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Multi-laboratory evaluation of the reproducibility of polymer biodegradation assessments applying standardized and modified respirometry methods

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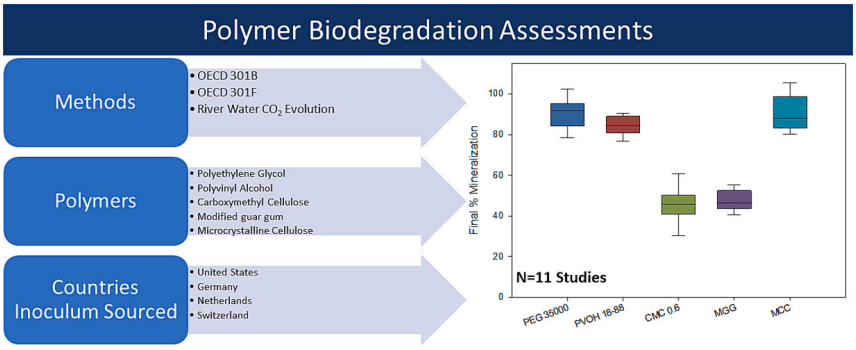
HIGHLIGHTS

- Eleven OECD 301 polymer mineralization studies produced highly reproducible results.
- Test extension yielded higher extent of mineralization for all polymers.
- Final mineralization was comparable between activated sludge and river water inoculum.
- Microcrystalline cellulose is a viable reference material for rapidly biodegrading polymers.

GRAPHICAL ABSTRACT



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ABSTRACT

This research evaluated the intra- and interlaboratory variability when applying OECD 301F and OECD 301B Ready Biodegradation respirometric test methods to quantify polymer biodegradation as well as the impact of method modifications including test duration, inoculum level and test substance concentration on results. This assessment synthesizes results of mineralization studies on 5 polymers of varying structural components, molecular weight, charge, and solubility, evaluated at 8 different laboratories in 4 different countries, providing significant geographic variation in inoculum source as well as lab to lab variations in test setup. Across all

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laboratories, intralaboratory variability was low (≤18 % absolute difference) indicating the reproducibility of results between replicates and uniformity of test setup in each laboratory. Interlaboratory variation was also low for all 5 polymers with extent of mineralization being comparable in all OECD 301F and 301B studies even when test methods were modified. Across all studies mean mineralization was 89 ± 5.5 % for polyethylene glycol 35,000, 85 ± 7.4 % for polyvinyl alcohol 18-88, 44 ± 13 % for carboxymethyl cellulose (DS 0.6), 48 ± 4.1 % for a modified guar gum, and 88 ± 6.2 % for microcrystalline cellulose (MCC) at study completion. Due to the lack of polymeric reference materials, MCC was evaluated and found to be a suitable reference material for polymers that biodegrade rapidly in screening studies. An additional respirometric study was conducted quantifying mineralization of the 5 polymers in river water to evaluate the relationship with OECD 301 results using activated sludge as the inoculum. A similar extent of mineralization was observed for all 5 polymers in the OECD 301 and river water studies but time to reach the maximum extent of mineralization was longer using river water as the inoculum source likely due to the lower microbial counts (10^6 CFU/L) in the test system.

1. Introduction

The OECD 301 Ready Biodegradation Test Guideline (TG) is a compilation of screening methods to assess chemical biodegradability and is used globally as part of environmental risk assessment schemes to conduct safety and persistence assessments including in the US, Canada, EU, and Japan (EC, 2005; USEPA, 2008; METI, 2011; ECHA, 2016; ECHA, 2017). The OECD 301 TG consists of 6 different methods to assess biodegradation using different analytical endpoints: dissolved organic carbon DOC (OECD 301A & E), carbon dioxide (CO_2) evolution (OECD 301B), and oxygen (O_2) consumption (OECD 301C, D & F), (OECD, 1992a, 1992b). The TG was developed for and has largely been applied to the evaluation of water-soluble, low molecular weight (MW) molecules (Kowalczyk et al., 2015). OECD 301 studies are batch tests conducted in aqueous phase mineral media to support microbial growth. The microbial inoculum is from activated sludge, sewage effluent, surface water, or soil with activated sludge being the most common inoculum source. The test substance is the dominant microbial food source (source of organic carbon) in the study. The studies are considered screening studies because the test substance concentration (1–100 mg/L) is typically many orders of magnitude above environmentally relevant levels (as needed to accurately quantify biodegradation using the nonspecific analytical methods discussed above) and because low levels of inoculum 10^4–10^8 cells/L are required (OECD, 1992a, 1992b). These relatively low levels of inoculum limit microbial diversity likely affecting the probability biodegradation is observed (Blok and Booy, 1984; Goodhead et al., 2014; Kowalczyk et al., 2015). The high-test substance concentration can cause experimental artifacts such as microbial toxicity further exacerbating the stringency of the test (Seyfried et al., 2015; Nabeoka et al., 2016). Because these studies are not simulating actual environmental conditions, the results do not indicate the rate of biodegradation in the environment but instead provide information on the possible extent of biodegradation. These studies are typically conducted for 28 d but at times must be extended to allow time for competent degrader populations to grow to a sufficient level that they are able to appreciably degrade the concentration of test substance added to the study (West et al., 2007; Bernhard et al., 2008; Kowalczyk et al., 2015; Gartiser et al., 2022; Menzies et al., 2023). The benefit of increased OECD 301 test duration to minimize the need for simulation testing has been acknowledged in the regulatory context with the adoption of the enhanced ready biodegradation test in REACH allowing test extension to 60 d to evaluate persistency (ECHA, 2017). In addition, increased microbial density in screening studies has been shown to lead to decreased variability in results and increased rates of biodegradation (Kowalczyk et al., 2015; Menzies et al., 2023).

As chemical persistence evaluations rise to the forefront of environmental concerns and changes in polymer regulations are being considered, there is renewed focus on understanding applicable test methods to assess polymer biodegradation. In comparison to water soluble, low MW test substances, there is a general lack of experience using the OECD 301 TG to assess polymer biodegradation. The OECD ring trial did not include polymers (Painter and King, 1985) and to date the intra- and interlaboratory variability has not been thoroughly assessed for polymers. While recent publications demonstrate the OECD 301 TG applicability to polymers (West et al., 2007; McDonough et al., 2017; Menzies et al., 2023), there is a need to understand whether the test methods are fit for purpose or whether potential modifications (such as test extension, increased levels of inoculum, and decreased test substance concentrations) are needed when assessing polymers (ECETOC, 2020). Polymers are unique in the challenge to assess biodegradation due to their complex composition and structural features. Polymers are by nature of their synthesis mixtures of structurally similar molecular species with varying MW and structural features; they are typically characterized as being high MW (e.g., >1000 Da) and behave differently from discrete low MW chemicals. Their MW limits many analytical options such as mass spectrometry for biodegradation assessments with specific analytical endpoints. In addition, the complexity of the mixture and synthesis routes can make it prohibitive to obtain representative samples of radiolabeled polymers for biodegradation studies (Menzies et al., 2023). This leads to environmental scientists relying heavily on methods that utilize non-specific or indirect measurements of biodegradation, such as those utilized in the OECD 301 TG. Research by McDonough et al. (2017) showed that the OECD 301B TG could be leveraged to assess the mineralization of non-soluble microparticles and test extension beyond the traditional 28 d duration would be needed to reach the maximum extent mineralization for many particulates including natural polymeric materials. More recently Menzies et al. (2023) utilized the OECD 301B to assess the mineralization of a variety of water-soluble polymers including polyethylene glycols (PEG), polyvinyl alcohols (PVOH), and carboxymethyl celluloses (CMC). As with the non-soluble microparticle study, in many cases test extension was needed to accurately evaluate mineralization of the water-soluble polymers especially the higher MW PEGs and the highly modified CMCs. Menzies et al. also found that when the test design was altered to increase the ratio of inoculum to test substance (in line with the OECD 302B TG, (OECD, 1992a, 1992b)) the time to reach the full extent of mineralization decreased for all polymers evaluated. Bernhard et al. (2008) evaluated biodegradation of PEGs (MW ranging from 250 to 57,800 Da) in an OECD 301A and found that for higher MW PEGs (MW > 14,600 Da) test extension was needed. Van Ginkel and Gayton (1996) evaluated a CMC (0.7 degree of substitution, DS) in an OECD 301D and like Menzies et al. had to significantly extend the study (>100 days) to reach the plateau phase of mineralization.

As briefly discussed above several studies have probed the usefulness of the OECD 301 TG to evaluate polymer biodegradation, but no study has thoroughly evaluated intra- and interlaboratory comparability when applying these methods to polymers nor the impact of test system modifications on results. This type of fundamental research is needed to understand if these methods can be useful screening tools to evaluate polymer persistence. The 11 OECD 301 Ready Biodegradation studies synthesized in this research were conducted by 8 different laboratories. Each laboratory used the same 5 polymers for their studies, but they independently developed test setup protocols and modifications. The aim of this analysis was to bring this information together to probe intra-

and interlaboratory variability from these different studies. In addition, one of the laboratories conducted studies using river water as the inoculum. For lower MW molecules, OECD 301 results have been correlated to mineralization in the freshwater compartment (Struijs and van den Berg, 1995; Aronson et al., 2006), the river water inoculum studies allow us to probe if this relationship also exists for polymers. Microcrystalline cellulose (MCC) was one of the polymers evaluated in these studies. MCC is a broadly available biodegradable particulate that is regularly used as a reference material in ISO biodegradation standards (ISO, 2019a, 2019b, 2021). The inclusion of MCC in these studies allowed for an evaluation of MCC as an alternate reference substance for future testing of polymer or microparticle biodegradation across OECD and ISO testing guidelines.

The objectives of this research were therefore to synthesize data and evaluate intra- and interlaboratory variability from OECD 301 Ready Biodegradation studies conducted by 8 different laboratories on the same suite of 5 polymers. The 5 polymers (which had varying monomeric structure, MW, substitution type, and charge) evaluated were: PEG, PVOH, CMC, modified guar gum (MGG) and MCC. The differences in polymer structures led to differences in solubility with PEG, PVOH and CMC all being water soluble, MGG being insoluble at dose solution concentrations and MCC being an insoluble particulate. This provided a range of scenarios for evaluation in testing. All of the laboratories used activated sludge inoculum which was sourced from different geographic locations allowing an evaluation of interlaboratory comparability with different inoculum sources. In addition, 5 of the laboratories conducted modified studies to evaluate the impact of 1) decreased test chemical concentration, 2) increased inoculum concentration, 3) increased inoculum and test chemical concentration on study results. An additional respirometric study using river water as the inoculum was evaluated and compared with results from studies with activated sludge as the inoculum to understand the correlation between results. Finally, results of these studies were probed to evaluate the usefulness of MCC as a future positive control in polymer biodegradation studies based on the positive control criteria in the current OECD 301 TG (60 % mineralization by day 14).

2. Materials and methods

As mentioned in the introduction, this research is an analysis of results from twelve studies independently designed and conducted at 8 different laboratories utilizing the same 5 polymeric test substances. To capture the key details of each study in a comprehensive and concise manner, a robust summary table was compiled and included in the supplementary data, Table S1. Study details will be briefly summarized below. For clarity each study was given a study number in the form of A-BC; where A indicates the test guideline followed (F=OECD 301F, B=OECD 301B, and RW = river water respirometry study with CO_2 production as the analytical endpoint), B provides the laboratory number (number 1–8 randomly assigned to each laboratory), and C denotes if the method closely followed the test guideline (G) only allowing for test extension or if the test guideline was intentionally modified (M) by altering the test substance concentration and/or inoculum levels (outside of guideline compliance) as well as extending the test duration. Per the TG, studies were conducted at 22 ± 2 °C.

2.1. Test substance characterization

The 5 polymeric test substances included polyethylene glycol (PEG 35000), supplier Sigma BioUltra, with a weight average molecular weight (M_W) of 33,207 Da; Mowiol polyvinyl alcohol 18-88 (PVOH 18-88), supplier Sigma, M_W = 130,000 Da and degree of hydrolysis of 87.8 mol%; carboxymethyl cellulose (CMC 0.6), supplier TCI America, with a degree of polymerization (DP) = 1050, and a degree of substitution (DS) of 0.6; 2,3-epoxypropyltrimethylammonium modified guar gum (MGG), supplier BASF, M_W = 1,270,000 Da and DS = 0.2–0.25; microcrystalline cellulose (MCC), supplier Merck, sieve analysis >160 μm ≤1 %, 20–160 μm = 90 %, <20 μm = 10 %. Independent elemental analysis of each test substance was conducted by many of the laboratories and the results were highly reproducible (Table S2).

2.2. OECD 301F test method

All 8 OECD 301F studies were conducted using activated sludge inoculum from domestic wastewater treatment plants (WWTPs) and following oxygen demand as the analytical endpoint. The inoculum was sourced from 5 different WWTPs located in 4 different countries (Switzerland, Germany, Netherlands, and the United States). Five of the studies generally followed the OECD 301F TG with the only modification being test extension beyond 28 d to ~60 d (56–76 d). These studies are labeled F-1G, F-2G, F-3G, F-4G, and F-5G (Table S1). Note, F-5G increased inoculum levels slightly out of guideline recommendations from 30 to 35 mg/L but the change was so small that it was deemed likely inconsequential on results and the test was labeled as guideline compliant. Three additional studies followed the OECD 301F TG but additional modifications beyond test duration were made to test substance concentration or inoculum levels. F-1M intentionally decreased test substance concentration 2X lower than the recommended guideline dose allowing for an assessment of decreased test substance concentration on polymer biodegradation. F-2M and F-3M both increased inoculum levels 4X and 2X above guideline recommendations respectively allowing for an assessment of increased microbial counts and diversity on polymer biodegradation. For studies F-2G and F-2M, the OECD 301F medium was prepared using higher buffer strength and additional vitamins and trace elements were added to the buffer (it is not anticipated that this would significantly impact test results).

2.3. OECD 301B test method

Like the OECD 301F studies, all OECD 301B studies were conducted using activated sludge inoculum from domestic WWTPs but the analytical endpoint was CO_2 evolution (3 studies in total). Study B-8G was conducted in the US for 28 d for test substances MCC and MGG and 60 d for PVOH 18-88, 147 d for CMC 0.6, and 160 d for PEG 35,000. Details for the MCC and MGG study can be found in Table S1. For PEG 35,000, PVOH 18-88, and CMC 0.6, study details and results are available in Menzies et al. (2023) and summarized in this manuscript for comparison purposes. Study B-6M was conducted in the US for 28 d at a test substance concentration of 40 mg C/L and an inoculum level of 60 mg TSS/L dry wt. Study B-7M was conducted in Germany for 52 d at a test substance concentration of 200 mg C/L and inoculum level of 120 mg TSS/L dry wt. Study B-7M was conducted following the ISO 14852 TG (ISO, 2021) which also follows CO_2 evolution as the endpoint of evaluation and for this analysis can be considered a modified OECD 301B as the major differences in the guidelines are the ISO guideline allows for higher inoculum levels and test substance concentrations. For both B-6M and B-7M increasing the inoculum level provided increased microbial diversity in the studies. Also increasing the test chemical concentration held the ratio of test chemical to inoculum concentration within typical guideline ranges ensuring a strong signal to noise between the blanks and test substance systems ensuring accurate quantification of test substance mineralization.

2.4. River water test method

Study RW-8 was the only study conducted using river water as inoculum (Table S1). River water was obtained from the bank of the Great Miami River in Colerain OH on the day of test initiation. River water was used as both the inoculum and test medium and amended with nutrient salts (magnesium sulfate, calcium chloride, ferric chloride, and ammonium chloride) based on OECD 301 TG levels. The analytical endpoint was CO_2 evolution and dissolved organic carbon, DOC

(Shimadzu Scientific Instruments, Columbia MD) was measured at study completion (95 d) to quantify the level of remaining dissolved organic carbon in the test media. For soluble test materials, DOC provides an additional analytical endpoint that allows understanding of test substance organic carbon final mass balance in the test system.

2.5. Data compilation and analysis

Each laboratory provided raw percent mineralization data for all replicates in each study. This replicate data was individually compiled for analysis and the mean percent mineralization was calculated to allow for easier comparison among studies and figure generation to graphically show results. For each laboratory 28 d and final extent of mineralization replicate data was compiled and mean and absolute difference were calculated. 28 d is a common test duration for an OECD 301 study. Final extent of mineralization at study completion is an important parameter as many studies were extended beyond 28 d to allow more time for polymer mineralization. Interlaboratory variation was evaluated by mean percent mineralization absolute difference comparison for replicates in each study and evaluated against the OECD 301 TG criteria which states that replicate variability should be <20 %. Comparison among laboratories and with test modifications were conducted by evaluating mean percent mineralization trends over the course of the study. The limited sample size prohibited the use of statistical evaluations beyond evaluating data trends and ranges observed.

3. Results and discussion

Figs. 1-3 contain the mean percent mineralization data for the MCC studies, guideline, and modified studies respectively. The replicate 28 d and final extent of mineralization are included in Tables S3-S7 and Table 1 contains the mean mineralization data and absolute difference between replicates for each study. For all OECD 301 studies following the TG, the validity criteria for blank respiration were met. For all OECD 301 studies, the replicate variation was <20 % meeting the OECD 301 validity criteria.

3.1. Microcrystalline cellulose study results

MCC was evaluated as a possible reference substance for biodegradation studies with the aim to provide information on viability of inoculum at longer test durations for polymeric test substances where test extension may be needed. Results for all MCC OECD 301 studies are compiled in Fig. 1 and Table 1. According to the OECD 301 TG validity criteria, a reference substance must meet 60 % mineralization in 14 d. In the OECD 301F and 301B guideline studies MCC reached 60 % mineralization in 14 d except for one replicate of F-4G which only reached 54 %. In the modified studies MCC reached 60 % mineralization in 14 d in all studies except for B-7M where replicates only reached 30–39 % on day 14. In the RW study, the MCC only reached 51 % mineralization on day 14 (Fig. 4, Table 2).

Intralaboratory reproducibility among replicates was strong with absolute differences between replicates ≤14 % in all studies. In the guideline studies, variation in final extent of mineralization between study replicates was <12 % absolute difference. At 28 d, replication was also strong with replicate absolute differences <7 %. In the modified studies, absolute difference between replicates was ≤7 % at 28 d and ≤ 14 % at study completion. Interlaboratory variability was low when considering the modified and guideline studies in total with 28 d mineralization ranging from 68 to 107 % with an average of 84 ± 10 % and final mineralization ranging from 81 to 101 % with an average of 88 ± 6.2 %.

3.2. Polyethylene glycol 35,000 OECD 301F & B study results

PEG 35000 significantly mineralized reaching >71 % at 28 d and >82 % at study completion for all guideline studies (Fig. 2A and Table 1) and reaching >79 % mineralization at study completion in the modified studies (Fig. 3A and Table 1). PEG 35000 mineralization was slower and reached a slightly lower extent in study F-5G than in the other studies reaching 71 % at 28 d and 82 % mineralization at study completion. In the modified studies, mineralization was slower and reached a slightly lower extent in B-7M than in the other studies with 46 % mineralization at 28 d and 79 % mineralization at study completion. Comparing mean mineralization data from all studies (guideline and modified), intralaboratory variability was low with replicate absolute differences ≤12 % at 28 d and at study completion. Interlaboratory variability of final mineralization was also minimal across all guideline studies with final mineralization ranging from 82 to 93 % with an average of 89 ± 4.9 % at study completion. When modified studies are also included in the evaluation, interlaboratory variability remained low with final extent of mineralization reaching 79 to 94 % with an average of 89 ± 5.5 %. Note: in the F-2M studies for PEG 35000 (1 of the replicates) and for PVOH 18-88 (2 of the replicates) had a significant increase in pressure near study completion (~day 40) after the plateau phase in biodegradation had already been reached indicating a system malfunction so this data was removed from the analysis.

3.3. Polyvinyl alcohol 18-88 OECD 301F and 301B study results

Like PEG 35000, PVOH 18-88 mineralized extensively in all studies. In the guideline studies, mineralization ranged from 57 to 81 % at 28 d and 67–89 % at study completion (Fig. 2B and Table 1). Across all studies (guideline and modified) mineralization reached on average 70

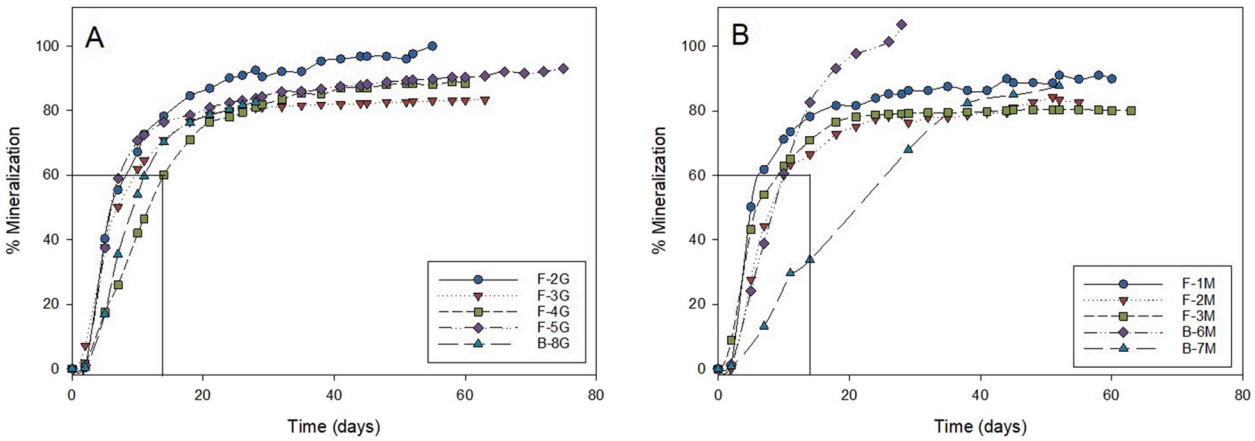

Fig. 1. Mean percent mineralization of MCC in A) guideline and B) modified OECD 301F and OECD 301B studies.

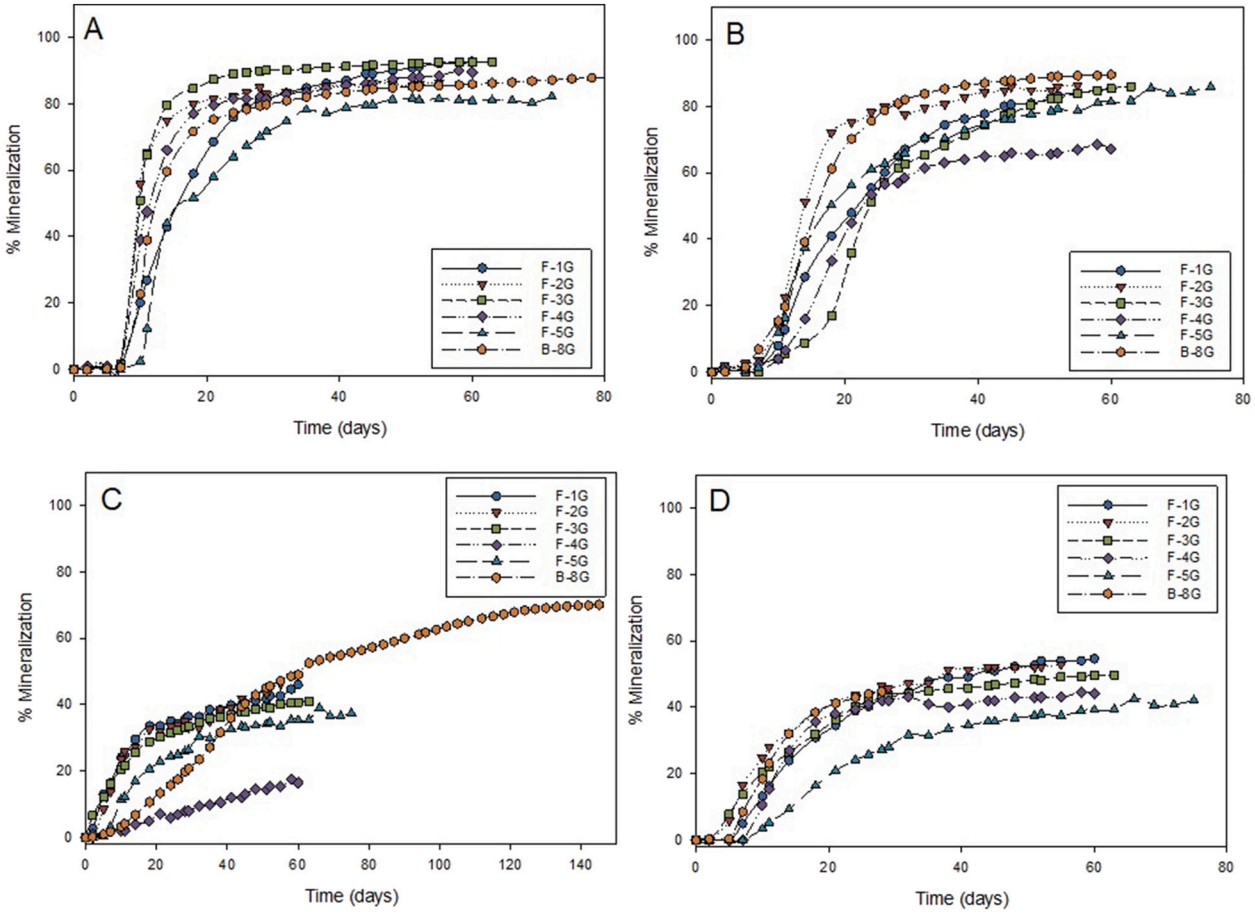

Fig. 2. Mean percent mineralization in OECD 301F and 301B guideline studies with test extension for A) PEG 35000, B) PVOH 18-88, C) CMC 0.6, and D) MGG. Data for PEG 35000, PVOH 18-88, and CMC 0.6 from study B-8G is adapted from Menzies et al. (2023).

± 11 % in 28 d and 85 ± 7.4 % at study completion, showing the value of test extension to evaluate the full extent of mineralization (Fig. 3B and Table 1). Intralaboratory variability was low with final mineralization absolute difference between replicates in guideline studies being ≤13 % at 28 d and ≤18 % at study completion. Intralaboratory variability was also low in the modified studies with absolute differences for final extent of mineralization ≤9 %. Comparing across labs in the guideline studies for PVOH, final extent of mineralization ranged from 67 to 89 % (average of 84 ± 8.7 %). The variability is driven by study F-4G which only reached 67 % while all other studies reached >86 % final mineralization (Fig. 3B). Evaluating the final extent of mineralization across all studies (guideline and modified) shows that PVOH exceeded 79 % mineralization in all studies except the F-4G study with the low replicate.

3.4. Carboxymethyl cellulose DS = 0.6 OECD 301F and 301B study results

CMC 0.6 mineralized to a similar extent across all guideline studies (38–46 % mineralization at study completion) except B-8G which reached 70 % and F-4G which only reached 17 % at study completion (Fig. 2C and Table 1). It is not obvious based on test design why the CMC 0.6 was slower to degrade in study F-4G but it is interesting to note that the mineralization was still increasing at 60 d when the study was terminated indicating that with test extension further mineralization would have likely been observed. Study B-8G applied test extension (study conducted for 147 d compared to the ~60 d duration of the other studies in this analysis) allowing CMC 0.6 to biodegrade further and reach 70 % mineralization (Menzies et al., 2023). It is important to note that 60 d data is available for study B-8G (Fig. 2C) which shows CMC 0.6 reached 49 % mineralization which is comparable to the other guideline studies conducted for a similar duration and evaluated in this assessment (except F-4G as discussed above). This data highlights the importance of test extension to allow time for higher MW polymeric materials to reach maximum extent of mineralization. In the modified studies, CMC 0.6 reached a similar extent of mineralization as the guideline studies ranging from 44 to 53 % mean mineralization at study completion (Fig. 3C, Table 1). Intralaboratory variability was low across all studies with replicate absolute differences for final mineralization of ≤12 %.

3.5. Modified guar gum OECD 301F and 301B study results

MGG showed a similar extent of mineralization across guideline studies (43–55 %), Fig. 2D and Table 1. When evaluating the modified studies, final extent of mineralization was similar ranging from 46 to 55 % with an average mineralization across all studies of 48 ± 4.1 % (Fig. 3D and Table 1). Intralaboratory variability of MGG data was low across all studies with replicate absolute differences ≤12 % 28 d and ≤16 % at study completion.

3.6. OECD 301F and 301B guideline study results comparison

Comparing results of the OECD 301F studies (4 studies with inoculum sources from different countries with variable test setups within guideline requirements) for all 5 polymers, final extent of mineralization was highly reproducible if test extension was employed to allow biodegradation to reach the plateau phase (Fig. 2). Interlaboratory

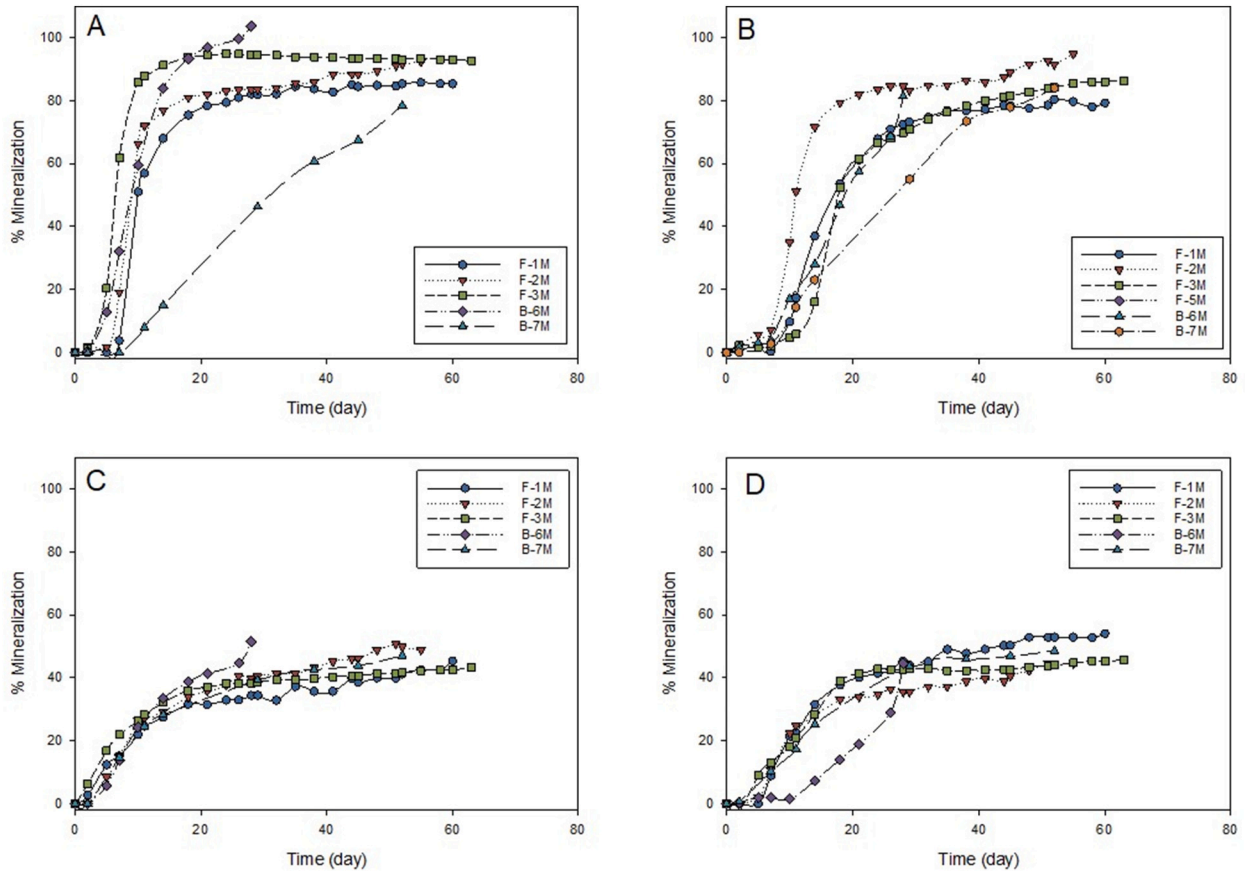

Fig. 3. Mean percent mineralization in OECD 301F and 301B modified studies for A) PEG 35000, B) PVOH 18-88, C) CMC 0.6, and D) MGG.

Table 1
Summary of 28 d and final mean percent mineralization and (replicate absolute difference) for PEG 35000, PVOH 18-88, CMC 0.6, MGG, and MCC for each study using activated sludge inoculum.

			PEG 35000		PVOH 18-88		CMC 0.6		MGG		MCC	
Study	Duration (days)	Location	28 day	Final	28 day	Final	28 day	Final	28 day	Final	28 d	Final
F-1G	60	Switzerland	82 (1)	93 (4)	65 (7)	86 (7)	36 (1)	46 (2)	43 (0)	55 (1)	NA	NA
F-2G	56	Germany	85 (12)	86 (12)	81 (13)	88 (4)	35 (0)	42 (5)	46 (5)	48 (5)	93 (7)	101 (1)
F-3G	65	Michigan, US	90 (0)	93 (2)	62 (5)	86 (5)	33 (2)	40 (6)	43 (2)	50 (4)	81 (0)	84 (1)
F-4G	60	Netherlands	82 (0)	90 (9)	57 (8)	67 (18)	8 (2)	17 (7)	42 (2)	44 (0)	81 (0)	89 (5)
F-5G	76	Netherlands	71 (1)	82 (5)	65 (3)	86 (6)	26 (1)	38 (8)	28 (3)	43 (3)	84 (4)	92 (12)
B-8G	28[a]	Ohio, US	79 (3)	93 (7)	81 (6)	89 (2)	20 (4)	70 (4)	45 (2)	NA	83 (1)	NA
F-1M	60	Switzerland	82 (0)	86 (5)	73 (5)	79 (4)	35 (5)	46 (9)	46 (3)	55 (3)	86 (5)	90 (7)
F-2M	56	Germany	83 (4)	94 (7)	84 (5)	95 (0)	46 (14)	53 (12)	35 (8)	47 (16)	79 (5)	85 (14)
F-3M	65	Michigan, US	95 (1)	93 (0)	70 (0)	87 (7)	39 (1)	44 (4)	43 (3)	46 (0)	79 (2)	81 (1)
B-6M	28	Maryland, US	103 (3)	NA	81 (3)	NA	52 (8)	NA	45 (12)	NA	107 (7)	NA
B-7M	52	Germany	46 (7)	79 (9)	55 (16)	84 (9)	39 (2)	47 (1)	43 (9)	49 (10)	68 (6)	88 (1)
Avg Guideline Studies ± St Dev			81 ± 7.2	89 ± 4.9	68 ± 9.1	84 ± 8.7	26 ± 12	42 ± 12	41 ± 7.3	48 ± 4.8	84 ± 5.5	91 ± 7.2
Avg All Studies ± St Dev			82 ± 15	89 ± 5.5	70 ± 11	85 ± 7.4	33 ± 12	44 ± 13	42 ± 5.5	48 ± 4.1	84 ± 10	88 ± 6.2

NA = Not applicable as study was only conducted for 28 d.

[a] Data for PEG 35,000, PVOH 18-88, CMC 0.6 from Menzies et al., 2023. The PEG 35000 study was conducted for 160 d and CMC 0.6 study was conducted for 147 d. Sixty-day mineralization data is also available and PEG 35,000 reached 86 ± 1.9 % and CMC 0.6 reached 49 ± 6.0 % CO_2 evolution.

variability was minimal except for one study (F-4G) which had lower results for PVOH 18-88 and CMC 0.6. In addition, when looking at the OECD 301B guideline study (B-8G) the 28 d and final mineralization values are comparable to results from the OECD 301F studies for all polymers with standard deviations across all guideline studies for each polymer of ≤12 % (Table 1). This is expected based on historic research with water soluble, low MW molecules which resulted in the allowance of 6 different test methods within the OECD 301 TG but it is important to verify that this relationship also holds true for polymeric test materials. The high level of reproducibility across both OECD 301F and OECD 301B guideline studies is especially interesting given the different types of polymeric test substances evaluated and geographic variation of inoculum sources. These test substances varied in structural monomeric components, MW, and charge including nonionic, anionic, and cationic molecules but the OECD 301 methods were still able to evaluate mineralization and generate reproducible results across 5 different laboratories with unique inoculums.

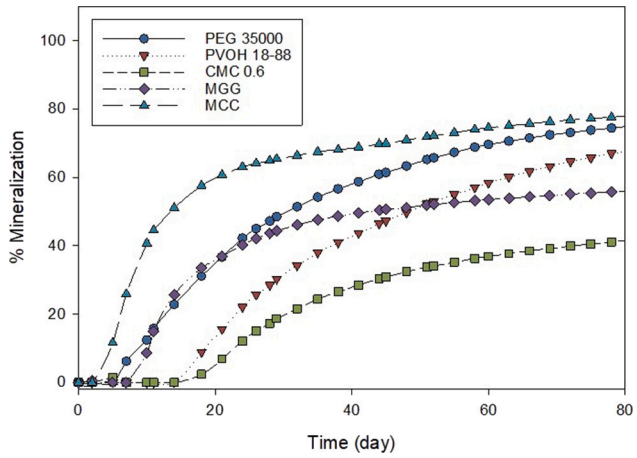

Fig. 4. Mean percent mineralization of PEG 35000, PVOH 18-88, CMC 0.6, MGG, and MCC in river water respirometry studies (RW-8).

Table 2
Mean percent mineralization (replicate absolute difference) of river water study (RW-8) at day 28 and study completion (90 d) and mean percent dissolved organic carbon (DOC) at study completion.

Polymer	28 d (Abs Dif)	90 d (Abs Dif)	DOC (%)
PEG 35000	47 (21)	77 (4)	0.4
PVOH 18-88	29 (13)	72 (9)	3.5
CMC 0.6	17 (5)	44 (2)	39
MGG	44 (2)	57 (5)	29
MCC	65 (0)	79 (5)	1.9

3.7. Modified OECD 301F and OECD 301B comparison

Studies F-1G and F-1M were 2 studies conducted by the same laboratory with the only difference being that in study F-1M all test substance concentrations were reduced to half of the F-1G study concentration (100 mg/L was the F-1G test substance concentration and 50 mg/L the F-1M study). The 28 d and final extent of mineralization were similar in both studies for all polymers (Table 1). Reducing the test substance concentration did not result in a change in the lag period for any of the polymers (Fig. S1). For PEG 35000, PVOH 18-88, and MGG there was a slight impact on the rate of biodegradation during the microbial growth phase with the most significant impact being observed for PEG 35000 (Fig. S1). Studies F-2G and F-2M were 2 studies conducted by the same laboratory with the only difference being a 4X increase in inoculum level in the modified study (30 mg/L TSS in F-2G and 120 mg/L TSS in F-3M). Increasing the inoculum level 4X correspondingly led to a 3-4X increase in blank respiration but even for the F-3M test systems with inoculum concentration of 120 mg/L TSS the test system blank respiration did not exceed the recommended blank respiration range in the OECD 301F TG of 20–30 mg/L even with test extension beyond 28 d. Fig. S2 is a comparison of the percent mineralization for each polymer evaluated in these studies. No differences were observed in length of the lag period or the extent of mineralization for any of the polymers but a slight increase in mineralization rate is observed for PVOH 18-88 during the growth phase (Fig. S2) in the test system with the higher inoculum level. Similarly, studies F-3G and F-3M were also conducted in the same lab with the only difference being the inoculum levels (30 mg/L TSS in F-3G and 60 mg/L TSS in F-3M), Table 1 and Fig. S3. Increasing the inoculum concentration 2X resulted in an increase in blank respiration of ~2X but the blank respiration remained below the OECD 301F recommended range of 20–30 mg/L for 28 d and below the maximum allowable level of 60 mg/L by study completion. For PEG 35000 a slight decrease in lag phase and for MGG a slight increase in rate of mineralization during the growth phase was observed in the modified test systems. For PVOH 18-88 an impact was observed during the growth phase of the curve with higher mineralization consistently being measured for the test system with increased inoculum levels, but the lag period and final extent of mineralization were not significantly impacted. Menzies et al. (2023) evaluated the impact of an increased ratio of inoculum to test substance concentration by increasing inoculum concentration from 17 to 200 mg/L TSS dry wt and test substance from 12 to 50 mg C/L and found that the increased ratio led to a decrease in time needed to reach full mineralization for all biodegradable polymers that were assessed. The improvement was more significant for the polymers that were slower to biodegrade in the OECD 301B studies such as high MW PEGs and highly substituted CMCs. Overall, the impact on rate and extent of mineralization with study modifications increasing the ratio of inoculum to test substance concentrations observed in the Menzies et al. research across all polymer classes was more significant than the limited impacts observed in this study for some of the polymeric test substances. More research is needed to fully understand test system modifications and impacts on test results.

Holistically looking across the guideline and modified studies for all 5 polymers, differences in extent of mineralization were not observed except for CMC 0.6 in B-8G where 70 % mineralization was reached when test duration was significantly extended to 147 d and in F-4G where only 17 % mineralization was measured at 60 d. For the other test systems, CMC 0.6 studies conducted for ~60 d only reported 40–53 % mineralization highlighting the importance of test extension when evaluating slowly degrading polymers. The strong reproducibility of these results across labs and polymer types is a very important finding given that activated sludge from 8 different treatment plants in 4 different countries located on multiple continents was used as the inoculum. In addition, different endpoints for analysis (CO_2 generation and O_2 consumption), aqueous test volumes, and ratios of microbial inoculum to test substance concentration were utilized across the studies. Even when the same lab intentionally increased inoculum or decreased test substance concentration (as discussed above), essentially the same final extent of mineralization was observed. This finding provides significant reassurance regarding reproducibility of OECD 301 studies conducted at different laboratories in different geographies with varying test conditions. The only variable that significantly impacted the results was test extension which can and should be readily employed for test substances when mineralization is occurring at a slow rate and quantification of maximum mineralization is the purpose of the study.

3.8. ISO 14852 study results

Study B-7M can be considered a modified OECD 301B (OECD 1992) but also follows the guideline for ISO 14852 (ISO, 2021) both of which utilize CO_2 evolution as the analytical endpoint. However, ISO 14852 uses a slightly different mineral media than the OECD 301 TG as needed for the higher levels of inoculum allowed in the TG. B-7M utilized higher test substance concentrations and higher inoculum levels than OECD 301B studies (in the range of the ISO 14852 guideline) but the inoculum to test substance concentration ratio matched that of an OECD 301F (~1:3). It is important to note that for respirometric biodegradation test methods the inoculum to test substance concentration ratio must remain within a narrow range to accurately quantify mineralization above the background level of respiration. Comparing the results of B-7M with the other activated sludge inoculum studies, the extent of mineralization was similar for all 5 polymers, but the 28-d data point was lower for PEG 35000, PVOH 18-88 and MCC indicating that in-test rates of mineralization were slightly slower with this study design and test extension beyond 28 d was critical to evaluate the full extent of mineralization.

3.9. River water results for all 5 polymers

In addition to the OECD 301F and OECD 301B guideline and modified studies utilizing activated sludge as the inoculum source, screening

respirometry studies were conducted on all 5 polymers utilizing river water as the inoculum source. Results of the river water studies are presented in Fig. 4 and Table 2. For all 5 polymers a similar extent of mineralization was reached in the river water studies as was observed in the studies utilizing activated sludge as the inoculum source (Tables 1 and 2). For PEG 35000 in river water studies the final extent of mineralization was 77 % with minimal DOC remaining (<1 %) and in the activated sludge inoculum studies the average extent of mineralization was 89 ± 5.5 % indicating significant mineralization of the test material with both types of inoculums. PVOH 18-88 reached 72 % (3.5 % DOC remaining) in the river water studies and 85 ± 7.4 % in the activated sludge studies again showing comparable results for both types of inoculums. Like the nonionic polymers, the charged polymers also showed similar extent of mineralization in river water studies compared to activated sludge inoculum studies reaching 44 % and 57 % in river water and 44 ± 13 % and 48 ± 4.1 % (CMC 0.6 and MGG respectively) in activated sludge studies. Of note, the slowly degrading CMC 0.6 reached 42 % in river water which is on par with the activated sludge studies conducted for ~60 d but not the B-8G study where CMC 0.6 reached 70 % mineralization after 147 d. MCC had a similar final extent of mean mineralization in river water and activated sludge systems with 79 % compared to 88 ± 6.2 % respectively, but time to reach maximum extent of mineralization was longer in river water (note the river water test was extended to 95 d to allow for each polymer to reach the plateau phase). The slower rate of mineralization observed in the river water studies could be due to the lower microbial inoculum level in the river water study (10^6 CFU/L compared to 10^7–10^8 typically in OECD 301F & B guideline studies). The slower rate and lower extent of mineralization detected was partially due to the pH of the river water study (starting pH = 8.2, test termination pH range 9–10 based on previous studies in this laboratory) which results in the retention of CO_2 in the aqueous test system due to equilibrium between aqueous phase and headspace of the test system. The OECD 301B studies which also evaluate evolved CO_2 as the analytical endpoint were conducted at pH 7.4 ± 0.2 which helped ameliorate the issue of CO_2 retention in the aqueous phase over the course of the study. Research is being conducted to optimize the test design for future river water screening studies.

4. Conclusions

With the increased pressure to quantify the biodegradation potential of polymers, the need to identify suitable screening methods becomes apparent. Environmental experts in academic, regulatory and industry environments have long used the OECD 301 TG as a screening test to evaluate chemical biodegradation potential, but little information was available on the applicability and reproducibility of the TG for polymeric test substances. In evaluating the results of studies performed on 5 high MW polymeric substances across 8 laboratories using activated sludge as the inoculum, the intra- and interlaboratory variability was low especially when evaluating final mineralization from tests extended beyond 28 d. Test extension was the modification that most significantly impacted biodegradation results while variation in inoculum levels and test substance concentrations did not significantly impact the results for the polymers evaluated in this study. It should be noted that these modifications in inoculum levels and test substance concentrations may not have been great enough to quantify a substantial difference in mineralization rate or extent. The OECD 301B and F test results were predictive of river water mineralization, but the river water studies took longer to reach maximum levels of mineralization likely due to lower microbial counts in the study setup. MCC was found to rapidly degrade and meet the reference criteria in 7 out of 8 labs using activated sludge inoculum suggesting it may be useful as a reference material for rapidly degrading polymers. Further research is needed to identify slowly degrading polymeric reference materials to provide a better assessment of inoculum performance over longer test durations. While more research is needed to represent the large chemical domain of polymers, this study indicates that OECD 301 TG studies can yield reproducible results when evaluating the biodegradation potential for this subset of polymers.

CRediT authorship contribution statement

Kathleen McDonough: conceptualization, study management, data curation, writing, review, and editing.

Jennifer Menzies: conceptualization, study management, data curation and visualization, review, and editing.

Glauco Battagliarin, Jared Bozich, Marlies Bergheim, Bjorn Hidding, Christian Kastner, Bahar Koyuncu, Georg Kreutzer, Hans Leijs, Yash Parulekar, Meera Raghuram, Nathalie Vallotton: conceptualization, study management, data curation, review, and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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