Quantifying the difference between CO_2-release and carbon conversion in aerobic aquatic biodegradation tests

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Abstract

Quantitative biodegradation measurement of polymers (plastic) is, usually, done by monitoring indirect parameter, such as CH_4- or CO_2-release or oxygen consumption, which are a result of microbial activity. The microorganisms in such a test bottle will metabolise a polymer only if they can use a certain part of the substrate for growth of new biomass (new cells). This part can not be measured with the indirect parameter. In our long time study we investigated a wide range of fast to slowly biodegradable polymers by measuring the biomass growth in addition to the CO_2-release. To that end we extended and improved the information about the metabolisation of the polymer for a full carbon balance. We quantified the biomass growth by measuring insoluble, therefore cellular, protein content from the inocula, from the suspensions during the biodegradation test runtime and at the end of each test. The procedure reduces the doubt about full biodegradability of all polymers, but especially that of mixtures and blends composed from different polymers. Our method evaluation is based on the results of 150 biodegradation tests.

Keywords: aerobic biodegradation, quantitative bioconversion, biomass growth, carbon balance

1 Introduction

According to the European Union Directive (2019/904/EU) there is meanwhile no doubt about the need to replace conventional persistent plastic with biodegradable materials in all applications where whole products or parts of it can end up in the environment [3]. There is also no doubt about the fact that biodegradable plastic does neither reduce unintentional loss nor littering quantities – the expected effect is the reduced harm such losses cause to the ecosystems because of the finite lifetime of biodegradable materials [1].

However, there is some doubt in the public but also among scientists about ultimate biological conversion of polymers [12][20]. It derives from the necessity to measure the biodegradation progress by indirect parameters, such as CO_2 evolution, biogas production, oxygen consumption or decay of total organic carbon (TOC) or dissolved organic carbon (DOC) in the test matrix [7].

Each of these methods has specific advantages and disadvantages, but all of them have in common that the conversion of polymer carbon into newly grown biomass carbon can not be measured [6].

The fate of a polymer‘s chemical elements can be monitored and quantified if it is synthesised from isotope marked components [16] or if the released CO_2 reflects the known distribution of natural isotopes [22]. While such investigations are of basic scientific interest for studying the degradation and assimilation dynamics, they are not applicable for testing of materials and products deriving from a real-scale production facility and which are intended for certification (e.g. DIN-certco or TÜV Austria-Belgium „OK compost“ label according to EN 13432) [4].

Textbooks about biochemistry and about microbiology have mentioned already in the early 20th century the general principle of substrate utilisation by microorganisms [19]. One of these general principles is the universal bioconversion path from extracellular polymer cleavage, uptake of smaller molecules (monomers, oligomers), inside the cell then continuing with katabolism to generate universal small molecules (acetate) and eventually the division into anabolism and energy production [14]. Because of the generation of new biomass for growth and replication, not all of the incorporated carbon can and will become fully oxidised and released as CO_2 (under aerobic conditions). Anaerobic metabolism may have different proportions between anabolism and energy generation but follows the same principal of life: a part of the substrate is used for growth.

< Figure 1 – Carbon mass flow diagram: from substrate to end products due to microbial metabolisation (=biodegradation) [6]. >

It is therefore obvious that the above mentioned indirect measurements, as they are required for biodegradation testing, can never result in a complete release of the substrate carbon (e.g. all substrate carbon released as CO_2) – measurement errors excluded. Scientists know about these principals of life and talk in casual speech about complete biodegradation of pure substances

(such as polymers) when at least 60% of the substrate carbon is released as CO_2. But the same scientists struggle with the interpretation of such CO_2-release results when the test specimen was a mixture or blend of multiple substances (e.g. multiple polymers plus additives). Doubt about a parallel full biodegradation of each of the components seems justified, as diauxic growth prohibits that [19]. Furthermore, a minor component may not be biodegradable but may be overlooked because of its small contribution to the overall carbon balance. This all combined is the reason why the well known test criteria standards can at one hand not request 100% CO_2 release rate but can not allow less than 90% as a pass level for assuring ultimate biodegradation.

The well known batch growth curve, as it is idealised shown in all textbooks, does not end with the stationary phase (the upper level plateau). Microbes can withstand starvation for a certain period of time but will eventually die out. When some bacteria cells disintegrate and release their content into the medium, others utilise this new substrate and start their metabolic activity again. Once more, the general path is followed, ending up in approx. 50-60% of the newly appeared carbon used for energy production and is released as CO_2. This cycle can repeat over and over again until the last living cell consumes the remains of the second last – theoretically [19]. In praxis, each cycle lasts a bit longer than the one before (because of Michaelis-Menten kinetic [14]) and a minimum of three cycles of growth and decay have to happen until more than 90% of the initial substrate carbon converted into energy (and CO_2) is reached – 55% with the first, 80% with the second, 91% with the third cycle. As a consequence, the 90% threshold level required by EN 13432 does not intend to allow 10% of the substrate polymer to remain undegraded, no, it requires significantly elongated test times for safety reasons (passing these three cycles of microbial growth and decay).

Even the approx. 50-60% carbon used for energy production is not a constant. The rate between anabolism and energy production is directly coupled with the required amount of maintenance energy [18]. In a summary, the less favourable the environmental conditions are for the biocenosis, the more energy is required to withstand the external factors and the more of the substrate carbon goes into energy production [9].

As mentioned before, only two situations allow a direct and quantitative measurement of the metabolic carbon pathways: (1) monitoring of isotope distributions from labeled substances or (2) measuring dissolved organic carbon in the test medium in case fully water soluble substances are investigated. For the usual routine biodegradation analysis of biodegradable plastic, none of these situations is applicable.

The draft standard ISO DIS 18957:2025 suggests to treat the solid fraction at the end of a test by dissolving the residual polymer material in organic solvents of choice and declare the insoluble remains as (newly grown) biomass [10]. Such a procedure can not be successful, as (1) parts of the biomass can be extracted with organic solvents and (2) typical polymer blends may not dissolve completely in one solvent. Using an extended series of solvent extractions under harsh conditions may completely destroy the biomass.

The draft standard ISO CD 23292:2025 suggests to quantitatively determine the newly grown biomass in a biodegradation test indirectly by the total DNA amount analysed via qPCR [11]. While this sounds reliable, the longer time presence of DNA which was released from dead cells must be considered [15][23]. Therefore, the measurement can be specific for the number of DNA strands, but is not necessarily reflecting the cellular mass, including a potentially variable amount of cellular storage substances [13].

There is a more promising option, which is the starting point of this study: Because of inability to separate living cells from residual insoluble polymer, another cellular component that is somehow correlating with the total cell mass has to be used. We followed the guideline of a singular publication that considers all those above mentioned facts and choose protein as indicating parameter [21]. By comparing the biomass at begin and end of a test and calculating the biomass growth, the amount of biodegraded but not as CO_2 released polymer carbon can be estimated [5].

Having measured a proportion between the average total organic carbon and the total protein content of an inoculum should, theoretically, allow to calculate a proportion factor. This number should be specific for a certain inoculum as long as neither the environmental factors nor the metabolic activity do change. However, collecting the microbial biocenosis from a complex real-life

ecosystem is not possible without also collecting a certain amount of habitat (water, minerals and other organic substances). Separating the microorganisms cells from other content is therefore a critical necessity. But even more, adapting the native population to the new laboratory conditions (without adding anything, especially no nutrients and no substrate) is required. By comparing the TOC to protein nitrogen proportion of the inoculum with the situation in the test blinds at the end of a test (which means several weeks of starvation) provides an additional information about the population composition after prolonged starvation – after going through multiple cycles of growth and decay.

Measuring the protein content of test vessels, which have gotten polymers or materials added, the grown biomass can be calculated by using the inoculum specific conversion factor. Adding the carbon content of the new biomass to the carbon which was released as CO_2 should directly give the biodegraded (=metabolised) amount of the polymer or material. This can be expressed as percent of the carbon which was added to the test and represents biodegradation much better.

In this study we combine the results obtained during 27 years of analysis from 16 biodegradation experiments with a total of 62 investigated substances analysed from 150 biodegradation bottles to prove or disprove the possibility and to evaluate advantages and limits of calculating a carbon balance in aerobic aquatic biodegradation tests.

2 Methods

2.1 Polymer element analysis

The elements C, H, N and S were determined on a VARIO EL elemental analyser (Heraeus). A 5 mg sample together with WO_3 catalyst (approx. 5 mg) was weighed into tin boats and carefully folded. The oxidative substance digestion was carried out at a combustion tube temperature of 1150 °C by explosive combustion of the sample in a highly oxygen-enriched (4.9) helium (5.0) atmosphere. In a downstream reduction tube (copper chips, 850 °C), nitrogen oxides (NOx), sulphur oxides (SOx) and volatile halogen compounds were chemically bound and eliminated. The gas components were quantified after selective adsorption and desorption on heatable separation columns using a thermal conductivity measuring cell (WLD).

Sulfanilic acid (Merck 100684) was used for instrument calibration and determination of the respective daily factor. The contents of the respective elements are given in mass percent [% w/w]. The oxygen content is calculated from the difference of the sum of the contents of C, H, N, S and ash to 100 %.

2.2 Inoculum preparation from sewage sludge

Sewage sludge was collected from the local 20000 EWG communal wastewater treatment plant (WWTP) in Tulln, Austria. The suspension was harvested from the pipe where enriched biomass was pumped from the sedimentation basin towards the biogas plant. The sludge was still aerobic.

The sludge was stirred in a 600 mL open glass baker in the laboratory at constant temperature of 21°C with passive aeration for a minimum of 5 days, but mostly 7 days prior to use. While the sludge did not contain any ready biodegradable solids after its retention time of 21 days in the WWTP, the additional time in the lab was provided for climate adaptation and stabilisation.

pH-value and solid dry matter was determined to characterise the inoculum, in some cases respiration activity was measured. However, the measured parameters did not have any significant influence on the biodegradability of biodegradable or not biodegradable polymers.

2.3 Inoculum preparation from compost

Compost was obtained from a local semi-industrial compost operation plant near our Institute. Compost is produced there from mixed communal, agricultural and green waste with typical seasonal variations. Still active and warm compost (Rottegrad 2, as defined in [2]) was collected for thermophilic biodegradation test, while ripe compost (Rottegrad 4-5) was used for tests at mesophilic conditions.

In both cases, approx. 1 kg compost was immediately submerged in approx. 4 litre warm or cold tap water, depending on the intended use, and manually stirred for one hour. Coarse particles were removed by sieving the suspension successively through sieves with 10, 2, 0,5 and 0,22 mm mesh widths. This suspension was allowed to sediment for 30 minutes. After this time swimming parts were removed from the top and sand and sediments were left behind when pouring the

172 suspension carefully into a glass baker. As such, the suspension was stirred at room temperature
173 or at 45°C, depending on the intended use, for a minimum of 3 and up to 5 days with passive
174 aeration. After that time a similar sedimentation step was done after which the biomass was
175 enriched and collected by a gentle centrifugation at approx. 500xg for 20 minutes. The pellet was
176 then re-suspended in mineral medium (Table 1) and equilibrated for another 5 to 7 days at the
177 intended test temperature.

178 pH-value and solid dry matter was determined to characterise the inoculum, in some cases
179 respiration activity was measured. However, the measured parameters did not have any significant
180 influence on the biodegradability of biodegradable or not biodegradable polymers.

181

182 ***2.4 Aerobic aquatic biodegradation test (modified Sturm test)***

183 Measurement of aerobic biodegradation via CO_2-evolution was done following the procedure of
184 OECD 301B, which is also called modified Sturm test [17]. We used 500 mL glass bottles (Schott)
185 with gas washing heads. The test setup consisted of 400 mL carbon free mineral medium (Table
186 1), 10 mL of inoculum and an amount of test substance that contains roughly 500 mg organic
187 carbon. The 250 mg ammonia sulfate equals approx. 53 mg nitrogen and allows for a synthesis of
188 approx. 330 mg/L protein due to microbial growth.

189 The bottles were placed in an incubation chamber for thermophilic conditions (58 ± 2°C) or were
190 operated in the laboratory at approx. 21 ± 1°C. Almost CO_2-free air was pumped through the
191 bottles in a rate of approx. 8-9 mL per minute and the exhaust gas was trapped in 0.2 M NaOH
192 solution for each bottle individually. Absorbed CO_2 was determined volumetrically by titration of an
193 aliquot volume with 0.5 M HCl and calculated from the volume HCl between pH 9
194 (Phenolphthalein, Merck 107233) and pH 5 (mixed indicator Merck 106130). Titrations were done
195 in intervals of two times per week at the begin and up to once every four weeks towards the end of
196 a biodegradation test run.

197 When enough sample polymer was available, threefold determinations were done, but in some
198 cases only double determination – and even then with reduced carbon content – was possible.

Reference substances were either amorphous cellulose from spruce (Fluka 22181) or pure PHB (Sigma P-8150).

Table 1: Mineral medium for the Sturm test, pH 7.1-7.2.

Substance	amount per L
K_2HPO_4	0.50 g
KH_2PO_4	0.20 g
$MgSO_4 \cdot 7H_2O$	0.20 g
NaCl	4.5 g
$(NH_4)_2SO_4$	0.25 g
$CaCl_2 \cdot 2H_2O$	0.40 g
Trace element solution [19]	1.00 mL

Evolved CO_2 was expressed as percent of the theoretically possible CO_2 calculated from the carbon content of the sample in each degradation bottle.

$$\text{sample TH-}CO_2 = SW \bullet \%\,C \bullet 36.6$$

% C	Carbon content of the sample (TC-analysis)
36.6	Factor % C to mg CO_2 per g
SW	Sample in the bottle, in g

$$\text{total } CO_2\ [mg] = V_M \bullet M \bullet 44 \bullet A$$

V_M	Volume HCl [mL] between pH 8.9 and 4.5
M	Molarity of the HCl
44	mg CO_2 per 1 mMol HCl
A	Aliquot of the NaOH absorber solution for titration

$$\text{sample } CO_2\ [mg] = \text{total } CO_2\ (\text{sample}) - \text{total } CO_2\ (\text{blind})$$

$$\%\ CO_2 = \text{sample } CO_2 \bullet 100 / \text{sample TH-}CO_2$$

2.5 Determination of insoluble TC and TOC and soluble DC and DOC

Total carbon (TC) of suspensions (inocula, samples from degradation tests) was analysed in the Solid Sample Module (SSM-5000 A) of the TOC Analyser (5000 A, Shimadzu). Clear solutions (centrifugation supernatants, filtered through 0.22 µm PTFE) were directly injected into the TOC Analyser for measurement of dissolved carbon (DC). For analysis of the respective organic carbon, 2M HCl was added to the samples and CO_2 gas was purged before thermal oxidation in the TOC Analyser – this was done by a procedure automatically run by the instrument.

225 TC and DC as well as TOC and DOC were analysed, TIC and DIC were calculated as the remains
226 after subtracting the inorganic carbon part from the respective total carbon. The measurement limit
227 for DOC and DIC was 2 mg/L.

228 After carefully examining the results from approx. 80 biodegradation tests (some not used as data
229 base for this manuscript), we never found amounts of dissolved inorganic carbon above approx.
230 0.2% of the sample polymer carbon introduced to the test. Furthermore, we never found significant
231 amounts of dissolved CO_2 in inocula or in the blind tests at the end of the biodegradation tests. In
232 those cases when we measured DC and DIC in samples taken while the biodegradation processes
233 were still going on, we found DOC and DIC concentrations above the measurement limit, but which
234 were still less than 1% of the carbon brought in with the polymer samples.

235 Some samples were analysed for TOC by using the ready-to-use test system of Hach-Lange (Art.
236 Nr. LCK380) according to the producers procedure. The principle is similar to the instrumental
237 determination in terms of organic substance digestion. The difference is in the photometric
238 quantification of the released CO_2 in an absorption tube adhered on top of the digestion tubet. The
239 LCK380 test system is less sensitive than the instrumental analysis, therefore we never measured
240 any concentration of DC or DIC above measurement limit (10 mg/L).

241 ***2.6 Determination of insoluble protein (total microbial cellular protein)***

242 For the determination of biomass protein 10 mL aliquots from the degradation suspension were
243 centrifuged (approx. 4500 g for 20 min), the supernatant was removed using a syringe with
244 cannula and the pellet was washed once with de-ionised water, centrifuged again and pellets were
245 stored frozen at -20°C until protein analysis.

246 400 µL of 0.25 M NaOH solution were added to each pellet and incubated in the water bath at
247 95°C for 15 minutes with gentle shaking, diluted by adding 600 µL deionised water and centrifuged
248 at 4500xg for 5 minutes. 50 µL of the supernatant of the sample or a BSA standard was incubated
249 with 1500 µL of Bradford reagent (Sigma-Aldrich No.: SLCG1879) for 30 minutes at room
250 temperature. Measurement was then taken with a UV-Spectrophotometer (Shimadzu, UV-1800) at

595 nm. To calculate the protein concentration of the pellets, a calibration function with BSA standard in the concentration range between 0.005 mg/ml to 1 mg/ml was prepared.

2.7 Calculation of TC to protein relation and carbon balance

The carbon to nitrogen relation can be expressed based on different levels of organisation, but most often either as total organic carbon to nitrogen (TOC/N or more often simply C/N) or biomass to protein (BM/protein). Based on what we analysed with our methods we decided to not introduce empirical conversion factors, neither to calculate organic substance from total carbon nor calculate organic nitrogen from protein. While there are empirical factors for the C to N ratios of microbial cell mass given in literature (4.2 by [8], 3.57 by [14]), they differ quite a lot. It is to assume that differently composed inocula biocenoses may have differently composed cellular proteins, at least different to a certain degree. We decided to calculate the relation directly as we measure the parameter and therefore the relation of total carbon to protein (and neither organic carbon / protein nor carbon / nitrogen) was used for all further calculations.

$$\text{C:Prot ratio} = TC_{inoculum}\ [mg/L] \ / \ protein_{inoculum}\ [mg/L]$$

$$\text{C-biomass}_{testend}\ [mg] = protein_{testend}\ [mg/L] \bullet \text{C:Prot ratio} \bullet 0.25$$

$$\text{C-residue} = (\text{sample TH-}CO_2 - \text{sample } CO_2) \ / \ 3.6 - \text{C-Biomass}_{testend}$$

$$\%\ \text{residue} = \text{C-residue} \bullet 360 \ / \ \text{sample TH-}CO_2$$

2.8 Statistics

All statistics (boxplots, outlier, error bars) and graphical representations were done with R-Studio (2024.12.1+563) with R version 4.4.3 (2025), using ggplot2, reshape2 and ggthemes libraries.

3 Results & Discussion

3.1 Biomass growth hypothesis test with water soluble substances

As described in the introduction, microbial metabolism prohibits a complete conversion of a substrate (not so for an inhibitory or toxic substance) into CO_2, water and minerals. The driving force for substrate metabolisation is cellular growth – which is a increase of biomass. The usual aquatic biodegradation tests have to work with the assumption, that a release of approx. 50-60% of

278 the substrate carbon as CO_2 is equal to a full metabolic conversion of the substrates organic
279 carbon.

280 For model experiments water soluble pure substances, namely starch (Sigma, S9765) and
281 cellobiose (Sigma, C7252) were used. The first pre-experiment of this study was to measure CO_2-
282 evolution and substrate decay (as DC-decay) in an aquatic biodegradation test. The result is
283 shown in Figure 2. Within two weeks both water soluble substrates were completely metabolised
284 (97.6 % starch and 98.0 % cellobiose) but only 77 % of the substrates carbon was released as
285 CO_2 (77.0 % starch and 77.2 % cellobiose).

286

287 < Figure 2 – Comparison of CO_2-evolution and DC-decay as measurement parameters in an
288 aquatic biodegradation test. Mean values from three parallels, each. >

289

290 CEN and ISO standard methods lack a description how to quantify the amount of organic carbon
291 that derives from a substrate and ends up as new biomass (new living microorganisms cells).
292 Biomass growth can be followed by usual microbiological and even by gravimetric methods in case
293 a water soluble substrate is used. Testing insoluble polymers and blends of unknown composition
294 disallows such simple biomass quantification measurements [6]. Other method principles have to
295 be found, where the measurement of biomass must not be disturbed by residual particles of not
296 biodegraded substrate. Therefore none of the more simple measurement methods, neither turbidity
297 (OD) nor gravimetric, can be used and limitations of quantitative DNA analysis must be considered.

298 Protein that is released from dying cells may not be present in the medium for a longer time, as it is
299 a highly valuable carbon-, energy- and nitrogen source for other cells. Active release of
300 extracellular enzymes can accumulate to elevated concentrations during exponential growth but
301 will be cleaved and absorbed in stationary and decay phase. Besides that, the protein content may
302 not appear in a fixed relation to the total biomass of living cells over different activity phases.
303 Biodegradation tests must be seen as batch cultivation with all the typical growth phases. During

exponential growth high enzyme activity and initially low but rising contents of lipid and carbohydrate reserve may significantly change carbon / protein ratios over time. When activity is reduced during deceleration- and especially during stationary phase the carbon / protein relations later approaches a certain value asymptotically, which should eventually be similar to the initial value of an inoculum stabilized in the starvation state.

We therefore measured the time dependency of protein content in the aquatic biodegradation test with water soluble starch and cellobiose. When comparing the time dependent protein content in Figure 3 with the degradation progress in Figure 2, a direct correlation can be assumed. It is also seen, that the maximum protein content did not exceed the sum of initial concentration plus 330 mg possible from inorganic nitrogen of the medium. Biomass protein has increased from 95.7 mg/L at test begin to 220 and 189 mg/L for starch and cellobiose, respectively, within 14 days. The highest protein contents were reached at day 3 for starch (409 mg/L) and at day 5 for cellobiose (314 mg/L). Protein content of the blind (no substrate added) slowly decayed to 85.8 mg/L almost linearily.

< Figure 3 – Protein content in the biodegradation bottles during starch and cellobiose degradation (same experiment as in Figure 2). Mean values from three parallels, each. >

Measuring biomass organic carbon (the soluble fraction was removed by centrifugation) and biomass protein at different times from the same test finally showed the microbial metabolisation dynamic of the substrate. The carbon / protein relations are shown in Figure 4. Biomass carbon is given as total carbon (TC), no inorganic carbon was measured separately. Microorganisms grew due to metabolisation of the substrates according to their ease of digestibility, changed their carbon / protein relation, presumably because of increased protein synthesis, and shifted the relation back to higher numbers over time. Because of the short test time, the stationary phase was barely reached and the biocenosis in the substrate bottles had putatively different conditions compared to those of the blinds. The blinds held their carbon / protein relation almost constant – next to no

331 biological activity was observed (17 mg CO_2 , equal to 4.6 mg C total released during these 14
332 days).

333

334 < Figure 4 – Relation of biomass carbon (TC) / biomass protein during a biodegradation test for a
335 blind and for the two water soluble substances (same experiment as in Figure 2). Mean values
336 from three parallels, each. >

337 ***3.2 TC / protein relations of microbial inocula before and after a biodegradation test***

338 Biomass carbon (expressed as TC) and biomass protein was measured for 18 inocula and 28
339 aquatic biodegradation test blinds. All samples were measured in double determination. At the end
340 of each biodegradation test six carbon- and six protein results were obtained. Only two generally
341 different inocula types were used, both in their native composition and without pretreatment or pre-
342 exposure, but after removal of coarse particles (sand, swimming things) and after an equilibration
343 time between 7 to 10 days at according temperature. The two types were: mixed microorganism
344 suspensions from sewage sludge at the outflow of the active basin and washed-off suspensions
345 from source separated communal biowaste compost.

346 The obtained carbon to protein relations of these inocula are shown in Figure 5. There was a
347 significant difference in the initial numbers between sewage sludge and compost inocula. Both
348 these inocula types contain living microorganisms and additional organics of unknown composition.
349 Because of the equilibration time, we assume having used inocula that were already in or close to
350 the stationary phase.

351 Total CO_2-evolution from the blinds was generally low, with exceptions, indicating a low content of
352 biodegradable organics which were brought into the test bottles with the inoculum. Bubbling the
353 inflow air through 2 M NaOH did not remove all CO_2 before entering the biodegradation bottles.
354 Finally, it was not possible to distinguish between CO_2-residues from the air inflow and CO_2-
355 evolution from microbial activity in the blinds. In one single experiment we used CO_2-free synthetic
356 air for aerating the biodegradation test (but did not do a full carbon balance analysis). In these tests
357 we measured a CO_2-release of 3.1 mg CO_2 per g inoculum DM per month compared to a range of

358 3.6 to 5.7 mg CO_2 per g inoculum DM per month in tests operated with compressed ambient air.
359 Cost and effort of running aquatic biodegradation tests with synthetic air does not seem justified.

360

361 < Figure 5 – Boxplots of suspended organic carbon (as TC) / protein relations in different inocula.
362 sewage sludge n = 5; compost n = 13 >

363

364 ***3.3 TC / protein relations after a biodegradation test***

365 The biomass carbon to biomass protein relations of blinds at the end of the tests showed a wide
366 range of values, graphically presented in Figure 6. Carbon to protein relations ranged from 1.2 up
367 to 5.7. Blinds from thermophilic tests had a not significantly lower mean value of 2.8 compared to
368 3.2 in blinds from mesophilic tests. We can only assume that not controlled and not analysed
369 factors had a bigger influence on the microbial biocenosis compositions than the test temperature
370 had.

371

372 < Figure 6 – Boxplots of TC / protein relations from inocula and from the respective resulting
373 biomass of blinds (no substrate added) after the according test duration. inokula n = 13; blind meso
374 n = 9; blind thermo n = 19 >

375

376 ***3.4 Carbon balance method test with water soluble substances***

377 After evaluating the prerequisites and specific analysis one-by-one, the generated data were
378 combined into a full carbon balance calculation. By using water soluble substrates their
379 disappearance during the biodegradation test could be followed analytically – which is impossible
380 when not water soluble samples of, maybe, unknown composition were tested. Figure 7 shows the
381 graphical representation of the special measurements for starch and Figure 8 shows the similar
382 representation for cellobiose. For both these graphs the biomass carbon was not calculated from

383 biomass protein measurement – the carbon content of the isolated biomass was directly
384 determined.

385

386 < Figure 7 – Biodegradation of water soluble starch expressed as CO_2-evolution, dissolved CO_2,
387 biomass carbon and residuals (difference to 100%) for a full carbon balance (starch data as in
388 Figure 2). Mean values from three parallels. >

389

390 < Figure 8 – Biodegradation of water soluble cellobiose expressed as CO_2-evolution, dissolved
391 CO_2, biomass carbon and residuals (difference to 100%) for a full carbon balance (cellobiose data
392 as in Figure 2). Mean values from three parallels. >

393

394 ***3.5 Method test with not water soluble, inherently biodegradable biopolymers***

395 Water soluble substances allow the formulation of the hypothesis, but in practical routine analysis,
396 next to all polymers are insoluble in water and have maybe unknown composition. There is no
397 possibility to analytically determine if insoluble organic carbon derives from residual polymer or
398 from newly grown biomass. A complete carbon balance, as shown before with the model
399 substances, was not possible. Analysing the carbon to protein relation from the used inoculum is
400 therefore a critical parameter, because the biomass carbon must be calculated from this relation,
401 assuming that it doesn't change much during the test time. The alternative – and therefore the
402 control parameter – is the carbon to protein relation of the biomass obtained from the blinds at test
403 end. However, due to prolonged starving conditions, compared to test bottles to which a
404 biodegradable polymer was added, the relation may be different between polymer and blind bottles
405 and different from the initial inoculum.

406 Results of an aquatic aerobic biodegradation test with starch and cellulose at mesophilic
407 temperature (21°C) are summarised in Table 2. The carbon / protein relation of the inoculum was
408 5.38, and it was 3.00 in the blinds at the end of the test, after 36 days. While the indirect calculation

409 of quantitative biodegradation via evolved CO_2 would had resulted in 63% for cellulose and 68%
410 for starch, carbon balance calculation increased these values to 92% and 77% degree of
411 metabolisation for the two substances, respectively. The conspicuously high gap of almost 23%
412 missing carbon for starch can be explained by a probably higher carbon to protein relation of the
413 biomass grown on starch than that of the biomass grown on cellulose. However, due to the inability
414 to measure the biomass carbon in these test suspensions directly, it remains a hypothesis. The
415 comparably slower metabolised cellulose resulted in a good congruency between theory and
416 praxis.

417

418 < Table 2 – Carbon balance for an aquatic aerobic biodegradation test with starch and cellulose at
419 mesophilic conditions. Only two of the three parallels were analysed for carbon balance. >

Bottle	Polymer	Polymer C in [mg]	C as CO_2 out [mg]*	C in biomass [mg]	C as CO_2 out [%]	Metabolised [%]	Residue [%]
2	Cellulose	413.8	257.0	-	62	-	-
4	Cellulose	479.0	322.7	116.1	67	92	8
8	Cellulose	367.6	219.4	118.7	60	92	8
3	Starch	469.7	336.1	40.1	72	80	20
5	Starch	363.6	248.7	-	68	-	-
6	Starch	429.7	270.9	50.1	63	75	25
1	Blind	-	82.1	8.9	-	-	-
7	Blind	-	75.6	-	-	-	-
9	Blind	-	80.7	9.5	-	-	-

420 *) values given as net CO_2-evolution from samples (individual sample minus mean value blinds)

421

422 ***3.6 Practical method check with a blend made from three biodegradable polymers***

423 The distribution of substrate carbon (anabolism, energy production) is comparably well predictable
424 when pure substances are investigated. It is much more challenging, if not impossible, to conclude
425 from the amount of released CO_2 to the degrees of metabolisation for each of the components of a
426 mixed substrate (of a polymer blend). Therefore we tested the carbon balance procedure with a
427 polymer blend consisting of three chemically different polymers, namely: 80%(w/w) PHBV (Enmat
428 Y1000P, TianAn), 13.3%(w/w) PBS (Bio-PBS FD92PM, Mitsubishi), and 6.7%(w/w) PLA
429 (Cordenka fibres 610F). Biodegradation is shown as diagram over time in Figure 9 and the data of

the carbon balance are listed in Table 3. PHBV was used as positive control for this test due to limited amount of bottles.

< Figure 9 – Biodegradation of PLA, PBS, PHBV and a blend of those three polymers in an aquatic aerobic biodegradation test at thermophilic conditions (58°C). Respective data in Table 2. All data are mean values from three parallels. >

< Table 3 – Carbon balance for an aquatic aerobic biodegradation test with a polymer blend consisting of three different biodegradable polymers at thermophilic conditions. >

Bottle	polymer	polymer C in [mg]	C as CO_2 out [mg]*	C in biomass [mg]	C as CO_2 out [%]	Metabolised [%]	Metabolised av. [%]	Residue [%]
1	Blend	657.7	518.0	67.7	79	89		11
12	Blend	515.4	407.2	28.4	79	85	89	15
15	Blend	548.0	383.7	126.0	70	93		7
3	PLA	457.5	39.1	9.3	9	11		89
8	PLA	537.6	34.6	11.1	6	9	12	91
11	PLA	457.0	66.7	15.9	15	18		82
4	PBS	599.3	0.0	5.8	0	0		100
6	PBS	558.0	0.0	10.0	0	0	3	100
7	PBS	516.2	36.1	9.8	7	9		91
5	PHBV	638.4	402.3	52.8	63	71		29
10	PHBV	508.9	310.5	63.6	61	74	76	26
13	PHBV	638.4	421.1	107.2	66	83		17
2	Blind	-	175.8	8.1	-	-	-	-
9	Blind	-	190.0	8.0	-	-	-	-
14	Blind	-	206.7	8.4	-	-	-	-

*) values given as net CO_2-evolution from samples (individual sample minus mean value blind)

PLA was slowly and PBS not at all metabolised within the 121 test days. PHBV was expected to biodegrade quickly at thermophilic conditions, the behaviour of the blend was unknown. Providing a biodegradation result solely based on the indirect measurement via evolved CO_2 would had let questions open: does the approx. 76% conversion of the blends carbon into CO_2 result from complete biodegradation of all it's components? The carbon balance results in an amount of residue (11% by average) that is lower than the 13% of PBS the blend consists of. Therefore the carbon balance is able to show the effect of co-metabolism in the investigated blend and allows the conclusion of complete biodegradability within 121 days at thermophilic conditions.

3.7 Practical method test with biodegradation at different temperatures

PLA is well known to be slowly biodegradable at ambient/mesophilic conditions but is rapidly metabolised at temperature above approx. 55°C. We set up parallel aquatic aerobic tests at both temperatures and compared biodegradation and biomass growth for Cellulose and PLA. The combined biodegradation diagrams are shown in Figure 10 as an overlay and the carbon balance calculation data are listed in Table 4. Cellulose was fully metabolised at both temperatures, as expected. There was one outlier in the thermophilic test (bottle #8) which showed reduced CO_2-evolution and reduced biomass growth. The reason for this deviation is unknown. Less than 3% of the PLA was metabolised during 62 days at mesophilic conditions, while 22% of the PLA was metabolised at thermophilic conditions during the same time. Biodegradation results based solely on CO_2-evolution would had been approx. 30% lower in all cases. Calculating a carbon balance increased the confidence in biodegradation result and reduced doubts and or speculations about not-degraded residues.

< Figure 10 – Biodegradation of PLA and cellulose via measurement of CO_2-evolution at mesophilic (21°C) and thermophilic (58°C) conditions. All tests were done in triplicates. >

< Table 4 – Carbon balance for two aquatic aerobic biodegradation tests with cellulose and PLA at mesophilic and thermophilic conditions. All data are average of triplicate analysis. >

mesophilic test							
Bottle	**Polymer**	**Polymer C in [mg]**	**C as CO_2 out [mg]***	**C in biomass [mg]**	**C as CO_2 out [%]**	**Metabolised [%]**	**Missing [%]**
1	Cellulose	458	286	155	62	96	4
4	Cellulose	523	363	129	70	94	6
7	cellulose	494	333	121	67	92	8
5	PLA	690	3.5	11.6	1	2	98
6	PLA	786	10.2	12.2	1	3	97
9	PLA	686	0.0	11.7	0	2	98
2	Blind	-	79	10.6	-	-	-
3	Blind	-	89	13.3	-	-	-
8	Blind	-	75	12.3	-	-	-

thermophilic test							
Bottle	**Polymer**	**Polymer C in [mg]**	**C as CO_2 out [mg]***	**C in biomass [mg]**	**C as CO_2 out [%]**	**Metabolised [%]**	**Missing [%]**
5	Cellulose	576	365	188	63	96	4
6	Cellulose	532	317	198	60	97	3
8	Cellulose	551	280	158	51	79	21
1	PLA	625	131	44.8	21	28	72
3	PLA	717	95	36.6	13	18	82
9	PLA	626	91	40.1	15	21	79
2	Blind	-	109	13.3	-	-	-
4	Blind	-	106	14.8	-	-	-
7	Blind	-	96	16.8	-	-	-

*) values given as net CO_2-evolution from samples (individual sample minus mean value blind)

3.8 Practical method test from routine biodegradation analysis done within 27 years

Measurement data of 16 aquatic aerobic biodegradation tests with a total of 150 bottles were calculated for carbon balance and are provided in the support information <data in the Annex>. A wide range of natural and synthetic polymers, some of them in pure form, others as blends with known or partly unknown components were investigated. It doesn't make sense to discuss each of those polymer biodegradation behaviours separately. Most interesting is, how much each of the quantitative biodegradation result was increased by adding newly grown microbial biomass carbon to the amount measured as evolved CO_2. Over the 150 bottles the biodegradation results

478 increased 17% by average and 22% for the cellulose references only. In only 12 of the 150 cases
479 the sum built from evolved CO_2 and biomass carbon exceeded 100% of the carbon introduced with
480 the test substance, with the highest single value of 108%. According to these results it seems
481 justified to claim that the information gain from doing a carbon balance exceeds by far the potential
482 additional error deriving from uncertainties of the exact carbon to protein relation of the microbial
483 biomass at a certain stage (at a certain day) of the biodegradation test. Carbon balance calculation
484 should be included in European and International standard methods.

485 4 Conclusion

486 Routine biodegradation analysis of 62 polymer samples showed possibilities and limits of the
487 aquatic tests method as well as allowed to evaluate the additional information gain from carbon
488 balance analysis. The analysis of organic carbon and protein from biodegradation suspensions,
489 which contain a mixture of residues from a water insoluble substrate (polymer) and microorganism
490 cells of different life stages, is technically simple but has some pitfalls. Attention and care during
491 biodegradation test operation and a general understanding of microbial dynamics is necessary for
492 obtaining reliable carbon balance results.

493 *4.1 Suggestions based on the data generated in this study*

494 Minerals, floating things (wood, plastic, whatever) and excess suspended or dissolved organics
495 should be removed from inocula. This can be achieved by sedimentation, sieving, gentle
496 centrifugation (and careful separation of pellet and supernatant) and by equilibrating the
497 suspension for several days at either laboratory environment or in the incubator at elevated
498 temperature, according to the respective intention of meso- or thermophilic test conditions.

499 Carbon and protein content in the inoculum are critical parameters for calculating carbon balances.
500 The initial relation of a fresh but stabilized inoculum reflects very well the biomass composition of
501 the microorganisms at a later time of a test when the test substance turned out to be
502 biodegradable. The inoculum parameters should be measured in three, better in five repetitions.

503 Measuring biomass carbon and biomass protein from the blinds of each test bottle at the end of the
504 biodegradation test is essential for obtaining a reliable carbon balance for all those substances that

are not or next to not biodegradable. The starvation situation of the blinds does not significantly differ from the starvation situation of a test bottle containing a not or next to not biodegradable substance. These measurements should be done with great care as well. Three parallel blind bottles are recommended and all parameters should be analysed in double determination from each of them separately. However, these analysis are less critical, as it doesn't change the biodegradation result much when only some single percent of the substrate carbon was released as CO_2 over many months.

Protein analysis is the base for calculating the biomass amount at test end and can be obtained by using the factor from either the inoculum or from the blinds at test end. The differences between those relations turned out to be not that big in a majority of tests but had exceptions in some, especially in tests operated at thermophilic conditions. All biodegradation tests should be done in triplicates and carbon balance should be calculated from each bottle individually. If the data are reliable, final values can be combined into average biomass growth, average CO_2-release and average residual substrate carbon. Data can also be presented for each bottle individually and provided as ranges (from – to). Explanations for bigger deviations should be given.

Polymer blends and products containing raw protein as one of their components will disturb the protein based carbon balance calculation. We investigated one such product (data are property of a private company and not part of this study) and made a carbon balance after three month test time. The disturbance disappeared almost completely after this prolonged starvation time – excess organic nitrogen was slowly converted to nitrate by the microbial activity. It is important to get full information about the composition of a polymer before deciding on the test setup.

4.2 Remaining questions and suggested work for the future

The carbon to protein relation of a microbial test biocenosis seems to be within a certain range, but is not predictable, neither for the inoculum origin nor for the development during a test runtime for one given test situation. That is not a big surprise considering varying compositions and abundances of certain species depending on the substrate availability before harvest and

depending on the chemical composition of the maybe unknown test specimen to be measured. No general rule, nor a general relation can be provided as of yet – each case is unique.

While we could demonstrate the general usefulness of measuring a carbon balance that justifies the additional effort in most of the cases, some mysteries remain, such as the gap differences shown in Table 2. In that case two known to be inherently biodegradable substances (starch and cellulose) were used as substrate with the more easily degradable showing a big amount of carbon missing in the combined analysis results. This should be taken as a hint about missing knowledge of metabolic reactions of a mixed microorganism community (in contrast to axenic single species cultures).

Despite presenting data from so many years of biodegradation measurement with 9 scientists and three technicians involved, the results were all obtained at one research institute and done with the varying instrumentations over time. Despite working with highest attention and care, we are looking forward to see other research groups repeating our methods and evaluating our data and findings. We would be pleased having suggested a valid method for calculating a carbon balance that is suitable for routine analysis and will, eventually, become the base for a recommendation in European and International standardisation.

5 Authors declaration and funding

The biodegradation tests were funded by many private companies in the form of ordered analysis to measure and evaluate their products. Main parts of the biomass analysis were funded freely with general money of BOKU University and special analysis of polymer blends were funded as part of the EU cooperative project upPE-T (H2020-NMBP-TR-IND-RIA Grant Agreement 953214).

Special acknowledgement is given to Mr. Uwe Link who did many of the chronologically early basic experiments as employee of our institute in the years 1996 to 2000.

The authors declare having no conflict of interest. The presented work does not rise any ethical issues.

6 Abbreviations used

BSA	bovine serum albumin (protein standard substance)
DOC	dissolved organic carbon
DC	dissolved carbon (organic and inorganic)
TOC	total organic carbon
TC	total carbon (organic and inorganic)
PCL	poly-caprolactone
PHB	poly-hydroxybutyrate
PHBV	poly-hydroxybutyrate-hydroxyvaleriate (biological random co-polymer)
PBS	poly-butyrate-succinate (synthetic co-polymer)
PLA	poly-lactic acid

7 References

[1] Degli Innocenti F., Breton T.: Intrinsic Biodegradability of Plastics and Ecological Risk in the Case of Leakage. *ACS Sustainable Chem. Eng*. 2020, 8, 9239−9249. https://dx.doi.org/10.1021/acssuschemeng.0c01230

[2] Dimambro M., Steiner H.-J., Rayns F.: Literature review: Compost stability – impact and assessment. Report WRAP OMK009-001, Banbury, 2016. www.wrap.or.uk

[3] Directive 2019/904/EU of the European Parliament and of the Council on the reduction of the impact of certain plastic products on the environment. Brussels, 2019.

[4] European Standardisation Organisation (CEN): EN 13432 Packaging – Requirements for packaging recoverable through composting and biodegradation. Test scheme and evaluation criteria for the final acceptance of packaging. Brussels, 2000.

[5] European Standardisation Organisation (CEN): EN ISO 14852 Plastic – Evaluation of the ultimate aerobic biodegradability of plastic materials in an aqueous medium - Method by analysis of released carbon dioxide. Brussels, 2021.

[6] European Standardisation Organisation (CEN): CEN/TR 17910 Biodegradable plastics - Status of standardization and new prospects. Brussels, 2022.

[7] Folino A., Pangallo D., Calabrò P.S.: Assessing bioplastics biodegradability by standard and research methods: Current trends and open issues. *Journal of Environmental Chemical Engineering* 11:109424, 2023. https://doi.org/10.1016/j.jece.2023.109424

[8] Garcia-Depraect O., Lebrero R., Rodriguez-Vega S., et.al.: Biodegradation of bioplastics under aerobic and anaerobic aqueous conditions: Kinetics, carbon fate and particle size effect. Biores. Tech. 344, 126265. https://doi.org/10.1016/j.biortech.2021.126265

[9] Gu W., Müller A.L., Deutzmann J.S., et.al.: Growth rate-dependent coordination of catabolism and anabolism in the archaeon *Methanococcus maripaludis* under phosphate limitation. *The ISME Journal* 16:2313–2319, 2022. https://doi.org/10.1038/s41396-022-01278-9

[10] International Standardisation Organisation (ISO): ISO DIS 18957 Plastics — Determination of the aerobic biodegradation of plastic materials exposed to seawater using accelerated conditions in laboratory. Geneva, 2025.

[11] International Standardisation Organisation (ISO): ISO CD 23292 Plastics - A method for measuring the amount of bacteria in the hydrosphere biodegradability assessment. Geneva, 2025.

[12] Lavagnolo M.C., Poli V., Zampini A.M., Grossule V.: Biodegradability of bioplastics in different aquatic environments: A systematic review. Journal of Environmental Science 142 (2024) 169-181. https://doi.org/10.1016/j.jes.2023.06.013

[13] Lima A., Franca A., Muzny C.A., et.al.: DNA extraction leads to bias in bacterial quantification by qPCR. *Applied Microbiology and Biotechnology* 106:7993–8006, 2022. https://doi.org/10.1007/s00253-022-12276-4

[14] Madigan M.T., Bender K.S., Buckley D.H., Sattley W.M., Stahl D.A.: Brock Biology of Microorganisms, 16th edition, *Pearson*, 2021.

[15] Monticolo F., Palomba E., Termolino P., et.al.: The Role of DNA in the Extracellular Environment: A Focus on NETs, RETs and Biofilms. *Front. Plant Sci.* 11:589837, 2020. doi: 10.3389/fpls.2020.589837

[16] Nelson T.F., Baumgartner R., Jaggi M., etal.: Biodegradation of poly(butylene succinate) in soil laboratory incubations assessed by stable carbon isotope labelling. nature communications, 13:5691, 2022. https://doi.org/10.1038/s41467-022-33064-8

[17] Organisation for Economic Co-operation and Development (OECD): Guideline for Testing of Chemicals 301 B – CO2 evolution (modified Sturm) test. Paris, 1992.

[18] Russel J.B., Cook G.M.: Energetics of Bacterial Growth: Balance of Anabolic and Catabolic Reactions. Microbiological Reviews, 3, 48–62, 1995.

[19] Schlegel H.G.: Allgemeine Mikrobiologie (Engl. General Microbiology), 7th edition, *Thieme*, Stuttgart, New York, 1992.

[20] Strotmann U., Thounad G., Pagga U., et.al.: Toward the future of OECD/ISO biodegradability testing-new approaches and developments. *Applied Microbiology and Biotechnology* (2023) 107:2073–2095. https://doi.org/10.1007/s00253-023-12406-6

[21] Urstadt S., Augusta J., Müller R-J., Deckwer W-D.: Calculation of Carbon Balances for Evaluation of the Biodegradability of Polymers. *Journal of Environmental Polymer Degradation*, Vol. 3, No. 3, 1995.

[22] Vavilin V.A., Lokshina L.Y.: Carbon and hydrogen dynamic isotope equations are used to describe the dominant processes of waste biodegradation: Effect of aeration in methanogenic phase of the landfill. *Waste Management* 166, 280–293, 2023. https://doi.org/10.1016/j.wasman.2023.04.027

[23] Zemb O., Achard C.S., Hamelin J., et.al.: Absolute quantitation of microbes using 16S rRNA gene metabarcoding: A rapid normalization of relative abundances by quantitative PCR targeting a 16S rRNA gene spike-in standard. *MicrobiologyOpen* 2020;9:e977. https://doi.org/10.1002/mbo3.977

Annex – Data from biodegradation tests

<Annex 1 Data table.xlsx>

Carbon balance calculation for 62 polymer substances (including references) in 16 aquatic aerobic biodegradation tests. Explanations in the main text.

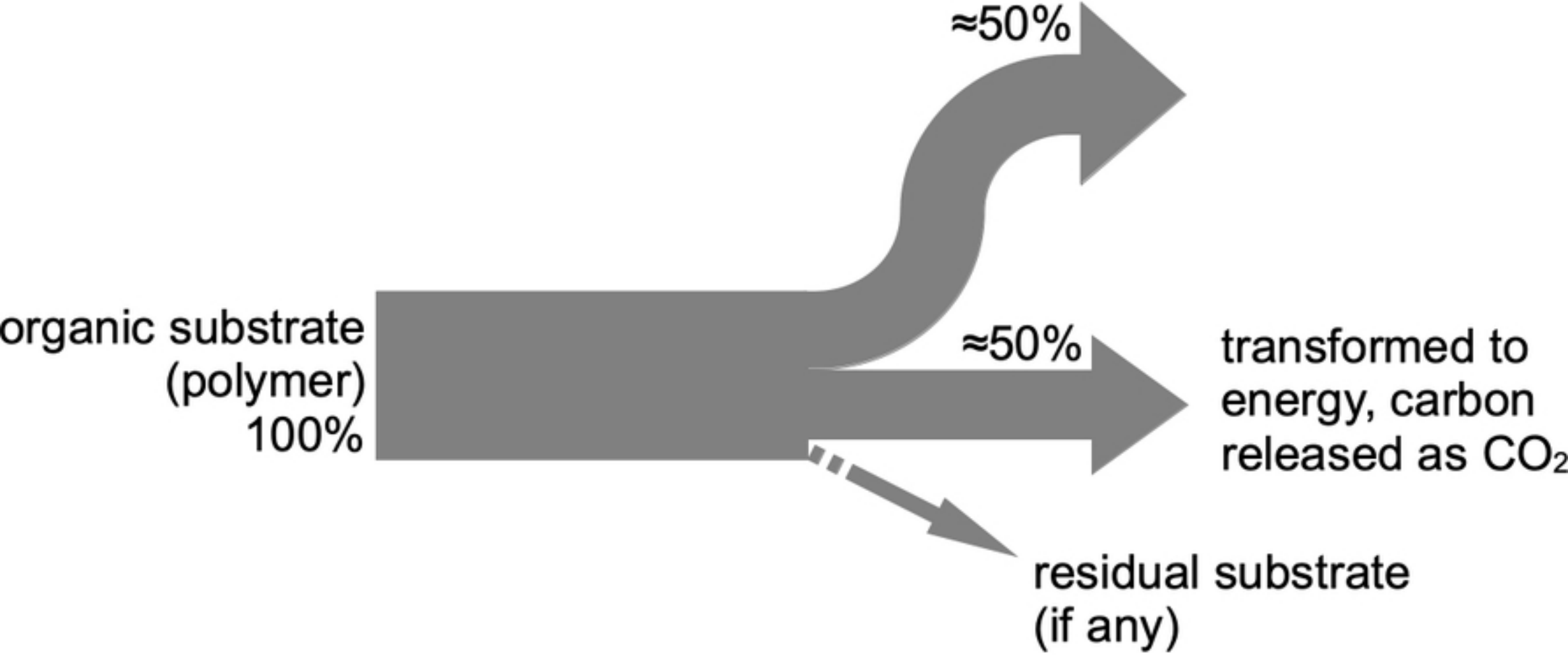
≈50%
organic substrate
(polymer)
100%
≈50%
transformed to
energy, carbon
released as CO_2
residual substrate
(if any)

Figure 1

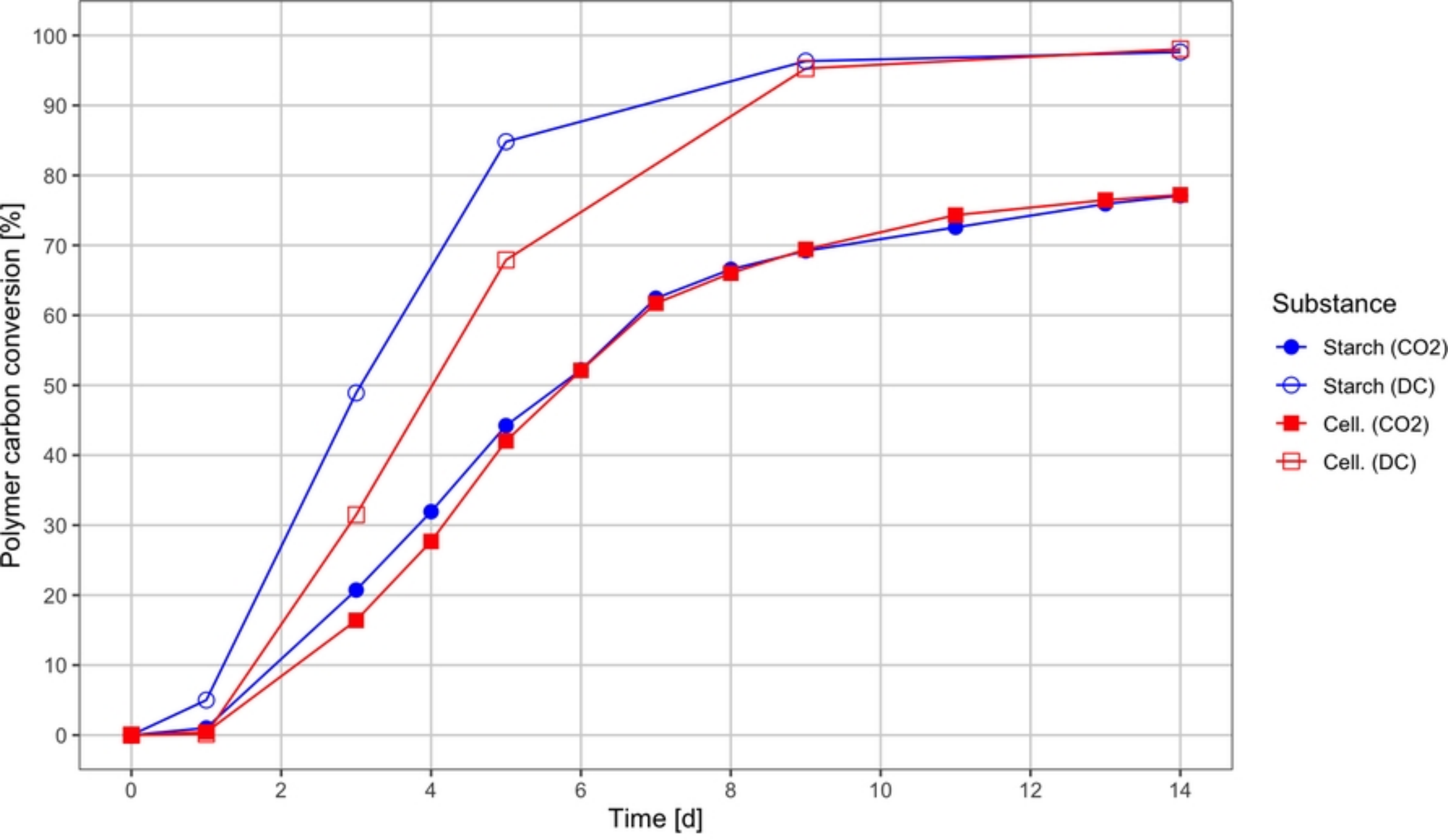
Polymer carbon conversion [%]
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Time [d]
Substance
Starch (CO2)
Starch (DC)
Cell. (CO2)
Cell. (DC)

Figure 2

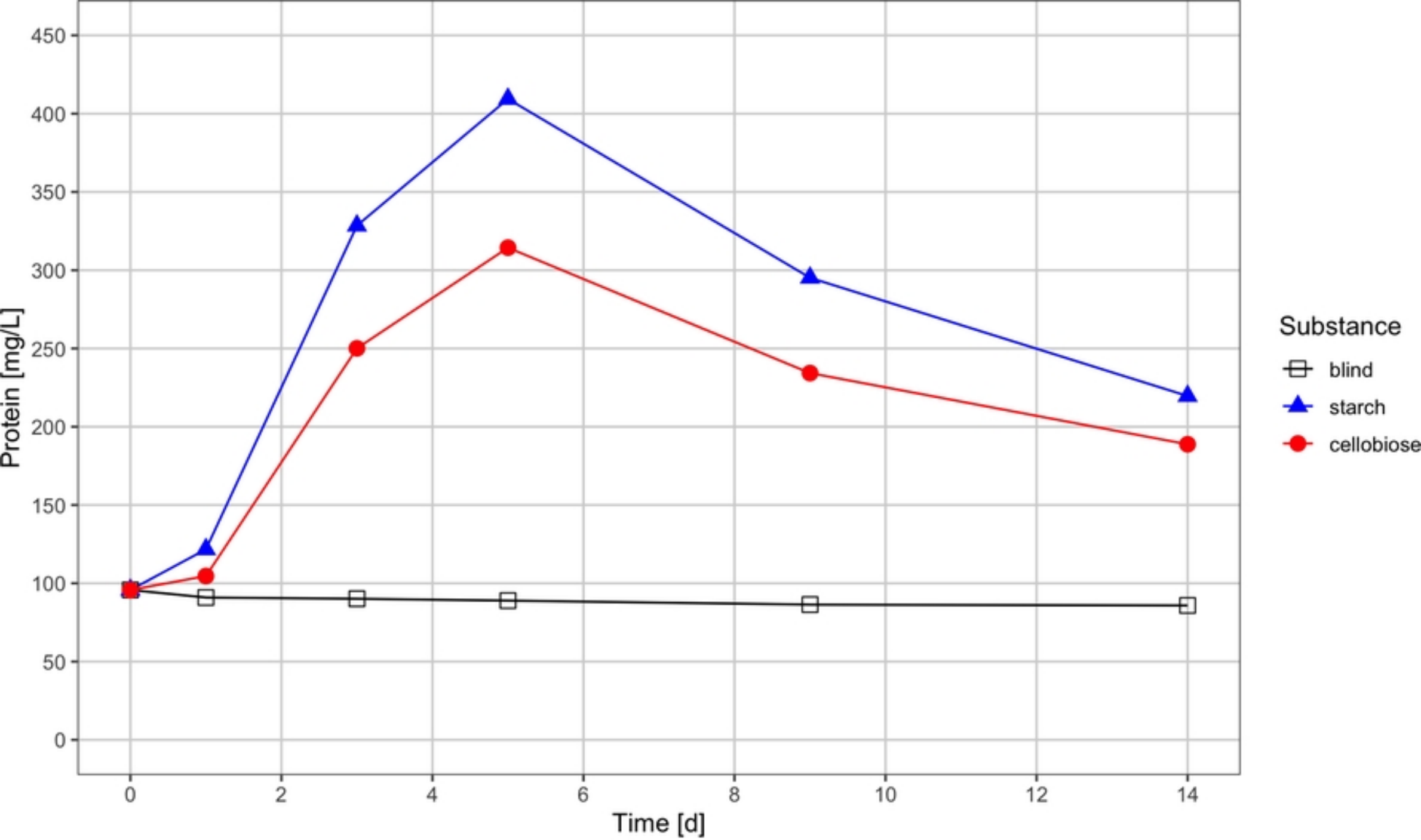
Protein [mg/L]
450
400
350
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
Time [d]
Substance
blind
starch
cellobiose

Figure 3

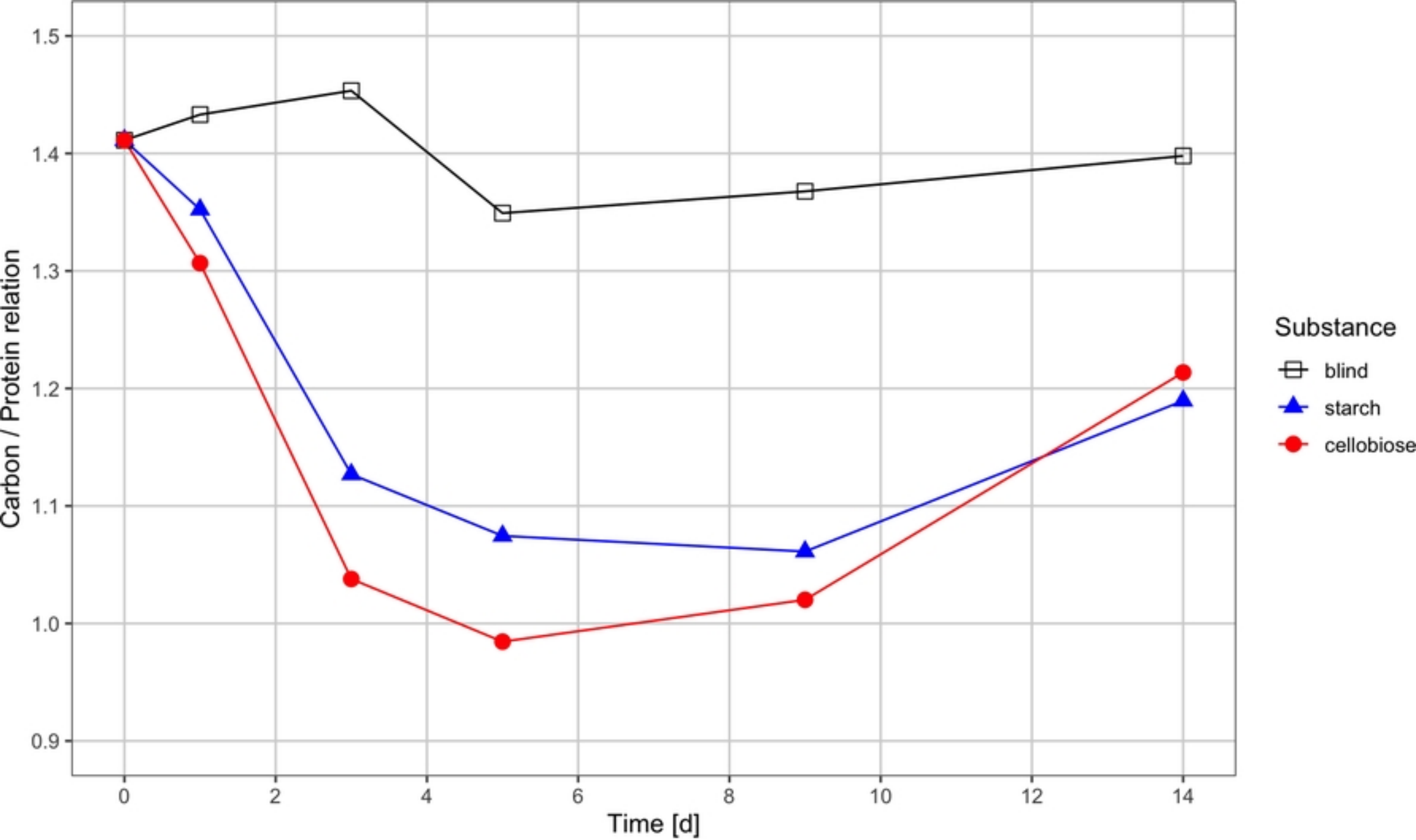
Carbon / Protein relation
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0
2
4
6
8
10
12
14
Time [d]
Substance
blind
starch
cellobiose

Figure 4

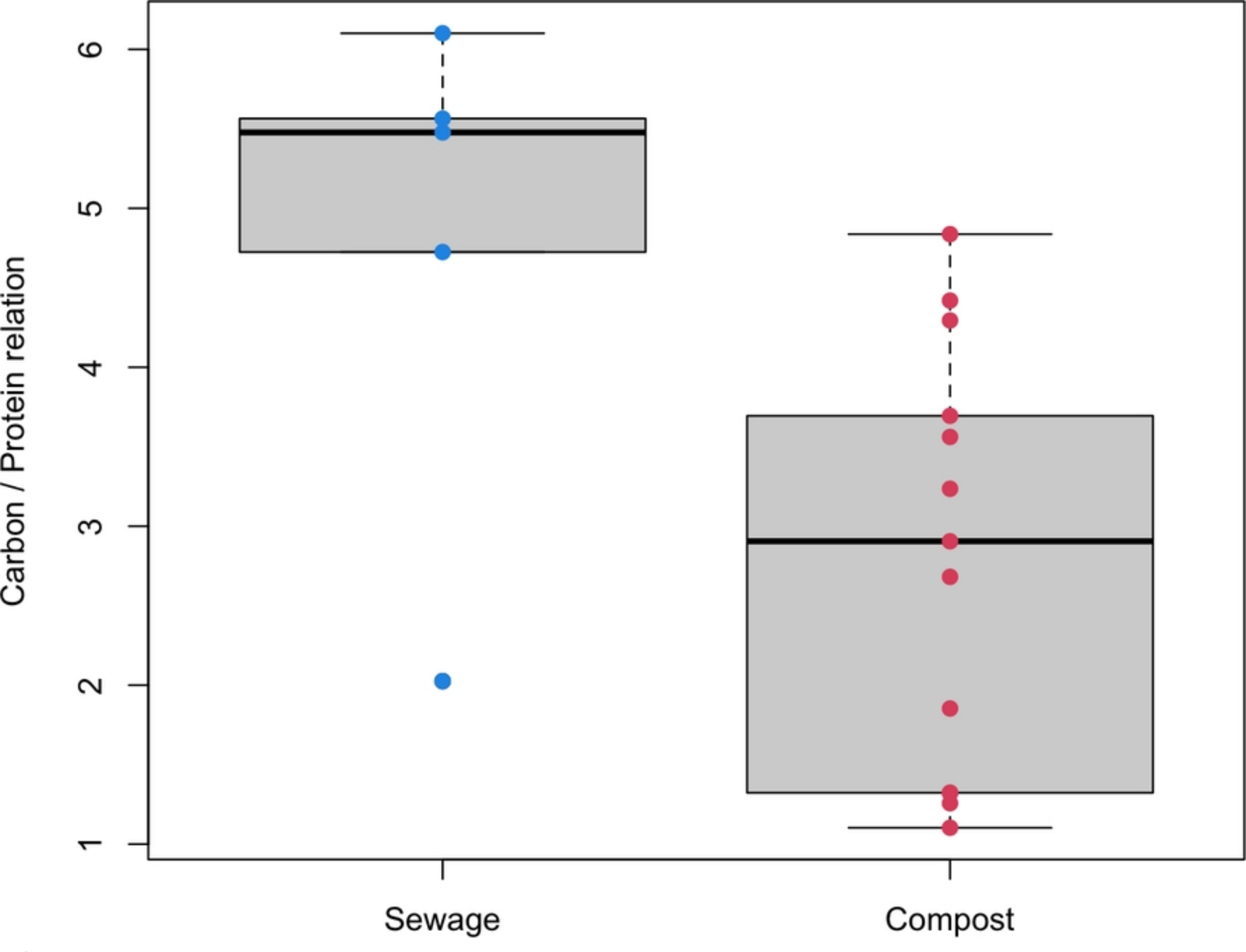

Figure 5

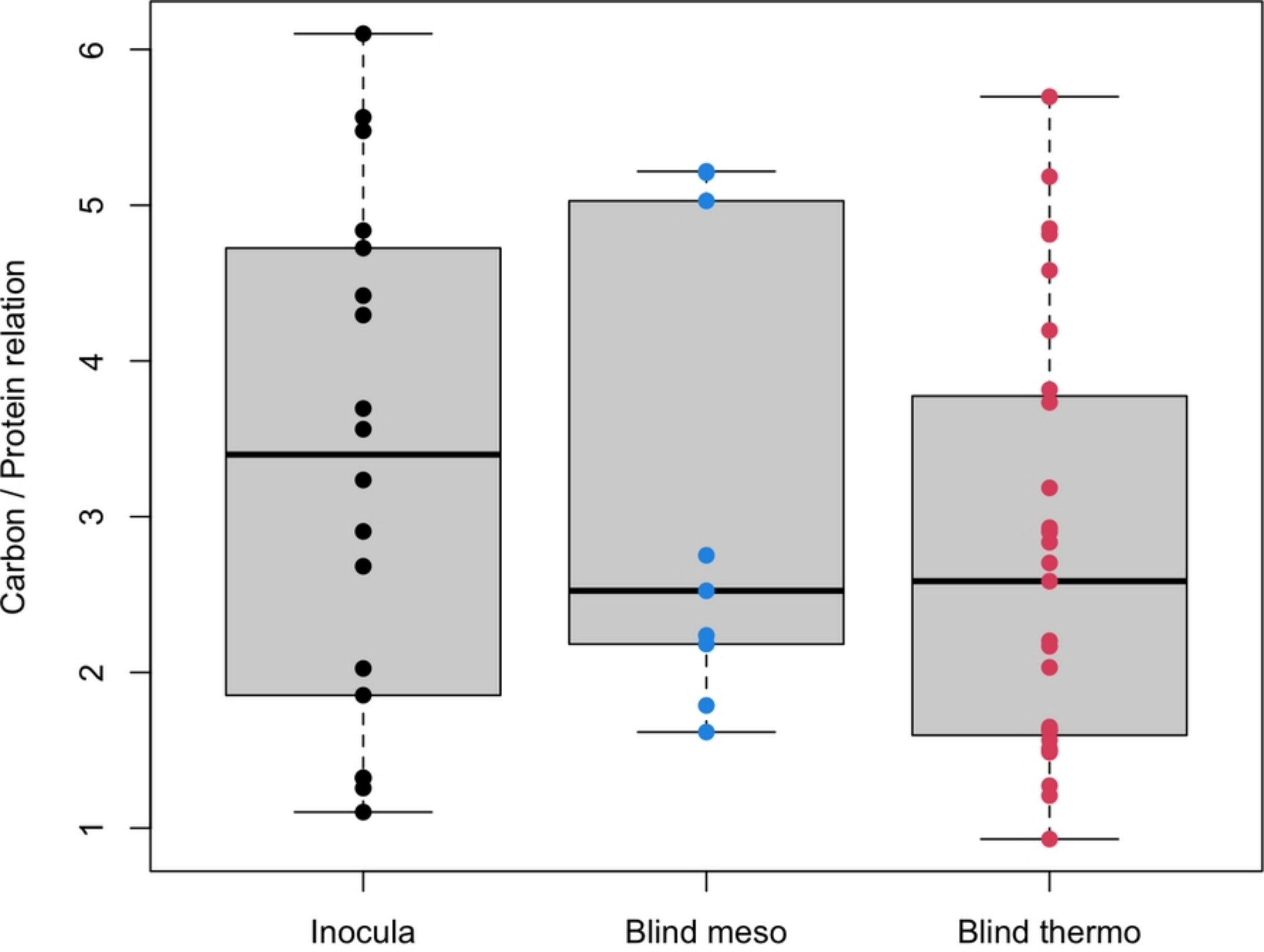
Carbon / Protein relation
1
2
3
4
5
6
Inocula
Blind meso
Blind thermo

Figure 6

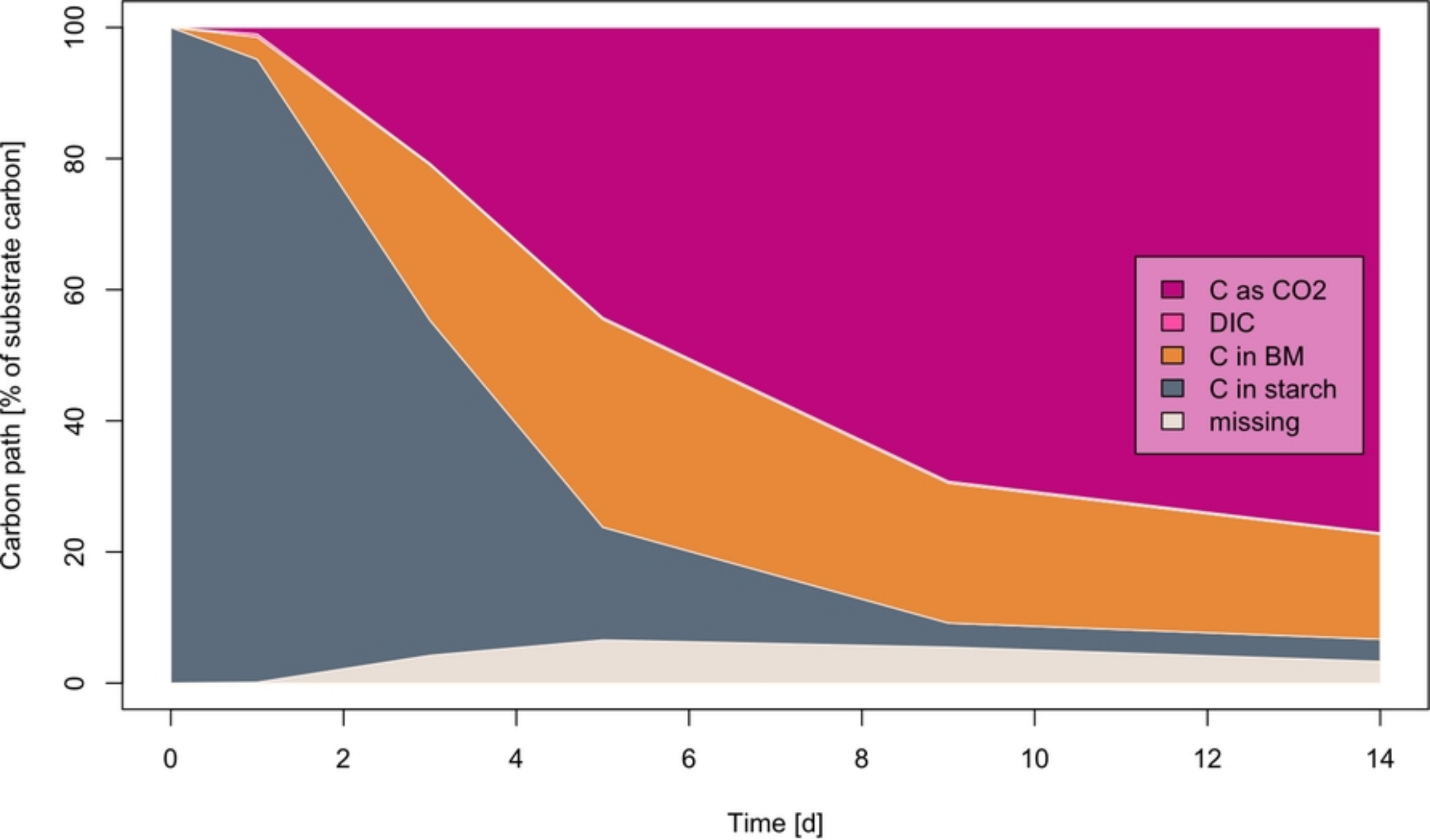
Carbon path [% of substrate carbon]
0
20
40
60
80
100
0
2
4
6
8
10
12
14
Time [d]
C as CO2
DIC
C in BM
C in starch
missing

Figure 7

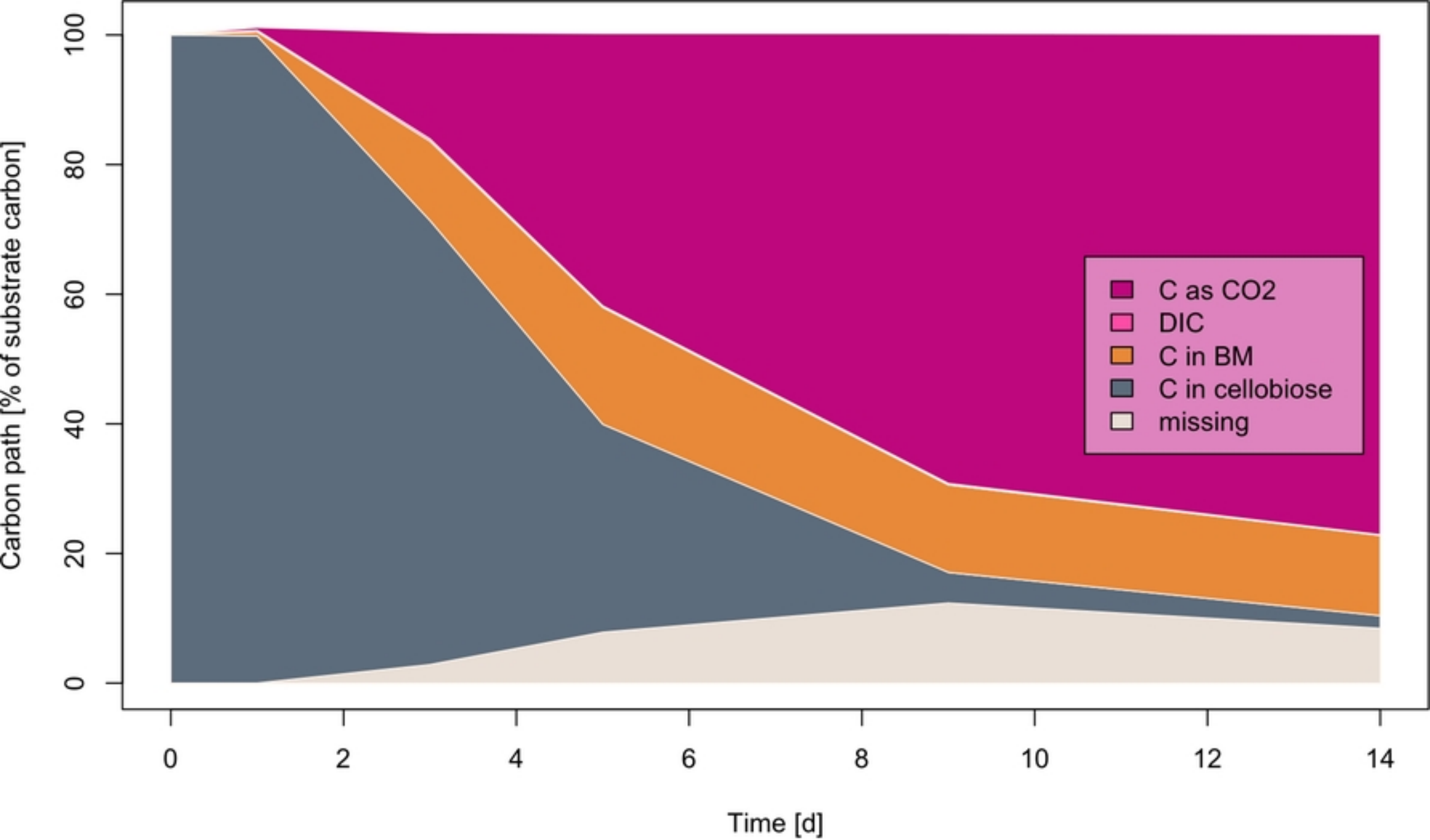
Carbon path [% of substrate carbon]
0
20
40
60
80
100
0
2
4
6
8
10
12
14
Time [d]
C as CO2
DIC
C in BM
C in cellobiose
missing

Figure 8

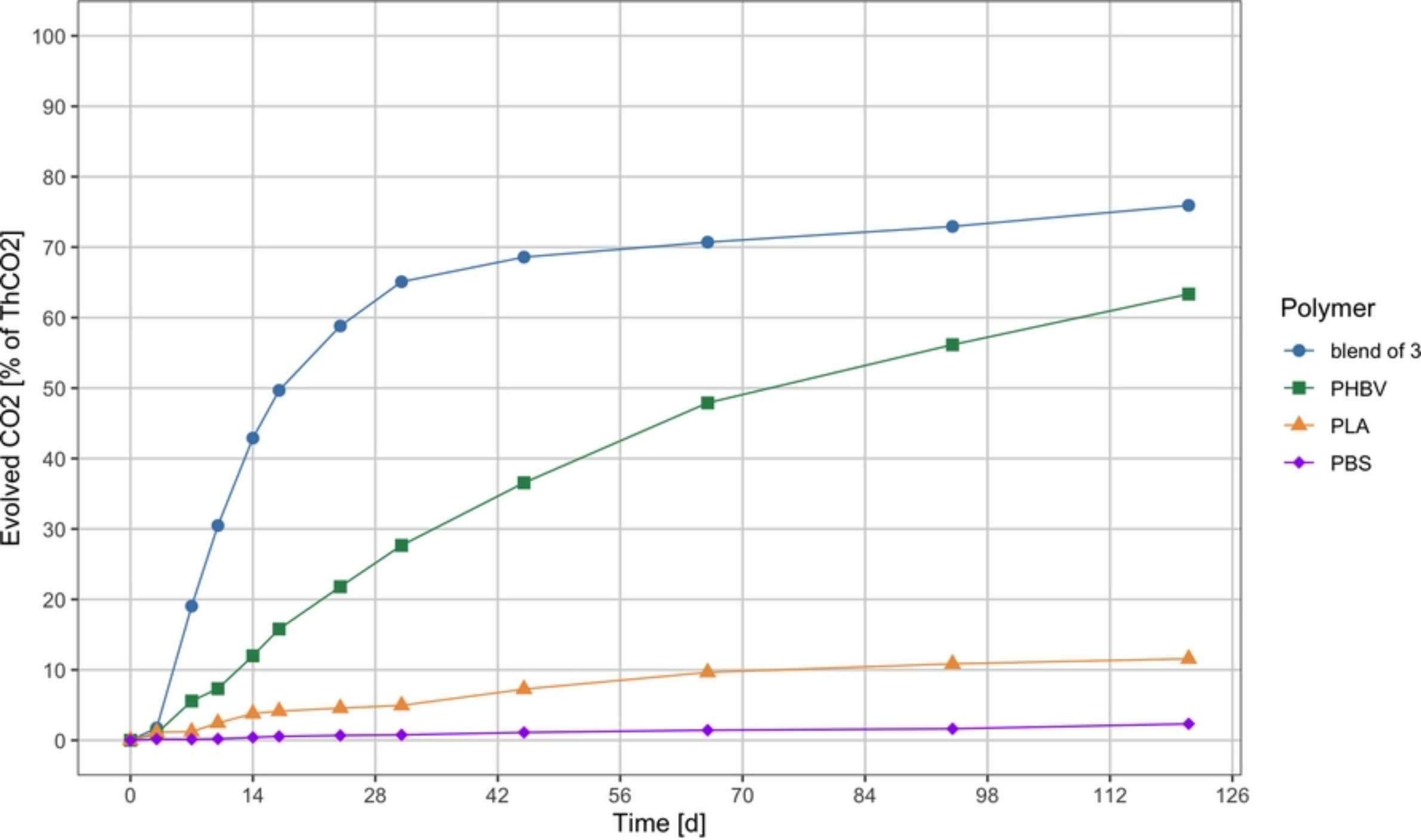
Evolved CO2 [% of ThCO2]
100
90
80
70
60
50
40
30
20
10
0
0
14
28
42
56
70
84
98
112
126
Time [d]
Polymer
blend of 3
PHBV
PLA
PBS

Figure 9

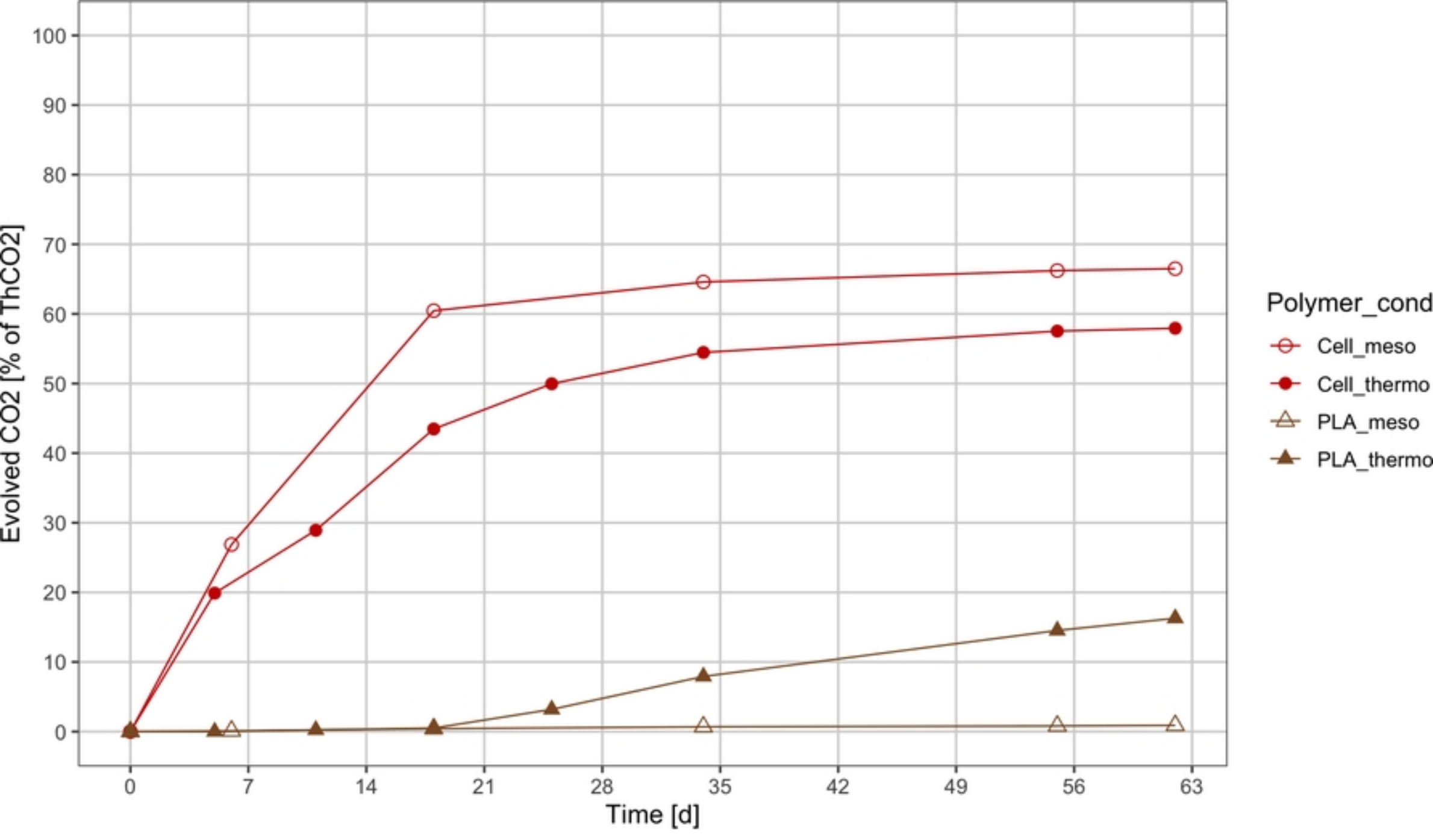
Evolved CO2 [% of ThCO2]
100
90
80
70
60
50
40
30
20
10
0
0
7
14
21
28
35
42
49
56
63
Time [d]
Polymer_cond
Cell_meso
Cell_thermo
PLA_meso
PLA_thermo

Figure 10