days later (Fig. 1). The observed pattern suggests that once the necessary biochemical mechanisms have been established and are operating, biodegradation can proceed fairly rapidly.

The long acclimation period witnessed in this investigation was not unexpected, as other studies on 2,4-D degradation have shown acclimation phases up to 80 d, under aerobic conditions (Orhon et al. 1989; Mangat and Elefsiniotis 1999). Moreover, anaerobic systems usually require longer acclimation times than aerobic ones (Speece 1996). It has been postulated that the length of the acclimation (lag) period in pure cultures can be linearly related to the initial 2,4-D concentration and the starting degrader population density (Greer et al. 1990).

As mentioned earlier, the reactor was originally seeded with an MLVSS concentration of 10 000 mg L⁻¹. However, a reducing trend in MLVSS was observed, resulting in a concentration drop below 2000 mg L⁻¹. The recommended MLVSS range for a low rate anaerobic reactor is between 2000 and 10 000 mg L⁻¹ (Speece 1996). After 30 d of operation, the reactor was reseeded to a MLVSS concentration of 10 000 mg L⁻¹. Following reseeding the MLVSS content decreased again fairly rapidly, until it stabilized at the approximately 2450 mg L⁻¹ level. It should be also noted that, during acclimation, the pH had a tendency to drop below 4.5; therefore, sodium hydroxide was added to the feed to maintain a reactor pH of about 4.7.
Effect of 2,4-D concentration and temperature
Following complete degradation of 2,4-D at 20 mg L\(^{-1}\) for about 3 weeks, the feed 2,4-D concentration was then increased to 100 mg L\(^{-1}\) (run 2A). The percent 2,4-D degradation pattern obtained during the first 2 weeks of this run is illustrated in Fig. 2. It is apparent that the reactor displayed a gradual adaptation behavior for about 10 d before a high 2,4-D removal (>90%) was achieved. The time required for the bacteria to adapt to the increased 2,4-D concentration may be an indication of the susceptibility of the system to shock loadings. Thereafter, the reactor consistently degraded more than 95% of the 2,4-D in the feed, exhibiting also a slight increase in the MLVSS content (Table 1).

During the second part of this run, the temperature was raised to 33 °C to explore its effect on degradation behavior (run 2B). Although the system continued to completely degrade 2,4-D at 33 °C, there appeared to be no apparent advantage in raising the temperature. Data from representative track studies shown in Fig. 3 reveal a small but consistent decline in the degradation performance between ambient temperature (run 2A) and 33 °C (run 2B), respectively, which is further reflected in the corresponding kinetic parameters (discussed later). This was accompanied by a modest decrease in the average MLVSS concentration (Table 1) and in the visually observed sludge settling rates as well. Although the preference of acidogenic systems for temperatures in the ambient range has not been explicitly documented, it would have an important practical implication, especially when the acid-phase digestion products such as VFAs are purposefully produced for other applications such as biological nutrient removal processes (Banerjee et al. 1999; Maharaj and Elefsiniotis 2001).

The next run was therefore conducted at an ambient temperature and a 2,4-D concentration of 200 mg L\(^{-1}\) (run 3). It was observed that during the first 2 d immediately after the introduction of the higher concentration, the system continued to degrade approximately the same amount of 2,4-D as it had achieved prior to the change (i.e., about 100 mg L\(^{-1}\)). This reinforces the observation made earlier regarding the reactors limited capability to handle shock loadings, as the bacteria appeared to require an adaptation period to concentration variations. However, within 1 week, a gradual increase in 2,4-D degradation was observed, reaching a plateau at approximately the 65% (or 130 mg L\(^{-1}\)) level, until the termination of the study (Fig. 4). Although, it is not possible to predict the long-term behavior of the reactor, this observation serves as an indication of an upper limit in the degradation potential of the system. Throughout this run the MLVSS values remained reasonably stable at about 2350 mg L\(^{-1}\).

Substrate utilization patterns
In all experimental runs, glucose was the preferred substrate and was always completely removed in less than 3 h, from the beginning of the study, regardless of the variations in 2,4-D concentration and temperature. A representative example of the sequential removal of glucose followed by that of 2,4-D is depicted in Fig. 5. The sequential utilization pattern observed during this investigation is in agreement with observations reported in the literature regarding the presence of glucose and 2,4-D in a dual-substrate environment (Papanastasiou and Maier 1982; Mangat and Elefsiniotis 1999). Other studies have also indicated such a trend involving simpler, nonchlorinated organic compounds and more complex, chlorinated ones (Klecka and Maier 1988; Sàez and Rittmann 1991). This phenomenon, known as diauxic growth, is due to catabolite repression, which denotes the repression of enzymes that degrade a less rapidly metabolized energy source in the presence of a more rapidly metabolized one (Chang and Alvarez-Cohen 1995).
During the later stages of run 2A, the potential of acclimated biomass to degrade 2,4-D in the absence of supplemental substrate was also investigated. To accomplish this purpose, the system was operated without adding glucose to the feed for a period of 5 d. On the last day of this period, a track study was performed; the results have been included in Fig. 5 (i.e., 2,4-D no glucose curve). It is apparent that in the absence of glucose 2,4-D was degraded rapidly following a short lag phase of about 1 h. However, the distinction between the two degradation patterns of 2,4-D, in the presence and absence of supplemental substrate, became less pronounced as time progressed and practically disappeared after 8 h of reaction time, when more than 80% removal had been already achieved. The ability of acclimated biomass to degrade 2,4-D (as a sole carbon source) without any appreciable lag phase has also been observed under aerobic conditions (Mangat and Elefsiniotis 1999). Following this exploratory diversion, regular operation was resumed to provide an adequate amount of substrate and maintain the biomass level in the reactor, with an overall performance identical to that observed for the rest of this run.

**Biodegradation kinetics and specific rates**

Data for the calculation of the kinetic rate constants associated with glucose or 2,4-D consumption were obtained through a number of track studies, and the results are summarized in Table 2. Biodegradation of glucose appears to follow very closely first-order kinetics, as indicated by the small standard deviation values calculated for each run, leading to an average coefficient of variance (CV) of 9%. It is also evident that the kinetic rate remained practically constant during all ambient temperature runs, with a mean value of $1.12 \pm 0.10 \, \text{h}^{-1}$. In a similar fashion, the 2,4-D biodegradation behavior can be reasonably well approximated by a first-order kinetic model, with an average CV of 27%. Although the rate constant was not affected by the variation in 2,4-D concentration up to 100 mg L$^{-1}$, a further increase in concentration resulted in a remarkable decline to about one third of the previous average value (Table 2). In addition, the increase in temperature during run 2B resulted in a decrease in the 2,4-D rate by about 25%, with respect to run 2A, which reinforces the remarks made earlier on the potential negative effect of temperature increase on 2,4-D biodegradation in acidogenic environments.

The specific glucose utilization rate (milligram of glucose consumed per milligram of reactor VSS per day) and the specific 2,4-D utilization rate (milligram of 2,4-D consumed per milligram of reactor VSS per day) have also been included in Table 2. Overall, the specific glucose utilization rate followed the same trend as the corresponding first-order rate constant regarding apparent independence from the 2,4-D concentration. Statistical evaluation of the results also revealed that there is no statistically significant difference in the glucose rates at the 95% confidence level. On the other hand, the 2,4-D rate improved dramatically with an increase in the initial concentration from 20 to 100 mg L$^{-1}$. However, a further increase in concentration did not seem to have an effect on the specific rate. Since specific rates reflect the ability of biomass to biodegrade the corresponding substrate, the existence of a plateau at higher 2,4-D concentrations suggests a possible limitation on the biomass to further enhance its performance under the conditions investigated.

**Acidogenic digestion products**

Throughout this study a favorable acidogenic environment was firmly established in the bioreactor manifested by the lack of gas production, a pH in the 4.5 to 5.0 range with minimal need for external adjustment, an oxidation-reduction potential (ORP) within the 80 to 150 mV range, and a steady VFA generation. Regarding the VFA production pattern, acetic, propionic, and butyric acids were generated on a constant basis, with an average percentage distribution (excluding the acclimation period) of 83, 11, and 6%, respectively. It is interesting to note that isobutyric acid was never detected, while valeric acid appeared on only two occasions. The VFA distribution (similar to glucose degradation) was reasonably consistent in all runs and appeared to be unaffected by the changes in 2,4-D concentration and (or) temperature. The predominance of acetic acid is a common characteristic of acidogenic digestion of municipal and industrial wastewaters. For example, it has been reported that during the acid-phase digestion of primary sludge, acetic acid accounted for 50% to 76% of the total VFAs formed (Elefsiniotis and Oldham 1994; Banerjee et al. 1999). The higher acetic acid percentage observed in this study can be largely attributed to the presence of glucose as the principal carbon source in the feed. Glucose, a simple carbohydrate, can be readily de-

### Table 2. Kinetic rate constants and specific consumption rates for glucose and 2,4-D.

<table>
<thead>
<tr>
<th>Run</th>
<th>First-order rate, $k_1$ (h$^{-1}$)</th>
<th>Specific rate (mg mg$^{-1}$ VSS d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>2,4-D</td>
</tr>
<tr>
<td>1</td>
<td>$1.15 \pm 0.08$</td>
<td>$0.21 \pm 0.06$</td>
</tr>
<tr>
<td>2A</td>
<td>$1.14 \pm 0.10$</td>
<td>$0.19 \pm 0.05$</td>
</tr>
<tr>
<td>2B</td>
<td>N/A</td>
<td>$0.15 \pm 0.04$</td>
</tr>
<tr>
<td>3</td>
<td>$1.08 \pm 0.13$</td>
<td>$0.07 \pm 0.02$</td>
</tr>
</tbody>
</table>
graded under anaerobic conditions to yield mainly acetic acid via the glycolytic pathway (Sawyer et al. 1994). In contrast, primary sludge contains complex carbohydrates, proteins, and lipids, which usually generate a mixture of two to five carbon atom VFAs under acidogenic conditions (Speece 1996; Maharaj and Elefsiniotis 2001).

The soluble compounds identified in the effluent from acid-phase digestion systems can be classified into three categories: soluble substrates, extracellular intermediate metabolites, and end products of this phase (Elefsiniotis and Oldham 1994). The main end products generated during the acidogenic digestion of municipal and industrial wastewaters are normally short chain VFAs, with minor amounts of other organic acids (formic and lactic), alcohols (ethanol, 2-propanol, butanol, and glycerol), aldehydes, and ketones (Gottschalk 1986; Speece 1996). Consequently, the soluble effluent COD in the form of VFAs, expressed as a percentage value or ratio, can be used as a performance indicator for an acidogenic system.

The corresponding VFAs contributing to effluent COD along with the measured soluble effluent COD values are portrayed in Fig. 6. For comparison purposes, the following three sets have been identified: acetic acid COD, other VFA COD (i.e., propionic and butyric), and COD in VFA form (i.e., the sum of the previous two sets of values). It is evident that, subsequent to the acclimation period, the reactor was characterized by a great degree of stability with reference to its acidogenic behavior. Overall, excluding acclimation, the percent soluble COD in the form of VFA averaged 65% of the total amount measured. This value compares favorably with other studies (conducted at a similar SRT of approximately 10 d) using primary sludge, which have shown a corresponding range between 60% and 75% (Elefsiniotis et al. 1996; Banerjee et al. 1999). Since it has been documented that changes in SRT can appreciably affect the COD in the VFA form value (Elefsiniotis and Oldham 1994), the above comparison is meaningful only among systems operated under similar SRT conditions. The remaining soluble COD can be principally attributed to the metabolic intermediates of the process, since both substrates were practically removed, except in run 3 where a residual 2,4-D amount of about 70 mg L$^{-1}$ was detected.

**Conclusions**

The application of an acid-phase ASBR proved to be an effective strategy for the treatment of 2,4-D contaminated wastewater. A long acclimation period of 100 d was required before the onset of 2,4-D degradation. Overall, the system was able to remove up to 130 mg L$^{-1}$ of 2,4-D at ambient temperature. However, the bioreactor appeared to be sensitive to shock loadings, since no additional 2,4-D removal was observed immediately following an increase in feed concentration. Ambient temperature (20 to 25 °C) resulted in a slightly better performance than that at 33 °C, in terms of 2,4-D degradation and reactor MLVSS content. Glucose, serving as a supplemental substrate, was completely degraded throughout the study in a sequential utilization fashion with respect to 2,4-D. In the absence of glucose, 2,4-D was used by the acclimated biomass as a sole carbon source in a manner similar to that observed in its presence. In addition, both glucose and 2,4-D consumption patterns followed closely a first-order kinetics behavior. Finally, successful acidogenesis resulted in a stable production of VFAs (mainly acetic acid, followed by propionic and butyric acids), which together accounted for 65% of the effluent soluble COD.

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