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Biogeosciences Discussions

Interactive comment on "Increased bacterial growth efficiency with environmental variability: results from DOC degradation by bacteria in pure culture experiments" *by* M. Eichinger et al.

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Reply to general comments :

We would like to thank reviewer 1 for his highly relevant questions regarding O2 consumption and potential limitation. This allowed us investigating an area that we have overlooked and therefore we have improved the manuscript. However, as we explain below, it will not change general conclusions of our manuscript. As suggested by reviewer 1, oxygen limitation is an issue that needs to be taken into consideration. We monitored oxygen concentration during several minutes at each sampling time (incubation time of approximately 15 minutes). However, as each sample was in contact with

atmospheric O2 before measurement, O2 depletion could not be detected from these measurements. Instead, we used the decreasing O2 concentration during the incubation period in the oxygraph chambers to estimate O2 consumption at each sampling time.

Magnetic stirrer was used to allow oxygen penetration in the culture flask, but no aeration. Bottles were opened and shaken to increase oxygen diffusion into the cultures at each sampling time and each pulse addition. Consequently, we could assume that, after each substrate pulse, biomass (POC) increase and substrate (L-DOC) consumption could be due to either substrate addition and/or oxygen input due to bottle opening. However, if the biomass increase was due to the input of oxygen into the culture, this phenomenon should occur after each sampling, which we did not observe in our results. Indeed, for the experiment P, biomass increased only after substrate addition. The outcome is the same for the experiment B where DOC accumulation started from approximately 45 hours. However, this culture was sampled/opened 6 times more after the start of DOC accumulation, but biomass did never increase from this time. If oxygen limitation occurred in this experiment, and because DOC concentration was still significant, we should observe a biomass increase after each sampling, which is not the case. The same conclusion can be formulated by the observation of O2 consumption rates time evolution: O2 consumption increased only after substrate addition, but not during other sampling times. Consequently, the possibility of oxygen limitation in these experiments is very unlikely. Another consequence is that DOC accumulating could not consist in L-DOC.

To support this theory, we compared values of bacterial respiration obtained from the oxygraph to those obtained with a calculation of mass balance. For the experiment P, oxygraph measurements gave respiration rates comprised between 100 and 350 μMO2.h-1 (Figure 2 in the original manuscript). We considered an average value of 250 μMO2.h-1 for the following calculations. Using a simple model we simulated the oxygen dynamics within the culture where only diffusion and respiration rates are taken

into account. The diffusion model is formulated as an equation of gas transfer (Maier and Büchs, 2001) from headspace to medium culture (where oxygen flux diffusion is proportional to oxygen saturation and measured oxygen concentration and where we experimentally estimated the diffusion rate as being 0.18 h-1). Using the oxygraph based respiration rates (250μMO2 h-1), we estimated that cultures should be oxygen depleted after 1 hour (see attached Figure 1). However, the cultured bacteria (Alteromonas infernus) are strictly aerobic and does not have fermentative metabolism (Raguénès et al., 1997). This means that, if cultures were effectively limited by O2 after 1 hour, growth (POC increase) would not be possible under oxygen limitation. Because of this property of strict aerobic metabolism, growth in our culture can only be due to substrate consumption and co-limitation between organic carbon and O2 is not conceivable.

If now we consider a broad mass balance on the whole culture experiment, where the apparent net DOC consumption (ΔDOC, e.g. carbon demand) resulted from the biomass increase (ΔPOC) and bacterial respiration (BR), we obtain the following equation: BR = (ΔDOC- ΔPOC)/ Δt (where t is time). For the whole experiment P, ΔDOC = 6.1 mMC and ΔPOC = 1.8 mMC over 240 hours, leading to BR=18 μMC.h-1. By considering a respiratory quotient (RQ) equals 1, we obtain an apparent O2 consumption rate of 18 μM O2.h-1. For the experiment B, ΔDOC=7 μMC and ΔPOC = 1.1 mMC over 87 hours, leading to an apparent O2 consumption rate of 65 μM O2.h-1. With the same equation of gas transfer than previously and by using these rates of apparent O2 consumption, cultures would be at equilibrium (in term of O2 flux between headspace and medium) after 30 hours for the experiment P (see attached Figure 2) and would be O2 depleted after 5 hours for the experiment B (see attached Figure 3). However, as cultures were frequently aerated and shaken, O2 depletion certainly occurs later, if it occurs. Moreover, as we demonstrated above, even if cultures were anoxic, growth can only be due to substrate consumption and co-limitation does not seem to be a relevant hypothesis.

By comparing both estimations of bacterial respiration (from oxygraph and from the mass balance equation) to our experimental results, we may assume that O2 consumption rates estimated with the oxygraph constitute a measurement of potential respiration or maximal respiration (Vmax). Indeed, oxygraph measurements showed that cultures were anoxic after 1 hour, which is not compatible with our experimental results (DOC consumption and POC increase) and A. infernus metabolism. Consequently, oxygraph outputs can not be used to perform a balance of batch functioning but can be used to estimate Vmax and compare their dynamics over an experiment. We may assume that co-limitation between oxygen and organic carbon does not occurred in our experiments and that observed growth and DOC consumption were only due to substrate limitation. However, it might be possible that at some point in time (for example few hours after substrate addition) O2 concentration dropped down to zero, and thus that growth stopped. But we do not have the accurate observations to demonstrate or refute this assumption. Another conclusion from this demonstration is that accumulating DOC could not consist in L-DOC but is refractory DOC (R-DOC) produced by cultured bacteria. Our estimated BGE thus really reflected efficiencies of growth on the considered substrate. As at some points in time cultures may be anoxic, POC production and DOC consumption might be underestimated, thus affecting BGE estimation the same way. Our global conclusions on BGE are thus still valid.

A shortened demonstration will be added in the revised version of the manuscript, to show why oxygen limitation is very unlikely to occur, why substrate is assumed to be the sole limiting factor and why accumulating DOC is R-DOC.

Cited references:

Maier, U. and Büchs, J. Characterisation of the gas–liquid mass transfer in shaking bioreactors, Biochemical Engineering Journal, 7, 99-106, 2001 Raguénès, G. H. C., Peres, A., Ruimy, R., Pignet, P., Christen, R., Loaec, M., Rougeaux, H., Barbier, G., and Guezennec, J. G.: Alteromonas infernus sp. nov., a new polysaccharide-producing bacterium isolated from a deep-sea hydrothermal vent, Journal of Applied Microbiol-

ogy, 82, 422-430, 1997.

Specific comments:

Introduction

Page 791 L.10: please indicate here the three models used.

Reply: We will add the names of the three models used.

Material and Methods

Page 792 L. 16: please indicate the vitamins used and concentrations.

Reply: We will add the list and concentrations of vitamins used for the culture in the manuscript: Cobalamin: 0.5 μg.L-1

Biotin: 5 μg.L-1

Riboflavin, Pyridoxine, Folic acid, Nicotinic acid, Para-aminobenzoic acid: 50 μg.L-1

Panthotenic acid, Meso-inositol: 500 μg.L-1

Page 792 L. 23: Most likely the oxygen was totally exhausted after L-DOC consumption. Was the time gap between pulsed substrate additions enough to replenish oxygen concentrations in the cultures? Please clarify.

Reply: As explained in the general comments, cultures were frequently opened (4 times a day when pulse was added) and thus cultures were aerated. To improve oxygen diffusion into the cultures, we also shaken incubation bottles before and after sampling. We explained previously that estimated O2 consumption rates with the oxygraph represent potential respiration and do not allow estimating when oxygen will be depleted in the cultures. We have no observation enabling to state that O2 concentration never reached zero, but we may assume that observed growth and DOC consumption are related to organic carbon limitation and not to oxygen limitation. This will be explained in the revised version of the manuscript.

Page 793 L. 3 : Bacterial cell density is written 6.106 cells cm-3 I am not familiar with this notation I am rather used to 6 x 106 cells cm-3.

Reply: According to reviewer comment, we will replace bacterial density notation 6.106 cells.cm-3 by 6 x 106 cells.cm-3

Page 793 L. 10 : Are you sure that bacteria were under starving conditions? Remaining organic substrate is substantial. Was there any oxygen limitation?

Reply: As shown previously (see general comments), due to the property of the studied bacterial strain, growth was only related to organic carbon limitation and oxygen limitation is not conceivable. Remaining DOC can not consist in substrate but consists in R-DOC. Consequently, during non-growth periods, bacteria were in starving conditions.

Page 794 L. 9: should read analyzer instead of analyser?

Reply: We will replace analyser by analyzer.

Page 794 L. 10 : Please provide information regarding the accuracy and precision of the oxygen determinations. It is not clear if a very high cell concentration is needed to get enough sensitivity.

Reply: According to the manufacturer, the sensitivity of the OROBOROS Oxygraph-2k is <2 pmol.s-1.cm-3, which is equivalent to 7.2 nmol.h-1.cm-3, at steady-state over 5 min at 20 - 40°C, including instrumental background correction (for more details please refer to http://www.oroboros.at). The minimal cell density in our cultures was 5.7 x 106 cells.cm-3 (at t0) and the minimal specific respiration rate was 0.2 fmol O2 .h-1.cell-1. Thus, the minimal signal was about 1.1nmol.h-1.cm-3, value slightly lower that the sensitivity of the device. However, cell densities and specific activities were generally well higher than these minimum values, which means that the estimated O2 consumption rates were just on the edge of accurate determination for the lowest bacterial densities, but highly significant for higher concentrations. This provides objective arguments that

high cell concentration is needed to get enough sensitivity. The concentration of cells (bacteria) used with the OROBOROS 2K is usually in the order of 1 million cells per mL. Several publications related to Microbiology, Biotechnology or Plant Sciences have used the OROBOROS Oxygraph-2k (see below).

This information will be added in the revised version of the manuscript.

- Jarolim S, Millen J, Heeren G, Laun P, Goldfarb DS, Breitenbach M (2004) A novel assay for replicative lifespan in Saccharomyces cerevisiae. FEMS Yeast Research 5(2): 169-177

- Grégori G, Denis M, Lefèvre D, Beker B (2002) A flow cytometric approach to assess phytoplank-ton respiration. Methods Cell Sci. 24: 99-106

- Wippich N, Peschke D, Peschke E, Holtz J, Brömme HJ (2001) Comparison between xanthine oxidase from buttermilk and microorganisms regarding their ability to generate reactive oxygen species. Int. J. Molec. Med. 7: 211-216.

- Guan YH, Kemp RB (1999) Detection of the changing substrate requirements of cultured animal cells by stoichiometric growth equations validated by enthalpy balances. J. Biotechnol. 69: 95-114.

- Nyström T, Larsson C, Gustafsson L (1996) Bacterial defence against ageing: Role of the Escherichia coli ArcA regulator in gene expression, readjusted energy flux and survival during stress. EMBO J. 15: 3219-3228.

- Guan Y, Kemp RB (1996) Medium design with the aid of heat flux measurement in mammalian cell culture. In: BioThermoKinetics of the Living Cell (Westerhoff HV, Snoep JL, Wijker JE, Sluse FE, Kholodenko BN, eds), BioThermoKinetics, Amsterdam: 387-395.

Page 794 L. 20 : Zero percent oxygen saturation: : instead to 0% oxygen saturation?

Reply: We will replace 0% oxygen saturation by zero percent oxygen saturation.

Page 794 L. 26 : What was the cell density in the oxygen measurements? It is not clear what is the sensitivity of the oxygen technique used.

Reply: The cell density in the oxygen measurements was exactly the same as the cell density in the culture. So, it ranged from 5.7 x 106 cells.cm-3 to 3.4 x 108 cells.cm-3. Information regarding the sensitivity of the oxygen technique used has been provided previously.

Page 795 L. 10: Epifluorescent microscope? Or Epifluorescence microscope?

Reply: We will replace epifluorescent microscope by epifluorescence microscope.

Results:

Page 800 L. 2 and 8: It is difficult to follow the respiration dynamics from the description given in the text. The description would be improved if the authors also refer to the incubation time.

Reply: According to reviewer comment, we will improve the description of O2 consumption measurements, especially in the Material and Methods part: the same protocol was followed for each sampling. It has to be noted that the oxygraph measured O2 concentrations, but as samples were in contact with atmospheric O2 before their introduction into the measurement chambers, we only used these data sets to extract O2 consumption rates at each sampling time. Incubations were realised at the same sampling times as for the other measurements and lasted approximately 15 minutes. For each incubation, the first 10 minutes of O2 concentrations recording were not utilised because we expected that the thermodynamic effects induced by the movement of the stopper when opening and closing the chambers were higher than bacterial respiration. These 10 minutes were chosen so that the estimated O2 consumption rate, by a linear regression on data points, was stable after this interval. After these 10 minutes, the incubation still lasted less than 5 minutes, where only the 200 first seconds (3.3 minutes) were used to calculate the O2 consumption rate. This information will be

added in the new version of the manuscript.

Discussion:

Page 804 L. 26 and 29 : the term jEM is not defined in text or in tables and the term jVM is not defined in text or in tables.

Reply: jEM and jVM are typos. In fact, jEM corresponds to jMEM and jVM to jMVM, which both were described in tables. However, according to reviewer 2 comments, we will change model abbreviations in the revised version of the manuscript: jMEM will be maint E and jMVM will be maintV.

Page 805 L. 10 : Please discuss in this section the possibility that substrate consumption might be limited by oxygen concentration.

Reply: In the revised version of the manuscript, we will add a paragraph dealing specifically with the problem surrounding oxygen concentration. This paragraph will mostly be based on the information and calculations provided in the general comments.

Page 806 L. 26 : Please explain what do you mean with "widely used methods underestimate BGE values" there are a number of methods that different authors use to calculate BGE.

Reply: By the term "widely used methods underestimate BGE values", we mean that the methods estimating BGE in typical batch systems or short incubation experiments, i.e. without considering the potential temporal variations of DOC/substrate, certainly underestimate BGE. Indeed, we demonstrated here that BGE was twice as high in a pulsed compared to a one dose substrate system. We also demonstrated that the consideration of the maintenance process in BGE calculation increased BGE values. However, in these "typical batch experiments", BGE is generally determined from bacterial production (BP) and respiration (BR) (from oxygen measurements, and converted into CO2 with an assumed respiratory quotient) measurements. By definition, BP only takes production into account and thus no process that would lead to biomass decrease

as maintenance. BR measurements are commonly made with incubations lasting few hours, thus before bacteria are starving and before that maintenance is the only remaining process. This will be better explained in the revised version of the manuscript.

Page 807 L.1 : Please explain what do you mean with the "overestimation of the role of bacteria as CO2 producers". If for instance ΔPOC and ΔCO2 production is measured how one can possibly underestimate the BGE. Please explain.

Reply: As explained above, this manuscript highlighted that BGE values might be underestimated with typical methods (systems with one dose of substrate) because of 2 results: BGE is higher in the pulsed system and BGE is higher when we considered maintenance in the calculation. So, even by using more appropriate methodology as by directly measuring ΔPOC and ΔCO2, instead of measuring BP and BR, BGE might be underestimated because temporal variation of the substrate was not considered and because experiments were too short to observe maintenance.

Page 808 line 6: I am not sure if a "threshold value" during starvation existed in the experiments because again, the oxygen concentration in the cultures is not reported.

Reply: Unfortunately, we do not have access to this information. Due to the constancy of the specific respiration between two pulses and to the fact that bacterial biomass increased after each substrate addition, we highly believe in the existence of a "threshold value" for respiration, which is related to maintenance. As stated previously, oxygraph measurements provide maximal respiration rates (Vmax) at the different sampling times. Consequently, the threshold value obtained in our experiments characterised the maximal maintenance rate (in term of O2 consumption).

Figures: In general I had a problem to distinguish the dotted lines from the continuous lines particularly in figures 1 and 2 but all the figures in general would be better if the legends and numbers are in a larger size.

Reply: Figure quality will be improved in the revised manuscript.

Please also note the supplement to this comment:
http://www.biogeosciences-discuss.net/7/C673/2010/bgd-7-C673-2010-supplement.pdf

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