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RESEARCH ARTICLE

Determination of growth kinetics of microorganisms linked with 1,4-dioxane degradation in a consortium based on two improved methods

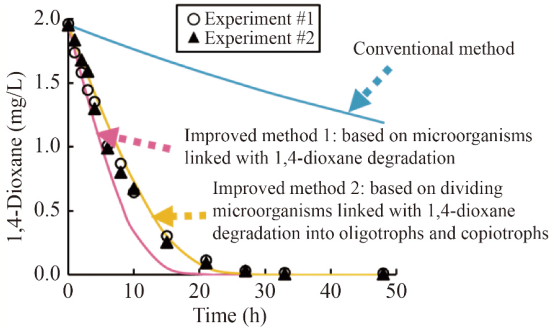
Yi Xiong[1,#], Boya Wang[1,#], Chao Zhou[2], Huan Chen[3], Gang Chen[1], Youneng Tang (✉)[1]

1 Department of Civil and Environmental Engineering, FAMU-FSU College of Engineering, Florida State University, Tallahassee, FL 32310, USA
2 Geosyntec Consultants Inc., Huntington Beach, CA 92648, USA
3 National High Magnetic Field Laboratory, Tallahassee, FL 32310, USA

HIGHLIGHTS

- Evaluated three methods for determining the consortia's growth kinetics.
- Conventional method is flawed since it relies on the total biomass concentration.
- Considering only selected bacterial taxa improved the accuracy.
- Considering oligotrophs and copiotrophs further improved the accuracy.

GRAPHIC ABSTRACT



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ABSTRACT

The conventional method for determining growth kinetics of microbial consortia relies on the total biomass concentration. This may be inaccurate for substrates that are uncommon in nature and can only be degraded by a small portion of the microbial community. 1,4-dioxane, an emerging contaminant, is an example of such substrates. In this work, we evaluated an improved method for determining the growth kinetics of a 1,4-dioxane-degrading microbial consortium. In the improved method, we considered only bacterial taxa whose concentration increase correlated to 1,4-dioxane concentration decrease in duplicate microcosm tests. Using PEST (Parameter Estimation), a model-independent parameter estimator, the kinetic constants were estimated by fitting the Monod kinetics-based simulation results to the experimental data that consisted of the concentrations of 1,4-dioxane and the considered bacterial taxa. The estimated kinetic constants were evaluated by comparing the simulation results with experimental results from another set of microcosm tests. The evaluation was quantified by the sum of squared relative residual, which was four orders of magnitude lower for the improved method than the conventional method. By further dividing the considered bacterial taxa into oligotrophs and copiotrophs, the sum of squared relative residual further decreased.

1 Introduction

1,4-Dioxane (dioxane) is an emerging contaminant identified at more than 66 sites on the National Priorities List (U.S. Environmental Protection Agency (USEPA), 2017). It was also detected in 19% of the US public water systems in the third Unregulated Contaminant Monitoring Rule (UCMR 3). To date, many states in the US have set

✉ Corresponding author
E-mail: ytang@eng.famu.fsu.edu

# These authors contributed equally to this work.

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guidelines or standards for dioxane in drinking water and groundwater at the range of 0.25–77 μg/L (USEPA, 2017).

With the increase of microorganisms found to be able to degrade dioxane (Table 1), microbial processes have great potential for dioxane treatment, but the application is limited by the dioxane biodegradation kinetics (Adamson et al., 2022). Based on Table 1, the half-maximum-rate concentration (K_s) ranges from 1.65 mg/L to 410.92 mg/L, which is a few orders of magnitude higher than the dioxane concentrations at most contaminated sites (usually < 100 μg/L) (Mohr et al., 2010). Therefore, it is very helpful to find microorganisms with a low K_s. Additionally, most of the K_s values reported in the literature are for pure cultures (See Table 1). These values may not represent the K_s in the real-world water treatment reactors since the real-world reactors always contain mixed cultures in which the 1,4-dioxane-degrading microbes interact with other microbes through a network that may alter the K_s. In a field-scale biological reactor for landfill leachate treatment, dioxane was removed from ~2,000 to ~10 μg/L (Zhou et al., 2018), suggesting that the microbial consortium in this reactor might have a low K_s. Therefore, the first objective of this study was to investigate the degradation of dioxane by this microbial consortium and determine the growth kinetics.

Conventionally, kinetics of microbial consortia can also be determined on the basis of the total microbial population, but this method is also problematic. The main drawback is that it potentially overestimates the portion of the biomass that are linked with the degradation and utilization of the substrate. With many advancements in molecular techniques, high-throughput 16S rRNA gene sequencing has become an efficient and inexpensive way to analyze the microbial composition. Although precise quantification of the active substrate-utilization portion of the consortium is still challenging for environmental samples (Props et al., 2017), estimated abundance of various microbial taxa can be obtained and correlated with contamination degradation. Hence, the second objective of this study is to test whether conventional method for estimating kinetics of microbial consortia can be improved by using only the portion of the total biomass statistically linked with substrate utilization.

Furthermore, it is common to have multiple degraders including both oligotrophs and copiotrophs in the microbial consortium (Koch, 2001). The conventional method based on the total biomass cannot differentiate

Table 1 Summary of reported dioxane-degrading microorganisms and their kinetics

Reported culture*	μ_{max} (d^{-1})	Y (mg protein/mg dioxane)	K_s (mg/L)	b (d^{-1})	Reference
Pseudonocardia dioxanivorans CB1190	2.40	0.02−0.09	160 ± 44	–	Mahendra & Alvarez-Cohen, 2006
Pseudonocardia dioxanivorans CB1190	0.74 ±0.06	0.45 ± 0.09	6.3 ± 0.2	0.05 ±0.01	Barajas-Rodriguez & Freedman, 2018
Mycobacterium dioxanotrophicus PH-06	–	0.16	78 ± 10	–	He et al., 2017
Pseudonocardia benzenivorans B5	0.07	0.03	330 ± 82	–	Kämpfer & Kroppenstedt, 2004; Mahendra & Alvarez-Cohen, 2006
Afipia sp. D1	1.20	0.19	25.8	–	Sei et al., 2013
Mycobacterium sp. D6	0.62	0.19	20.6	–	Sei et al., 2013
Mycobacterium sp. D11	0.22	0.18	69.8	–	Sei et al., 2013
Pseudonocardia sp. D17	0.48	0.22	59.7	–	Sei et al., 2013
Acinetobacter baumannii DD1	–	0.41	–	–	Zhou et al., 2016
Rhodanobacter sp. ASY5	7.68	–	–	–	Pugazhendi et al., 2015
Xanthobacter flavus DT8	3.60	0.35	17.5	–	Chen et al., 2016
Xanthobacter sp. YN2	0.6	0.27	410.91	–	Ma et al., 2021
Rhodococcus aetherivorans JCM 14343	0.18	0.03	59.2	–	Inoue et al., 2016, 2018
Pseudonocardia sp. N23	5.52	0.32	79.9	–	Yamamoto et al., 2018
Enriched culture FS	0.01	0.58	93.9	–	Nam et al., 2016
Enriched culture AS	0.19	0.34	181.3		Nam et al., 2016
Industrial activated sludge	0.24−1.03	0.18−0.50	9.9	–	Grady et al., 1997
Industrial activated sludge	0.12	–	181.9	–	Zenker et al., 2002
Industrial activated sludge	1.49	0.54	1.65	0.312	Zenker et al., 2002
Enriched culture	1.99	0.3	12.6 ±7.6	–	Zenker et al., 2002

* All values were reported in the temperature range of 25–30 °C, except for Zenker et al. (2002), which were reported at 35 °C.

the kinetics of the oligotrophic and copiotrophic fraction of the consortium. However, with the 16S rRNA sequencing, it is now possible to separate those two fractions and determine separate sets of kinetic parameters for each of them. Therefore, the third objective of this study is to test if the conventional method can be improved by further separating the biomass linked with 1,4-dioxane degradation into oligotrophs and copiotrophs.

2 Materials and methods

Three methods for determining the kinetics of a consortium were compared in this study: the conventional method based on total biomass, the improved method based on the portion of the biomass linked with dioxane degradation, and the improved method based on two sets of kinetics for the portion of the biomass linked with dioxane degradation — one for oligotrophs and the other for copiotrophs. The following five steps were included in the two improved methods, which differed slightly in Steps 3 and 4. Only Steps 1, 4, and 5 were needed for the conventional method. See Table S1 in supporting information (SI) for a summary of the procedures and differences of the three methods.

First, we conducted a set of microcosm experiments in the batch mode and tracked the concentrations of dioxane and total biomass. Second, we tracked the microbial community change during the tests by 16s rRNA gene sequencing and estimated the concentrations of various groups of bacteria (i.e., taxa) in the community by the product of the total biomass concentration and the relative abundance (%) of the corresponding groups of bacteria. While realizing that this method of determining bacterial concentration is inaccurate, our objective is to find how this improve the kinetics determination compared to the conventional method. Third, we determined the bacterial groups likely linked with dioxane degradation by correlation analysis between the concentration of degraded dioxane and the concentration of various groups of bacteria: groups of microbes whose concentrations increased while dioxane was degraded were considered as the portion of the total biomass linked with dioxane degradation. This portion could utilize dioxane or products associated with dioxane-degradation (e.g., intermediates) as growth substrate. Fourth, the concentrations of dioxane and the portion of the biomass correlated with dioxane degradation were used to estimate the dioxane degradation kinetics. Finally, the estimated parameters were evaluated by comparing the simulated results to a new set of experiments based on the same culture but different initial dioxane concentrations.

2.1 Microcosm experiments for constants determination (Step 1)

Three one-liter serum bottles (A, B, and C) containing 600 mL synthetic nitrate mineral salts medium were inoculated with a dioxane degrading consortium (see detailed medium composition in Xiong et al. (2019)). In the medium, dioxane was the sole carbon source and electron donor, and oxygen was the electron acceptor. The inoculum was from a field-scale biological reactor that reduced dioxane from ~2,000 to ~10 μg/L in a landfill leachate (Zhou et al., 2018). The mixed liquor of the consortium was centrifuged at 10,000 g for 10 minutes at 4 °C to pellet the biomass. After removing the supernatant, the pellet biomass was re-suspended in the synthetic medium; this was repeated three times to remove dissolved chemicals from the mixed liquor. The "washed" pellet biomass was transferred to the three serum bottles with the synthetic medium after the bottles with the medium were autoclaved at 121 °C for 45 min. The biomass concentration in the bottles at the beginning of the experiments was ~20 mg protein/L. Bottles A and B were duplicates, and Bottle C was autoclaved after the inoculation as the sterile control. ~50 mg/L dioxane was spiked into Bottles A, B and C. To minimize contamination but maintain oxygen supply, all bottles were sealed with sterile cotton at the bottleneck. All bottles were placed on a shaker (Model # SHKE 2000, Thermo Scientific, Dubuque, Iowa, USA) at 120 revolutions per minute. Every two or three days, samples were taken for measurement of dioxane and total biomass.

Dioxane was measured by solid-phase micro-extraction coupled with gas chromatography/mass spectrometry, and the details were described in Xiong et al. (2019). The quantification limit was ~5 μg/L, which was five times higher than that in Xiong et al. (2019) due to a dilution factor five times higher. To measure total biomass concentration as protein, 1 mL of sample was centrifuged at 10,000 g for 10 min to remove the supernatant. One mL of 0.1 mol/L NaOH was then added to the centrifuge tube for resuspension of cells and freezing (−20 °C). Thawed samples were heated at 100 °C for 10 min and measured for protein by a Pierce BCA Protein Assay Kit (Ras et al., 2008). The limit of quantification was ~5 mg protein/L. Each sample was measured four times to calculate the average protein concentration and standard deviation. Using protein to estimate the total biomass may be less accurate than methods such as flow cytometry, quantitative PCR (qPCR) and fluorescence in situ hybridization (FISH) (Props et al., 2017), but it is easier to implement and thereby widely used.

2.2 Microbial community analysis (Step 2)

The biomass samples taken from Step 1 were subsampled for DNA extraction in Step 2. The subsamples were first centrifuged at 10,000 g for 10 min to remove the supernatant. The pellets were preserved in a −20 reezer and extracted for DNA within a month. One set of

samples from Bottle A and duplicate sets of samples from Bottle B were used for DNA extraction; this resulted in three samples at each time point for calculation of the average relative abundance of different taxon. Fast DNA™ SPIN Kit for Soil (MP Biomedicals, USA) was used for DNA extraction according to the manufacturer's instructions. The extracted DNA was quantified with NanoDrop 2000 (Thermo Fisher Scientific, USA) before downstream sequencing.

The DNA samples were analyzed with 16S rRNA gene-targeted amplicon sequencing by an Illumina MiSeq sequencer. Basic processing of the raw data was performed by the University of Illinois at Chicago Core for Research Informatics (UICCRI). Primer set 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) was used in the 16S rRNA gene amplification (Pylro et al., 2014). After that, a two-step PCR amplification was carried out following the protocol modified from Ionescu's report (Ionescu et al., 2016). Raw sequences were then joined, demultiplexed and quality filtered using QIIME version 1.8. Sequences were then clustered into operational taxonomic units (OTUs) with a cutoff of 98% identity using USEARCH. USEARCH and the Silva 132 reference database were used to assign taxonomic annotations for each OTU.

2.3 Analysis of correlation between dioxane and various groups of bacteria (Step 3)

To understand how the dioxane degradation shaped the microbial community over time, the OTU relative abundance was first analyzed using non-metric multidimensional (NMDS) scaling in R with metaMDS function of the Vegan package (Oksanen et al., 2007). Bray-Curtis dissimilarity was used as the dissimilarity index. To fit the dioxane concentration onto the ordination, the Vegan function envfit was used. For each OTU with a relative abundance > 1% in at least one sample, we performed regression analysis between the concentrations of degraded dioxane (i.e., the difference between the initial concentration and concentration measured on the day of sampling) and the concentrations of biomass. The biomass concentration corresponding to an OTU was estimated as the product of the OTU's relative abundance and the total protein concentration. A p-value smaller than 0.05 indicates a strong correlation between dioxane degradation and the analyzed OTU, and a positive correlation coefficient indicates that the OTU's biomass concentration increased when dioxane was degraded. Oligotroph can live in an environment that offers very low levels of nutrients and copiotrophs prefer nutritionally rich environments (Koch, 2001). Therefore, if regression analysis of an OTU throughout the experiments (Day 0 to Day 16) shows p-value < 0.05 and a positive correlation coefficient, this OTU is considered oligotroph. If regression analysis of a OTU shows p-value < 0.05 and a positive correlation coefficient only when dioxane was > 20 mg/L (Day zero to Day 11), this OTU is considered copiotroph. These OTUs were selected for the kinetic estimation in Steps 4 and 5.

2.4 Estimation of constants in kinetics (Step 4)

The Monod kinetics was used to simulate dioxane removal and biomass growth in the microcosm experiments. The equations for the conventional method and the method with one set of kinetics (with copiotrophs and oligotrophs as one group) were as follows:

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -\frac{\mu_{\max}}{Y}\left(\frac{S}{S+K_s}\right)X, \tag{1}$$

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \mu_{\max}\left(\frac{S}{S+K_s}\right)X - bX, \tag{2}$$

in which, K_s is the half-maximum-rate concentration, $\mu_{\max}$ is the maximum specific growth rate of the culture, b is the biomass decay rate, S is the concentration of dioxane, Y is the yield coefficient, and X is the total biomass concentration in the conventional method and the fraction of biomass linked with dioxane degradation in the improved method with one set of kinetics, respectively.

In the improved method based on two sets of kinetics, to incorporate the kinetic parameters of copiotrophs and oligotrophs for the fraction of the total biomass linked with dioxane degradation, Equations (1) and (2) were slightly modified as follows:

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -\frac{\mu_{\max 1}}{Y_1}\left(\frac{S}{S+K_{s1}}\right)X_1 - \frac{\mu_{\max 2}}{Y_2}\left(\frac{S}{S+K_{s2}}\right)X_2, \tag{3}$$

$$\frac{\mathrm{d}X_1}{\mathrm{d}t} = \mu_{\max 1}\left(\frac{S}{S+K_{s1}}\right)X_1 - b_1X_1, \tag{4}$$

$$\frac{\mathrm{d}X_2}{\mathrm{d}t} = \mu_{\max 2}\left(\frac{S}{S+K_{s2}}\right)X_2 - b_2X_2, \tag{5}$$

in which, $\mu_{\max 1}$, Y_1, K_{s1}, and b_1 are the kinetic constants for copiotrophs; $\mu_{\max 2}$, Y_2, K_{s2}, and b_2 are kinetic constants for oligotrophs; X_1 and X_2 are the concentrations of copiotrophs and oligotrophs, respectively.

PEST, a model-independent parameter estimation and uncertainty analysis package (Doherty et al., 2014), was used to estimate the kinetic constants in Eqs. (1) to (5) (e.g., $\mu_{\max}$, Y, K_s, and b). To use PEST, we first coded a FORTRAN program to predict the concentrations of dioxane and fraction of biomass linked with dioxane degradation in the microcosm experiments following Eqs. (1) to (5). PEST compared the simulated concentrations of dioxane and the fraction of biomass linked with dioxane degradation with the experimentally measured concentrations and then adjusted the kinetic constants until the discrepancy between the models and

experiments reached the minimum. The discrepancy was quantified using the sum of squared relative residual (SSRR):

$$\mathrm{SSRR} = \sum_{i=1}^{n}\left(\frac{S_i - S_i^*}{S_i}\right)^2 + \sum_{i=1}^{n}\left(\frac{X_i - X_i^*}{X_i}\right)^2, \qquad (6)$$

in which, n is the number of sampling time points in the microcosm experiments, S_i and X_i are the concentrations of dioxane and fraction of biomass linked with dioxane degradation in experiments at various time points, and S_i^* and X_i^* are the corresponding values in the model.

2.5 Evaluation of the estimated constants in kinetics (Step 5)

After the initial 50 mg/L of dioxane was completely degraded to below the detection limit in the Bottles A and B, 2 mg/L dioxane was added to them to start a set of experiments for evaluating the constants obtained in Step 4. The initial concentration of dioxane in the experiments for evaluation was much lower than that in the experiments for constants determination. The two bottles were sampled on an hourly basis at the beginning of the experiments and less frequently when the dioxane concentration decreased to < 0.5 mg/L. Because of the short test duration (< 2 d) and low dioxane concentration, we assumed that there was no significant change in the total biomass concentration and the microbial composition. Microbial samples were taken from both bottles at the beginning of the tests for protein measurement and 16S rRNA gene sequencing.

The estimated constants from Step 4 and the initial concentrations of dioxane and the fraction of biomass linked with dioxane degradation were used to predict the dioxane concentrations during the experiments for constants evaluation.

3 Results and discussion

3.1 Microcosm experiments for constants determination (Step 1)

In the microcosm experiments for constants determination, ~97% dioxane was degraded within 16 d in Bottles A and B, as shown in Fig. 1(a). The degradation curves for Bottles A and B were reproducible. No significant degradation was observed in the sterile control Bottle C. The protein concentrations of the three bottles were shown in Fig. 1(b), and they showed minor day-to-day variations during the tests.

3.2 Microbial community analysis (Step 2)

The 16S rRNA gene sequencing data from the triplicate sets of biomass samples taken on different dates were compared based on NMDS ordination in Fig. 2. The

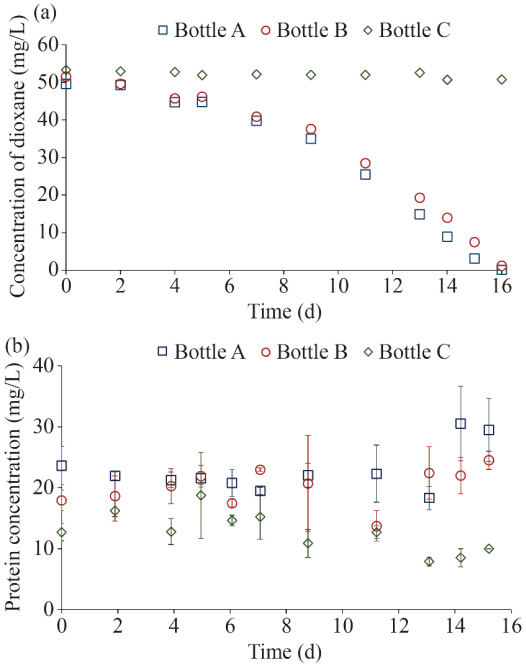

Fig. 1 Experimental results from the microcosm experiments for constants determination: (a) Concentrations of dioxane; (b) Concentrations of proteins (error bars represent the standard deviations based on quadruplicate sampling and analysis).

NMDS analysis showed the significant shift in microbial community structure when dioxane concentration was decreasing. The microbial community composition at the same sampling day and of similar dioxane concentrations are closely clustered. These results reveal that dioxane shaped the microbial community. To focus on the microbial groups that were linked with dioxane degradation, we first excluded the groups whose relative abundance never exceeded 1% during the microcosm

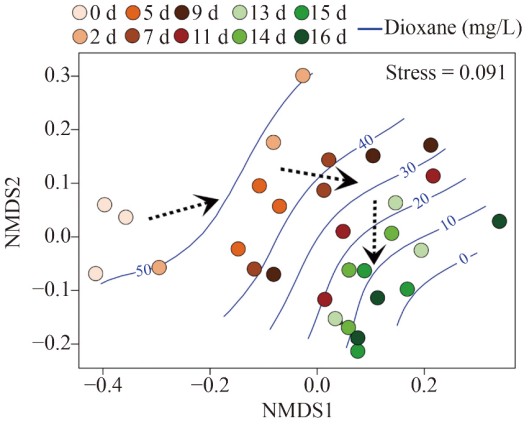

Fig. 2 NMDS analysis: an overview of the change of microbial community in the microcosm experiments for constants determination. (Note: Different colors represent different dates of sampling. For each date, one set of samples from Bottle A and duplicate sets of samples from Bottle B were analyzed. Typically, the analysis is believed to be reliable if the stress level is smaller than 0.1 (Kruskal, 1964)).

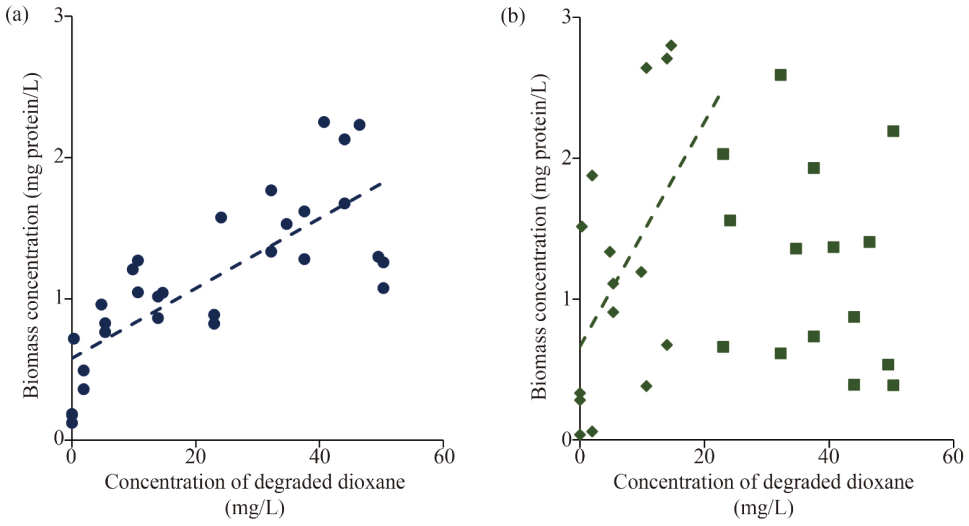

Fig. 3 Linear regression analysis for the concentration of degraded dioxane and the concentration of microbial groups from Bottles A and B in the microcosm experiments for constants determination: (a) An example oligotrophs: *Afipia* (p-value = 4.22×10^{-7}, correlation coefficient = 0.025); (b) The only copiotrophs: *Pseudonocardia dioxanivorans* CB1190 (p-value = 0.027, correlation coefficient = 0.098). (Note: in Fig. (b), the diamonds and squares represent the samples taken before and after the dioxane was degraded from 50 to 30 mg/L, respectively. Only the data corresponding to the diamonds (higher dioxane concentrations or less dioxane degradation) were used for regression analysis.)

tests. 42 OTUs out of 1781 were selected and further considered. The 42 OTUs and their relative abundance were shown in a heatmap (see Fig. S1 in supporting information (SI)) for one set of samples from Bottle A and duplicate sets of samples from Bottle B. The triplicate sets of samples demonstrated high similarity and reproducibility.

3.3 Analysis of correlation between dioxane and various groups of bacteria (Step 3)

After regression analyses of the 42 OTUs for their correlation with dioxane degradation throughout the experiments, we found that 14 microbial groups fell intro the category of oligotrophs. Figure 3 (a) shows *Afipia* as an example: Its p-value was 4.22×10^{-9} (< 0.05), which suggested a strong correlation between dioxane degradation and the growth of *Afipia*. Its correlation coefficient was 0.025 (> 0), which suggested that the *Afipia* concentration increased while dioxane was decreasing. 13 additional taxa showed positive and significant correlation based on the same method and the analysis results are shown in Fig. S2 of SI. The name, p-value, and correlation coefficient of the 14 microbial groups are summarized in Table 2. We further evaluated the (28 = 42–14) OTUs for their correlation with dioxane degradation at the first 11 days of the experiments and found only one microbial group (*Pseudonocardia dioxanivorans* CB1190) fell into the category of copitrophs (see Fig. 3(b) and Table 2). It should be noted that claiming the microbial group to be *Pseudonocardia dioxanivorans* CB1190 only suggest this microbial group has at least 98% similarity of the target region sequence with *Pseudonocardia dioxanivorans* CB1190.

As shown in Table 2, among the 15 microbial groups, four groups including *Pseudonocardia dioxanivorans* CB1190, *Afipia*, *Xanthobacteraceae,* and *Burkholderiaceae* were reported as dioxane-degrading bacteria in previous pure-culture studies. Three additional groups among the 15 groups, including *Saccharimonadales*, *Rhodopseudomonas,* and *Dokdonella*, were enriched in dioxane-degrading consortia in previous studies (See Table 1). The other eight groups may be dioxane-degrading bacteria that have not been reported before. Since dioxane was the only electron donor in our study, all the 15 groups were metabolizing dioxane or products associated with dioxane-degradation (e.g., intermediates). Therefore, they were collectively defined as the fraction of biomass linked with dioxane degradation in the consortium.

To evaluate which microorganisms other than the 15 groups might have participated in the 1,4-dioxane degradation, we conducted LEfSe (Linear discriminant analysis Effect Size) analysis of the 16S rRNA sequences corresponding to Day 0 and Day 16. The results are shown in Fig. S3. The largely overlapping between the correlation analysis results (14/15 = 93%) and the LEfSe analysis results with high Linear Discriminant Analysis (LDA) scores suggests that we have captured most of the abundant taxa (> 1%) linked with 1,4-dioxane degradation by using the correlation analysis method. The inconsistence between the correlation analysis results and the LEfSe analysis results suggests that we may have neglected many taxa that played a role in 1,4-dioxane degradation but had low abundance (< 1%).

Table 2 Microbial groups likely responsible for dioxane degradation in this study

Name	Category	Correlation coefficient	*p*-value	Reported as dioxane-degrading bacteria in pure culture studies	Reported as potential dioxane-degrading bacteria in mixed culture studies	Reference
Pseudonocardia dioxanivorans CB1190	Copiotrophs*	9.8×10^{-2}	2.7×10^{-2}	Yes	Yes	Aoyagi et al., 2018; Mahendra & Alvarez-Cohen, 2006
Afipia	Oligotrophs†	2.5×10^{-2}	4.2×10^{-7}	Yes	Yes	Nam et al., 2016; Sei et al., 2013
Xanthobacteraceae		1.2×10^{-2}	3.5×10^{-7}	Yes	Yes	Chen et al., 2016, 2021; Nam et al., 2016
Burkholderiaceae		2.9×10^{-3}	6.1×10^{-3}	Yes	Yes	Mahendra & Alvarez-Cohen, 2006; Nam et al., 2016
Saccharimonadales		9.6×10^{-3}	8.9×10^{-7}	No	Yes	Chung et al., 2019
Rhodopseudomonas		3.5×10^{-3}	8.2×10^{-7}	No	Yes	Aoyagi et al., 2018
Dokdonella		4.6×10^{-3}	3.9×10^{-2}	No	Yes	Nam et al., 2016
Pedomicrobium		2.9×10^{-3}	1.1×10^{-2}			
Mesorhizobium		3.2×10^{-3}	4.4×10^{-4}			
Blastocatella		2.8×10^{-3}	2.4×10^{-2}			
Chlorobi bacterium OLB5		1.9×10^{-3}	3.0×10^{-3}	No	No	–
JGI 0001001-H03		2.9×10^{-3}	1.6×10^{-2}			
Bauldia		1.3×10^{-3}	8.3×10^{-3}			
Ellin6067		1.6×10^{-3}	6.4×10^{-4}			
Mine drainage metagenome		3.5×10^{-3}	3.5×10^{-12}			

* Based on the regression analysis of the data of the first 11 days.
† Based on the regression analysis of the data of the first 16 days.

3.4 Estimation of constants in kinetics (Step 4)

The estimated constants based on the three methods are summarized in Table 3, and the simulation results based on the estimated constants are compared to the results from the microcosm experiments for constants determination in Fig. 4 for dioxane and Fig. 5 for biomass. Table 3 shows that considering the fraction of biomass linked with dioxane degradation instead of the total biomass significantly improved the estimation of the kinetic parameters (SSRR = 5.6 compared to 61,900). Inclusion of two sets of kinetic parameters, one for oligotrophs and one for copiotrophs, for the fraction of biomass linked with dioxane degradation achieved an even smaller SSRR (4.0). It also correctly predicted the decrease of CB1190 (the only copiotroph) in the second half of the experiments (Fig. 5(c)).

Copiotrophs are expected to grow fast under high substrate conditions (Koch, 2001), and as expected, our estimate showed that they had a higher μ_{max} in this consortium. However, oligotrophs are characterized by slow growth but are able to survive the oligotrophic conditions (Koch, 2001), and thus, our analysis found that they had a much lower K_s in the consortium. The K_s for oligotrophs in our study (0.44 mg/L) is smaller than all the K_s (1.65–330 mg/L) reported in literature (see Table 1). Other constants for oligotrophs, including μ_{max}, Y, and b, are in the reported ranges. The highly oligotrophic nature of the overall culture is consistent with its origin — an oligotrophic environment in the bioreactor where only ~ 10 μg/L of dioxane is present. Similarly, the consortium with low K_s (1.65 mg/L) reported by Zenker et al. (2002) was from a continuous bioreactor receiving low concentration of dioxane. The kinetic parameters for the only copiotrophs (CB1190) were remarkably similar with those reported in the literature based on pure culture studies: 2.5 d^{-1} in our study and 0.74–2.4 d^{-1} in the literature for μ_{max}; 0.44 mg protein/mg dioxane in our study and 0.02–0.45 in the literature for Y; and 160 mg/L in our study and 6.3–160 mg/L in the literature for K_s (See Table 1 for details).

As shown in Fig. 3, copiotrophs and oligotrophs had very different growth patterns. The major difference occurred in the last one third of the experiments when the dioxane concentrations dropped to < 20 mg/L. The concentration of copiotrophs (CB1190) was decreasing since the dioxane concentration was much lower than their K_s (160 mg/L), while the concentration of oligotrophs continued to increase since the dioxane concentration was still much higher than their K_s (0.44 mg/L).

The constants determined by the improved method based on one set of kinetics (μ_{max} = 0.33 d^{-1}, Y = 0.45, K_s = 0.56 mg/L, b = 0.22 d^{-1}) were much closer to the constants of oligotrophs (μ_{max} = 0.26 d^{-1}, Y = 0.40, K_s = 0.44 mg/L, b = 0.16 d^{-1}) than those of copiotrophs (μ_{max} = 2.50 d^{-1}, Y = 0.44, K_s = 160 mg/L, b = 0.28 d^{-1}). This indicates the oligotrophs had more influence on the

Table 3 Summary of the constants determined by the three methods

Methods		μ_{max} (d^{-1})	Y (mg protein/mg dioxane)	K_s (mg dioxane/L)	b (d^{-1})	Total SSRR
Conventional method		0.07	0.08	69.71	0.01	61,900
Improved method based on one set of kinetics		0.33	0.45	0.56	0.22	5.6
Improved method based on two sets of kinetics	Copiotrophs	2.50	0.44	160.00	0.28	4.0
	Oligotrophs	0.26	0.40	0.44	0.16	

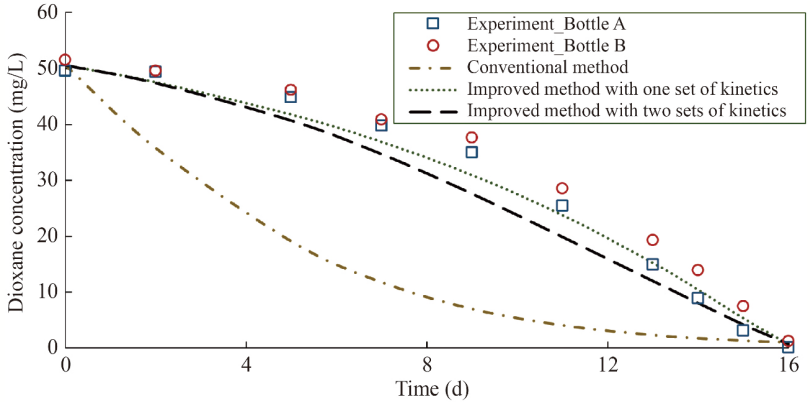

Fig. 4 Comparison of dioxane concentrations from the microcosm experiments for constants determination and the three methods for determining kinetics: a) the conventional method, b) the improved method with one set of kinetics, and c) the improved method with two sets of kinetics.

overall apparent kinetic characteristics of the microbial consortium compared to the copiotrophs. This can be explained by the higher concentrations of oligotrophs than copiotrophs in the consortium (Fig. 5(c)). However, the drastic difference in the growth kinetics of the two groups of bacteria suggests that the only copiotroph would be the most likely isolate from this culture using conventional series dilution isolation techniques with the oligotrophs potentially being overlooked siginicantly or even entirely. It is therefore possible that the kinetics determined from the pure-culture approach would be skewed towards the copiotrophs.

3.5 Evaluation of the estimated constants in kinetics (Step 5)

As shown in Fig. 6, the improved method with two sets of kinetics best predicted the microcosm experiments for evaluation (SSRR = 2.7). Prediction from the improved method with one set of parameters (SSRR = 4.9) was slightly higher. Both SSRRs were much smaller than that based on the conventional method (SSRR = 61890).

4 Conclusions

This work compared three methods for estimating the kinetic constants of a microbial consortium that degraded dioxane: 1) a conventional method that is based on the total biomass of the consortium, 2) an improved method with one set of kinetics for the fraction of biomass linked with dioxane degradation in the microbial consortium, and 3) an improved method with two sets of kinetics - one for oligotrophic fraction of biomass linked with dioxane degradation in the microbial consortium, and the other for copiotrophic fraction of biomass linked with dioxane degradation in the microbial consortium. For the second and third methods, we used protein measurement combined with the relative abundances of bacterial taxa to estimate the concentrations of the fraction of biomass linked with dioxane degradation. The second and third methods showed much higher accuracy than the first method (the conventional method), with the third method giving the best results in terms of comparison to two sets of experiments: one for determining the constants, and the other for evaluation of the constants. Based on the third method in our study, the constants for the copiotroph (CB1190) were consistent with the reported values in the literature that were obtained from pure culture studies. The constants for the oligotrophs had a smaller K_s (0.44 mg/L) than the range reported in the literature (1.65–330 mg/L). Almost all of the pure cultures reported in previous studies were isolated with 1,4-dioxane at very high concentrations (e.g., 500 mg/L). This favors the isolation of copiotrophs, which usually have large K_s and μ_{max}, and outcompete oligotrophs in these isolation experiments. However, they do not necessarily represent the kinetics of the dominant cultures in real-world *in-situ* remediation sites and *ex-situ* treatment reactors since the 1,4-dioxane concentrations in most of the real-world contaminated water are less than 100 μg/L. The kinetics parameters of the oligotrophs

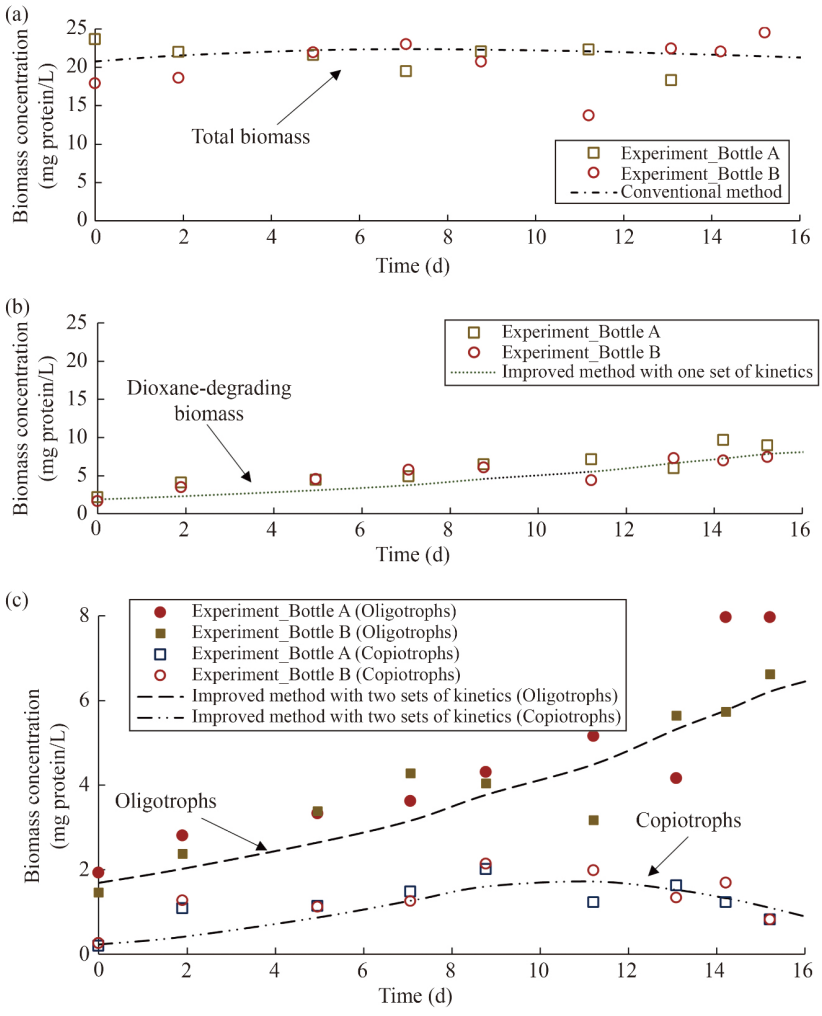

Fig. 5 Comparison of biomass concentrations from the microcosm experiments for constants determination and the three methods for determining kinetics: (a) the conventional method, (b) the improved method with one set of kinetics, and (c) the improved method with two sets of kinetics. (Note: The symbols for experiments represent the average. For standard deviation of the quadruplicate measurements of proteins and variation among the triplicate measurements of taxa relative abundance, refer to Fig. 1(b) and S1, respectively).

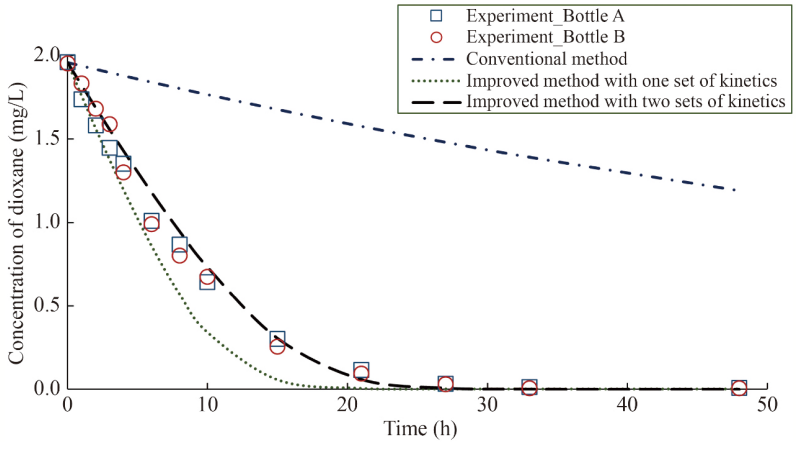

Fig. 6 Comparison of results from the microcosm experiments for evaluation by the three modeling methods.

reported in this study are likely closer to the kinetics of real-world applications.

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