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Reductive Dechlorination of Methoxychlor by Bacterial Species of Environmental Origin: Evidence for Primary Biodegradation of Methoxychlor in Submerged Environments

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ABSTRACT: Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] is an organochlorine insecticide that undergoes dechlorination in natural submerged environments. We investigated the ability to dechlorinate this compound in seven environmental bacterial species (*Aeromonas hydrophila*, *Enterobacter amnigenus*, *Klebsiella terrigena*, *Bacillus subtilis*, *Achromobacter xylosoxidans*, *Acinetobacter calcoaceticus*, and *Mycobacterium obuense*) and the enteric bacterium *Escherichia coli* as a positive control. In R2A broth at 25 °C under aerobic, static culture, all species except *Ach. xylosoxidans* were observed to convert methoxychlor to dechlorinated methoxychlor [1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane]. The medium was aerobic at first, but bacterial growth resulted in the consumption of oxygen and generated microaerobic and weakly reductive conditions. Replacement of the headspace of the culture tubes with nitrogen gas was found to decrease the dechlorination rate. Our findings suggest that extensive bacterial species ubiquitously inhabiting the subsurface water environment play an important role in the primary dechlorination of methoxychlor.

KEYWORDS: *methoxychlor, facultative anaerobic bacteria, reductive dechlorination, oxidation−reduction potential, submerged environment*

INTRODUCTION

Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)-ethane, MXC] is an organochlorine insecticide used as an alternative for DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)-ethane]. Like DDT, the metabolism of methoxychlor in higher organisms has been well investigated in mammals, birds, fish, bivalves, and fungi.[1−4] The metabolism of this compound is suggested to be initiated by *O*-demethylation catalyzed by cytochrome P450 (CYP) enzymes[1] in what is known as a phase I reaction. In most cases, the demethylated metabolites undergo subsequent conjugation in reactions known as phase II reactions.

It is also accepted that microbial reductive dechlorination is an essential primary step for degradation of both aliphatic and aromatic organochlorine compounds.[5−10] With regard to DDT, *Klebsiella pneumoniae* (formally *Aerobacter aerogenes*),[11,12] *Escherichia coli*,[11] *Alcaligenes denitrificans*,[13] and *Eubacterium limosum*[14] have been reported to produce a dechlorinated metabolite known as DDD. Among the bacterial species listed above, *Eu. limosum*[14] and *K. pneumoniae*[15] were also found to convert methoxychlor to the dechlorinated metabolite [1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane, de-Cl-MXC], which corresponds to DDD. These bacterial species (with the exception of *A. denitrificans*) inhabit the intestinal tract, and it is likely that they contribute to the complete metabolism of methoxychlor in mammals.

Compared to metabolism in higher organisms, there have been few reports on the subject of the environmental fate of methoxychlor.[16−20] Moreover, the microbial species actually responsible for the dechlorination of methoxychlor in the natural environment are not well-known. However, primary degradation of methoxychlor is found to occur at a much faster rate under submerged conditions than under aerobic upland conditions.[16−18] Baczynski et al.[21] found that organochlorine pesticides, including methoxychlor, were removed rapidly by addition of anaerobic, methanogenic granular sludge. These led to the expectation that anaerobic bacterial species largely contribute to the first step of biodegradation in the submerged environment, in a manner similar to that of the biodegradation that occurs in the intestinal tract of mammals.

More recently, an earlier study by us[20] demonstrated that degradation of methoxychlor in a water−sediment model produces de-Cl-MXC as a metabolite. It should be noted that the dechlorination was not observed under strong reductive conditions, such as methanogenic or sulfidogenic conditions. Thus, we presume that dechlorination of methoxychlor is not limited to obligate anaerobes and that more oxygen-tolerable bacteria could play a major role. We also found that the dissipation started immediately after dosing, and the rates were high, with half-lives within the range of 4−7 days. This suggests that the dechlorination reaction is mediated not by inductive enzymes of specific microbes but by constitutive enzymes of microbes ubiquitously distributed in the surface water or terrestrial environment.

To test these assumptions, we investigated the microbial dechlorination of methoxychlor with various bacterial species, including facultative anaerobes. The strains we selected are of environmental origin or usually occur in terrestrial and/or surface water environments.[22] We believe the results of the present study will help to improve the current understanding of

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Table 1. ATCC Number and Characteristics of Bacterial Species Used in This Study[a]

species	ATCC no.	class	source	respiration	main habitat
Ae. hydrophila	7966	γ-proteobacteria	canned milk	FA	surface water environment
En. amnigenus	33072	γ-proteobacteria	soil	FA	aquatic and soil environment
K. terrigena	33257	γ-proteobacteria	drinking water	FA	aquatic and soil environment
E. coli	49980	γ-proteobacteria	−	FA	intestine of warm-blooded animals
B. subtilis	6501	bacilli	−	MA	widely distributed in nature
Ach. xylosoxidans	15173	β-proteobacteria	soil	A	aquatic and soil environment
Aci. calcoaceticus	23055	γ-proteobacteria	soil	A	soil, water, sewage
M. obuense	27023	actinobacteria	soil	A	soil environment

[a]Definitions of symbol and abbreviations: −, unknown; FA, facultative anaerobic, MA, microaerobic; A, aerobic.

the microbial degradation of methoxychlor in the natural environment.

MATERIALS AND METHODS

Chemicals. [Ring-U-^{14}C]MXC ([R-U-^{14}C]MXC, [^{14}C]MXC; radiochemical purity >99%, specific radioactivity 5.84 MBq/mg) was purchased from BlyChem Ltd. (Billingham, U.K.). Nonradiolabeled MXC was obtained from Wako Pure Chemical Industries (Osaka, Japan; purity >97%). All other chemicals were purchased from commercial suppliers.

Bacterial Species and Culture Conditions. Seven bacterial species, namely, *Aeromonas hydrophila*, *Enterobacter amnigenus*, *Klebsiella terrigena*, *Bacillus subtilis*, *Achromobacter xylosoxidans* (formally *Alcaligenes xylosoxidans*), *Acinetobacter calcoaceticus*, and *Mycobacterium obuense*, were obtained from the Japanese Collection of Microorganisms (JCM) maintained at the Institute of Physical and Chemical Research (RIKEN, Saitama, Japan). *E. coli* was obtained from the National Collection of Industrial, Marine and Food Bacteria (NCIMB, Scotland, UK). The ATCC numbers, phylogenetic classifications, and isolation sources are listed in Table 1. The bacteria were pregrown without addition of methoxychlor in R2A broth (Wako), comprising 0.5 g of peptone, 0.5 g of yeast extract, 0.5 g of casamino acids, 0.5 g of glucose, 0.5 g of soluble starch, 0.3 g of K_2HPO_4, 0.3 g of sodium pyruvate, and 0.05 g of $MgSO_4{\cdot}7H_2O$, per liter of Milli-Q water, pH 7.0−7.4. The species were cultured for 4 days at 25 °C under static, aerobic conditions.

Preparation of Test Medium and Inoculation. The test medium was prepared as follows: an aliquot of [^{14}C]MXC dissolved in acetone was poured into the bottom of a glass tube (inner diameter 15 mm and length 13 cm) and air-dried. A 5 mL volume of R2A broth was poured into the glass tube and allowed to stand overnight to dissolve the MXC. The depth of the medium was approximately 3 cm, and the final concentration of [^{14}C]MXC was determined to be 0.29 mg/L. The growth of each pregrown bacterial species was monitored by obtaining spectrophotometer measurements at 600 nm (OD_{600}) to determine the volume for inoculation. The initial cell density of the test culture broth was adjusted to OD_{600} = 0.005 by transferring the appropriate volume of pregrown medium (10.0−49.5 μL). After inoculation, the tubes were capped with custom-fabricated, air-permeable silicon sponge plugs (Silicosen; Shin-Etsu Polymer, Osaka, Japan) for aerobic culture. In addition, for anaerobic culture, a nitrogen stream was introduced into the headspace of the tubes for 30 s, and then the tubes were immediately plugged with butyl rubber stoppers (Sanshin Industrial, Kanagawa, Japan) to prevent aeration. Triplicate tubes were prepared for both culture conditions and for each of the eight species investigated. Noninoculated control medium was also prepared. The growth (OD_{600}) of each culture was monitored periodically for one selected tube. The OD_{600} measurement was performed immediately after agitation of the medium. Therefore, the values are partially attributed to resting and/or dead cells. The oxidation−reduction potential (ORP) of the culture medium was measured immediately before inoculation and 17 days after inoculation using a SevenMulti pH/ORP meter with an InLab Redox combined electrode (Mettler-Toledo, Tokyo, Japan). The electrode was soaked in the medium and kept for at least 5 min to stabilize the measured value.

Sampling and Thin Layer Chromatography Analysis. Methoxychlor and its metabolite were periodically analyzed and quantified by thin layer chromatography (TLC). An aliquot (approximately 20 μL) of each culture medium was spotted on a silica gel F_{254} precoated aluminum plate (Merck, Darmstadt, Germany) and developed for 15 cm with *n*-hexane/ethyl acetate (8/2, v/v). The plate was kept in close contact with an Imaging Plate for 2 days, and a latent image was scanned using a BAS-2500 Bioimaging analyzer (GE Healthcare, Tokyo, Japan). The TLC chromatogram of each sample lane (culture tube) was fractionated in ladder form, and the pixel density in each fraction was measured by the software provided by the instrument manufacturer. The percentage distributions of fractions (after subtraction of background pixels) corresponding to MXC and its metabolite were calculated.

Identification of Metabolites. The metabolite de-Cl-MXC was identified by TLC and HPLC cochromatography. [^{14}C]de-Cl-MXC was used as a reference standard. This compound had been obtained in our preceding study[20] and was identified using gas chromatography−mass spectrometry with electroionization (GC/EI-MS/MS) (Agilent 7683; Agilent Technologies, Tokyo, Japan).

For TLC cochromatography, samples were spotted adjacent to the reference standard on the same plate. The identity of each compound was confirmed by development with two different solvent systems. The developing solvents were benzene (100%) and *n*-hexane/ethyl acetate (8/2, v/v).

For HPLC cochromatography, identification was confirmed by matching the retention times between the metabolite and its reference standard. A Shimadzu LC-10A HPLC system (Shimadzu Corp., Kyoto, Japan) with a Ramona Star radioactive flow scintillation analyzer (Raytest Isotopenmessgeraete, Straubenhardt, Germany) was used for HPLC analysis of radioactive metabolites. The ^{14}C compounds were separated using a Capcell Pak C18 ACR column (4.6 mm i.d. × 250 mm, 5 μm; Shiseido Co., Ltd.) with mobile phases of acetonitrile (solvent A) and 10 mM ammonium acetate buffer (pH 4.6; solvent B). Elution was achieved using the following two-step linear gradient program: 0−20 min, A/B = 30/70 (v/v) to 90/10 (v/v), and 20−25 min, leading to 100/0, with a flow rate of 1.0 mL/min at a column temperature of 40 °C.

RESULTS AND DISCUSSION

ORP in Culture Medium. As shown in Figure 1, we confirmed that the microaerobic—reductive condition (ORP < 100 mV) was sufficiently established in both aerobic and anaerobic culture by measuring the ORP at the last sampling point (day 17). The initial ORP in the medium immediately after preparation was 97.6 mV. For the control, consisting of medium without microbes, the ORP rose to 189.6 mV under aerobic conditions and decreased to 24.6 mV under anaerobic conditions. The proliferation of the microbial cells consumed oxygen and resulted in the generation of reductive conditions in both culture tubes. For most of the species tested, ORP in anaerobic culture was almost the same as or lower than that in

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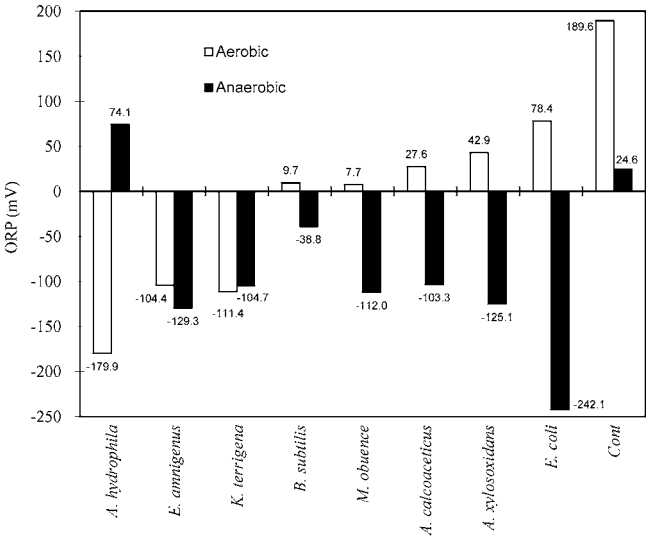

Figure 1. ORP in culture medium incubated under both aerobic and anaerobic conditions measured 17 days after inoculation of each bacterial species.

aerobic culture. Only the medium inoculated with *Ae. hydrophila* showed a contrasting result. The reason for this remains unclear.

It is generally accepted that the reductive conditions provided by low levels of oxygen are essential for reductive dechlorination of organochlorines.[5−10] In particular, dechlorination of aromatic organochlorines is known to require anaerobic conditions. Hiraishi[9] summarizes reductive dechlorination of polychlorinated biphenyl and dioxin by dehalorespiring bacteria. Stuart et al.[23] reported that an ORP lower than −100 mV is necessary for reductive dechlorination of pentachlorophenol. In contrast, Logan et al.[24] found that significant rates of reductive dechlorination of polychlorinated ethanes by *Pseudomonas putida* occur under partially aerobic conditions. Furthermore, Bouwer and McCarty[25] suggested that certain chlorinated aliphatic compounds can be reductively dechlorinated under denitrification conditions, which are generally accepted to be ORP values ranging approximately from −100 to +100 mV. The results of our investigation of *B. subtilis*, *Aci. calcoaceticus*, and *E. coli* in aerobic culture medium (described below) support this hypothesis. The findings by Muir et al.[19] using methoxychlor also confirm our results. The authors made weakly reductive conditions (ORP = −56 to −156 mV) in sediment by nitrogen aeration. They observed a higher degradation rate and a larger amount of the dechlorinated product than those in static or air-flow conditions (ORP > 100 mV).

Metabolism of MXC in Aerobic Culture. The time-course quantification of MXC and de-Cl-MXC in aerobic culture is shown in Figure 2, together with cell-growth curves. All eight of the species tested proliferated, and the R2A medium became turbid within 1−3 days. The cell density was maintained at an essentially constant level or slightly decreased thereafter, and 5−7 days after inoculation, the cells began to precipitate. At this point, the culture medium gradually became transparent again. The dechlorination reaction proceeded at a constant rate, which was not linked to the rapid cell growth in the early stage of the incubation. This suggests that the reductive dechlorination is a coupled reaction with an oxidative reaction.

As shown in Figure 2, four facultative anaerobes (*Ae. hydrophila*, *K. terrigena*, *En. amnigenus*, and *E. coli*) and *B. subtilis* significantly dechlorinated MXC to yield de-Cl-MXC. In particular, *Ae. hydrophila* transformed more than 90% of MXC to de-Cl-MXC during 17 days of incubation. Furthermore, two aerobes (*Aci. calcoaceticus* and *M. obuense*) also mediated the dechlorination, but the rate was slower than the rates of the other five species, despite greater cell growth.

Interestingly, although the cell density was sufficient, and the ORP appeared to be acceptable, *Ach. xylosoxidans* did not transform MXC, at least during the incubation period. It is likely that *Ach. xylosoxidans* does not harbor an enzyme system involved in the dechlorination of MXC that can be expressed under microaerobic conditions (42.9 mV, Figure 1). This observation implies that dechlorination of MXC requires not only reductive conditions but also an enzyme system to catalyze the reaction. Our previous study[20] revealed that humic acid in river sediment could act on the dechlorination of MXC. In this study, we can be fairly certain that microaerobic facultative anaerobic microbial enzymes catalyzed the dechlorination reaction. Anaerobic bacteria are known to use various compounds (in this case, MXC) other than oxygen as electron acceptors.[5−10] In contrast, it appears reasonable to assume that the obligate aerobes, which can only use oxygen as an electron acceptor, cannot mediate reductive dechlorination.

Metabolism of MXC in Anaerobic Culture. Figure 3 indicates the quantification result in anaerobic culture performed according to the conditions indicated in Figure 2. Among the seven species that mediated dechlorination in aerobic culture, six species also dechlorinated MXC, but the levels of dechlorinated MXC produced by these six species were obviously lower. We could not detect dechlorination by *M. obuense*. We had originally expected that the reaction would occur at a faster rate in anaerobic culture than in aerobic culture. However, the opposite result was observed. This is probably due to the low cell density in the medium. As described above, the growth was faster and more cells were present in the aerobic culture medium. This suggests that reductive conditions are an essential factor but not sufficient to promote the dechlorination reaction. The number of cells would be an important factor for facilitating reductive dechlorination of MXC.

B. subtilis has been reported to include both aerobic and facultative anaerobic species. In this study, the cells grew at slow rates in anaerobic culture, and we therefore considered it to represent a microaerobic bacterium. The other three species (*Aci. calcoaceticus*, *Ach. xylosoxidans*, and *M. obuense*) have been described as obligate aerobes.[22] These species showed very little growth in anaerobic culture in this study. These growth patterns are considered to reflect the respiratory characteristics of these microbial species.

One unexpected result was that *Ach. xylosoxidans* did not convert MXC under aerobic conditions. In the later period of anaerobic incubation, the dechlorination of MXC by the species was clearly recognized. This phenomenon remains unclear, but certain enzyme(s) may be induced under strict anaerobic conditions (−125.1 mV, Figure 1).

Biodegradation of MXC in the Natural Environment. As shown in Table 1, we selected bacterial species that can be considered as representatives of terrestrial and aquatic environments, except for *E. coli*, which is an intestinal bacterium. In conclusion, all bacterial species convert MXC to de-Cl-MXC under microaerobic to slightly anaerobic conditions which

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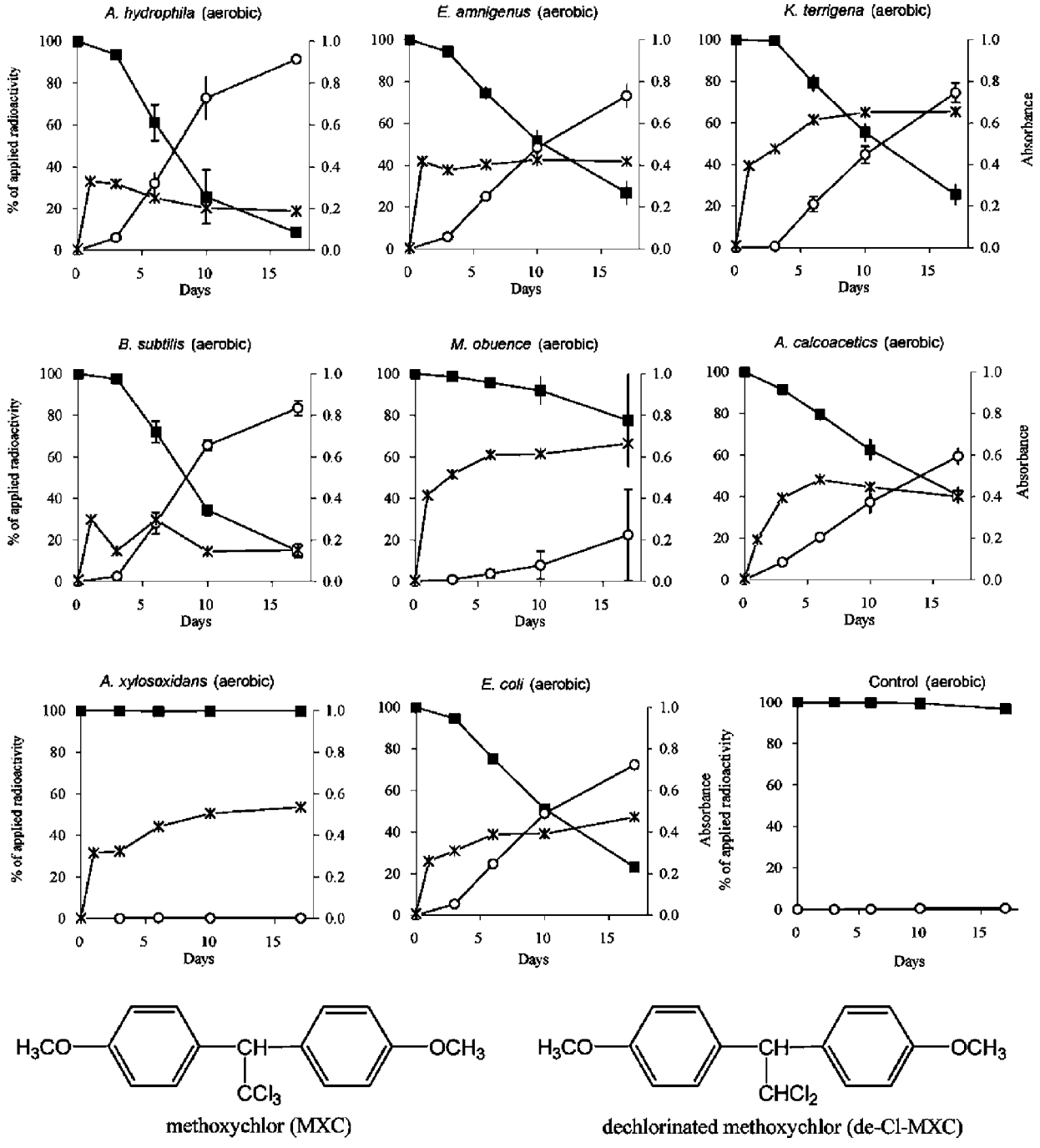

Figure 2. Transformation of [R-U–^{14}C]MXC (solid squares) to yield dechlorinated metabolite (open circles) by eight bacterial species and noninoculated control incubated under aerobic conditions. Asterisks represent cell growth (right axis) consecutively monitored in one culture tube at OD_{600}.

approximately range from ORP = +100 mV to ORP = −200 mV. In the submerged environment, MXC has been found to primarily undergo dechlorination to yield de-Cl-MXC.[20] It is generally accepted that a less chlorinated metabolite is more susceptible to oxidative biodegradation. In fact, several researchers have indicated that sequential anaerobic/aerobic transformation processes are most effective for complete biodegradation (mineralization) of organochlorine compounds. Fogel et al.[17] revealed that the rate of $^{14}CO_2$ evolution from soil treated with [^{14}C]MXC under anaerobic/aerobic sequential conditions is much greater than that under aerobic conditions alone. For the other organochlorines, including polychlorinated biphenyls[26] and perchloroethylene,[27,28] the advantage of the anaerobic/aerobic sequence has also been proposed.

Golovleva et al.[18] screened 17 microbial strains capable of dechlorinating MXC out of 709 strains isolated from MXC-applied soil. Although the authors described the neither classification of the isolates nor quantification of de-Cl-MXC, the presence of microorganisms which potentially dechlorinate MXC was certainly indicated. In the present study, we found that ubiquitous facultative anaerobic and microaerobic bacterial species inhabiting the soil/water environment could mediate the dechlorination of MXC in a manner similar to that of the previously reported dechlorination of MXC by enteric

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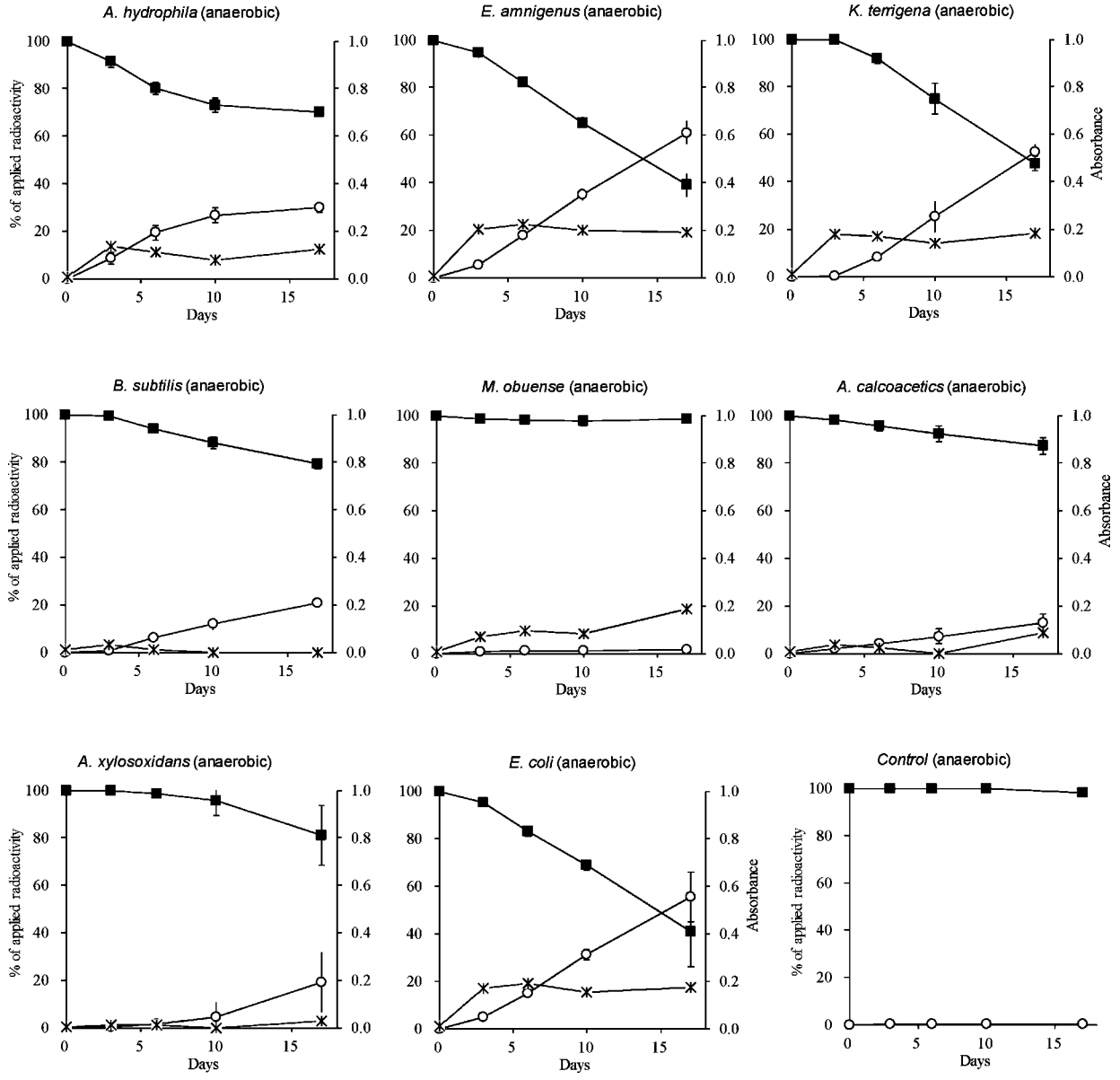

Figure 3. Transformation of [R-U-^{14}C]MXC (solid squares) to yield dechlorinated metabolite (open circles) by eight bacterial species and noninoculated control incubated under anaerobic conditions. Asterisks represent cell growth (right axis) periodically monitored in one culture tube at OD_{600}.

bacteria.[14,15] Furthermore, the bacterial species do not always require a strict anaerobic environment for dechlorination of MXC. Flooded soils and submerged sediments have complex heterogeneous structures and provide microhabitats with various oxygen concentrations for environmental bacteria. The oxic−anoxic interface in the microhabitat could play an important role for facultative anaerobes that dechlorinate MXC, as well as aerobes that contribute to the subsequent oxidative metabolism. Therefore, it is likely that complete biodegradation of MXC occurs without difficulty in oxygen-deliverable, lotic environments or shallow surface water environments.

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Notes

The authors declare no competing financial interest.

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