Water, Air, & Soil Pollution

Putative role of Flavobacterium, Dokdonella and Methylophilus strains in paracetamol biodegradation

--Manuscript Draft--

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Abstract:	Paracetamol, the most widely and globally used analgesic and antipyretic, is easily accumulated in aquatic environments. In the present study, the biodegradation of paracetamol in different media (one for general growth, one specific for sulphate reducing bacteria, a mineral salts medium and municipal wastewater) inoculated with two types of sludge (from anaerobic lagoon and from oxidation ditch) under different oxygenic conditions (anoxic; moderate oxygenation in open flasks and high oxygenation by aeration) was investigated. In addition, bacteria with relative abundances increasing simultaneously with paracetamol degradation, when this drug was the only carbon source, thus with a putative role in its degradation, were identified using 16S rRNA gene sequences. The results show that aerobic microorganisms had a major role in the degradation of paracetamol, with 50 mg/L totally removed from municipal wastewater after two days incubation with aeration, and that the metabolites 4-aminophenol and hydroquinone plus one compound not identified in this work were produced in the process. The identification of bacteria with a role in the degradation of paracetamol revealed a strain from genus Pseudomonas with the highest final relative abundance of 21.2%, confirming previous works reporting strains of this genus as paracetamol decomposers. Besides, genera Flavobacterium, Dokdonella and Methylophilus were also in evidence, with initial relative abundances of 1.66%, 1.48% and 0.00% (not detected) in the inoculum and 6.91%, 3.80% and 3.83% after incubation, respectively. Therefore, a putative role of these genera in paracetamol biodegradation is suggested for the first time.
Response to Reviewers:	Dear reviewer First of all we are very thankful for the time you have spent in revising this manuscript. We consider that all the comments and questions raised are extremely helpful in order to improve the manuscript. Thus, aiming to clarify all doubts and to meet the quality demanded of this scientific journal, we have made the suggested corrections and answered all the questions posed.

Below are listed, one by one, the corrections made in the manuscript and the answers to the comments and questions posed.
In the manuscript, new sentences and corrected sentences are written in green color.

Cordial regards
Jorge Dias Carlier

Reviewer #1:

General Comments:

This work focused on biodegradation of paracetamol in wastewater and identifies three new genera with a putative role in paracetamol degradation based on their enrichment in cultures when that pharmaceutical was provided as the sole carbon source. The dominant phylotype identified in the cultures investigated was Pseudomonas, which had previously been shown to degrade paracetamol; however, the other phylotypes identified had not previously been linked with degradation and were shown here to likely play a role in biodegradation of paracetamol metabolites. The manuscript offers some novel findings on a relevant topic; however, I have some concerns.

1) A general concern relates to the lack of biomass measurement and biomass normalization between conditions. Before the authors conclude that biodegradation does not occur under certain conditions, they should demonstrate that biomass was not limiting, at least to the extent possible. For example, it would have been appropriate to at least document that initial biomass levels were the same across conditions. How biomass was normalized is not clear.
Authors answer:
It can be considered that a rough normalization was guaranteed in the beginning of all experiments because: (1) similar bacterial growth rates were observed in all inocula enrichments, (2) at the end of all enrichments the bacterial growth was at a roughly equivalent stage and (3) the inocula proportions were in all cases the same: 10% (v/v). The bacterial growth was evaluated by optical densities measurements in the beginning and at the end of the enrichment cultures (see also point 9 of specific comments below).

2) Second, it is not clear how replication was handled for DNA sequencing; thus, the reproducibility of key findings is not clear. Were the three phylotypes listed in the title enriched to the same degree over time in all biological replicates?
Authors answer: This issue has been clarified.
Corrections were made in the first sentence of section 2.7 to clarify that large-scale biodiversity analysis by DNA sequencing was performed along the experiment on samples from just one of the triplicated cultures.
Moreover, the following sentences were added to section 2.7:
- “As the three replicate cultures were inoculated exactly with the same microbial community (source: enriched culture from WWTP’s oxidation ditch sludge; quantity: 10% (v/v)) and were maintained under exactly the same conditions (aeration: a shared air pump; temperature and light: flasks maintained side by side), it was assumed that studying the microbial dynamics through massive sequencing of 16S rRNA genes on just one of the cultures would provide enough robust evidences to identify taxa putatively involved in the degradation of paracetamol and/or its metabolites.”

The manuscript did not have line numbers making it difficult to provide detailed comments on the sentence level, although I did note some issues with grammar in several places. The manuscript is generally thorough in approach and details and is generally well written.
Authors answer: Line numbers were added and the text revised.

Specific comments:

1. The highlights should be reviewed for proper grammar.
Authors answer: done.

2. The graphical abstract is illegible and includes too many minutia related to

experimental design. I suggest reworking the graphical abstract to display overarching approaches and findings.
Authors answer: done.

Introduction

3. The Introduction should more clearly discuss the role of redox in paracetamol degradation. Are all the known pathways aerobic? What was the specific knowledge gap being addressed?
Authors answer: done
The following sentences were added to Introduction:
- “Although paracetamol stability decreases in acidic or alkaline conditions due to be slowly degraded via a base- or acid-catalyzed hydrolysis of the amide bond into acetic acid and 4-aminophenol, stability studies in purified water at room temperature demonstrated that after incubation for more than one month this drug was completely stable due to the high energy needed to overcome the barrier for the cleavage of its amide bond (Karaman et al. 2016). In addition, being 4-aminophenol a photo and thermal sensitive compound, like other aromatic amines, the results obtained by Khan et al (2006) showed the occurrence of its autoxidation after 7 days but at low rates: 0.8%, 26.6%, 20% and 13% at 50, 100, 150 and 200 mg/L, respectively. In any case, the authors of both works concluded that biodegradation was more effective. Indeed, several studies have demonstrated that microorganisms play a major role in the environmental degradation of paracetamol and during the past decades research related to the biodegradation of this drug has allowed the identification and characterization of metabolic intermediates involved in the catabolic pathways of aerobic bacteria (Wu et al. 2012).”
...
Minor corrections were made in the sentence of the Introduction starting by:
- “In this regard, biodegradation may represent a low-cost effective solution (Wu et al. 2012), though...”
...
The following sentence was added to the Introduction:
- “Thus, to our knowledge all the microbial catabolic pathways known to be involved in paracetamol biodegradation were studied in a small number of aerobic organisms; therefore, it is possible that unidentified mechanisms with a role in paracetamol biodegradation exist in other organisms.”
...

4. In the introduction, page 2, the statement the microorganisms degrade organic compounds is overly general and vague. Further, please also mention that some metabolites do not represent molecules that are particularly "simple". The issue of toxic metabolites should be addressed in the Introduction.
Authors answer: done
The following sentences were added to Introduction:
- “When paracetamol is used in excess, it can cause liver failure and necrosis due to N-Acetyl-p-benzoquinone imine (NAPQI), a highly toxic paracetamol´s metabolite formed by enzymatic oxidation in the liver (Bessems and Vermeulen 2001). About 30% and 55% of administered paracetamol is excreted in urine as conjugates paracetamol sulfate and paracetamol glucuronide, respectively (Thomas 1993). Moreover, a glutathione conjugate (1,4-Michael adduct) of NAPQI, the corresponding cysteine conjugate and mercapturic acid breakdown products have also been found in urine after ingestion of paracetamol (Prescott 1980). NAPQI is known to be fairly unstable, however, in aqueous solution it readily hydrolyzes into 1,4-benzoquinone, which is another toxic metabolite (Dahlin and Nelson 1982; Snyder 2000; Bedner and Maccrehan 2006). On the other hand, 4-aminophenol (the hydrolytic product of paracetamol) has highly genotoxic and mutagenic effects (Majeska and Holden 1995; Yoshida et al. 1998). The potential toxic effects of paracetamol in aquatic systems, mediated by its reactive oxygen species, have been reported. For example, Antunes et al. (2013) studied the effects of paracetamol exposure on physiological traits of bivalves and the results showed a significant increase in all oxidative stress biomarkers, evidencing the onset of deleterious effects.”
...
The following sentences were removed from the Introduction:
- “Microorganisms, particularly bacteria, have been demonstrated to play an important

role in the biodegradation of organic compounds in WWTPs. Biodegradation can be partial or complete."
...
Minor corrections were made in the sentence on the Introduction starting by:
- "When biodegradation is complete (mineralization), the substances are converted to inorganic..."

5. How long were cultures enriched?
The following sentences were added to section 2.2:
- "To enrich the inocula from the lagoon system, 2 g of sludge sediment were inoculated on each of two separate 250ml culture flasks containing 200 mL of TGM medium. In one flask 10 mL of paraffin-oil was added to prevent gas diffusion and the flask was sealed with a butyl rubber stopper and an aluminum crimp seal to avoid air intake in order to create anaerobic condition. In the other flask, cotton was used to serve as lid, thus allowing the maintenance of aerobic conditions. To enrich the inoculum from the oxidation ditch, 2 mL of water with sludge was inoculated in just one flask with 200 mL TGM medium with cotton as lid. The anaerobic flask was kept without shaking and the two aerobic flasks were placed in an orbital shaker at a speed of 150 rpm; all were maintained at room temperature and grown for 24 hours. The anaerobic enriched culture was used to inoculate all tests under anaerobic conditions. The aerobic enriched cultures from each type of sludge were used to inoculate the respective tests under moderate and highly aerobic conditions."
...
Minor corrections were made in the remaining sentences of section 2.2.

Materials and Methods

6. Please provide more details and references on the liquid paraffin method used for promoting aerobic conditions.
Authors answer: The use of liquid paraffin is to promote anaerobic conditions, not aerobic.
The following sentence was added to section 2.4:
"The liquid paraffin prevents gas diffusion through the medium surface and the flask sealed with a butyl rubber stopper and an aluminum crimp seal avoids air intake into the flask; thus the oxygen initially present is rapidly consumed and anaerobic conditions are created."

7. How did oxygen concentration vary between the "moderate" and "full" oxygenation conditions? What was the redox measurement? For moderate oxygenation cultures, were they stagnant? Or mixed? If mixed, how so?
Authors answer: additional corrections were made in section 2.4 to address these issues.
Minor corrections were made in the sentence of section 2.4 starting by:
- "To maintain aerobic conditions with moderate oxygenation, the cultures were incubated in open batch flasks..."
...
The following sentences were added to section 2.4:
- "The averages and standard deviations of dissolved oxygen (DO) percent saturation in the various tests under aerobic conditions were calculated using measurements made in the respective replicate cultures with a portable CD650 meter (Eutech Instruments) after 24 hours of incubation since inoculation. For moderate oxygenation conditions in open flasks without aeration, the DO percent saturation was: 9.3±1.8% in the tests with TGM, 31.1±2.3% in the tests with MSM and 27.5±2.2% in the tests with MWW (24 hours after both first media were inoculated with the anaerobic lagoon sludge and the third medium inoculated with the oxidation ditch sludge). For high oxygenation conditions in aerated flaks, the DO percent saturation was: 94.8±1.6% in the tests with MSM and 95.5±1.3% in the tests with MWW (24 hours after both these media were inoculated with the oxidation ditch sludge)."

8. Please describe any pre-acclimation. Biodegradation rates are typically improved by pre-acclimating cultures.
Authors answer: It was not performed any pre-acclimation with paracetamol.

9. How were biomass levels normalized between conditions? How was biomass

measured?
Authors answer: It can be considered that a rough normalization was guaranteed.
The following sentences were added to section 2.2:
- “In the inocula enrichments, initial and final optical densities were measured at 600 nm (OD600) as absorbance using a Hach-Lange spectrophotometer DR-2800 (Sköndal, Sweden). The initial OD600 values of enrichment media immediately after inoculation with the anaerobic lagoon and the oxidation ditch sludges were similar: 0.286 and 0.264, respectively. Thus, the also similar OD600 values measured after the 24 hours of incubation at room temperature in all enrichment conditions (anaerobic lagoon sludge/anaerobic growth = 0.838; anaerobic lagoon sludge/aerobic growth = 0.831; oxidation ditch sludge/aerobic growth = 0.833) roughly indicates similar bacterial growths.”
...
- “The initial and the final OD600 values in the SRB inoculum enrichment were 0.165 and 0.657, respectively.”

10. I'm unconvinced that the italicized subtitles are necessary. The first sentence of the DNA sequencing methods section is redundant with earlier text.
Authors answer:
We consider that the italicized subtitles are helpful for a more clear separation of all experiments and of all the several steps along the methodology; therefore we would like to maintain them.
The first sentence of the DNA sequencing methods (section 2.7.2) was removed.

11. In 2.6.1, the medium list does not have to put each medium on a separate line.
Authors answer: Correction done as suggested.

12. Why were the tests only run for ~72 hours? This time might have been too short.
Authors answer:
We agree that it would have been better to run the tests for longer periods. The first set of tests, with the anaerobic lagoon sludge, was run just for 72 hours because it was thought that comparing paracetamol removals in the inoculated and the non inoculated cultures under the two conditions (anoxic vs low oxygenic) during that time could be enough to evaluate which major metabolic pathways contribute for the biodegradation of this drug in WWTPs: anaerobic, aerobic or both. The second set of tests, with the oxidation ditch sludge, was in fact maintained for more 3 than days (that is why in the bacterial dynamics study there is a sample in day 6). However, the samples collected on that day were not analyzed for the presence of paracetamol and respective degrading metabolites due to a prolonged failure in the HPLC equipment.
The following sentences were added to the beginning of section 3.2:
- “The results obtained clearly indicate that the major routes for the biodegradation of this drug in WWTPs involve aerobic organisms; some already reported previously and others identified in this work for the first time, as discussed further below. However, some slow biodegradation seems to occur under anaerobic conditions, which was not more evident due to the short time of the assays.”

Results

13. OD600 is not a measure of bacterial "viability". You can have a high OD600 in killed cultures. I suggest changing the axis label for Figure. 2.
Authors answer: The vertical axis label of Figure 2 is not OD600. In fact it is “Bacterial viability %”, defined as the percentage of the optical density measured in a culture with a certain paracetamol concentration in relation to the optical density in the control culture without the drug.
Corrections were made in section 2.3, in the sentence starting by:
- “Based on the OD600 values, the percentage of bacterial viability...”
...
Corrections were made in section 3.1, in the sentences starting by:
- “The studies started by the determination of paracetamol IC50...”
...
- “Estimates were based on...”
...
The following sentence was added to caption of Figure 2:
- “Bacterial viability % is the optical density at 600 nm (OD600) measured in a culture

with a certain drug concentration divided by the OD600 in the control culture without drug, multiplied by 100."

14. Do you have biomass data for the differing redox conditions? How is it known that biomass was not simply limiting at lower redox? Could low biomass have limited rates such that at anaerobic redox more time would have been required to observe statistically significant degradation?
Authors answer: We agree that this issue is important.
The following sentences were added at the end of section 3.2.5:
- "Though the biomass growth was not analyzed during the biodegradation tests, it was evaluated by optical density measurements at the beginning and at the end of the inocula enrichment in TGM (a rich medium for anaerobic and aerobic microorganisms) and during that period roughly similar growths were observed in the different redox conditions (as described in section 2.2). Taking that into account, together with the fact that roughly similar biomasses were inoculated in all tests, it can be assumed that at least for the tests with TGM the bacterial growth under anaerobic conditions was not a limiting factor. However, when MWW was used, the lower paracetamol degradation rates observed under low oxygenic conditions (27.5±2.2% DO) compared with those observed under high oxygenic conditions (95.0±1.3% DO) were probably the result of slower biomass growth rates in the former compared with the second. This can be considered expected due to the fact that in high oxygenic conditions the complex organic molecules present in MWW are faster oxidized and transformed into simpler carbon sources suitable for bacteria."

15. Which abiotic mechanisms account for the observed losses in un-inoculated controls?
Authors answer: this issue is now addressed.
The following sentences were added to section 3.2.5:
- "One non biological mechanism that can account for the observed losses of paracetamol in all tested media is its oxidation by SO42- ions. It is known that compared with hydroxyl radical (•OH), SO42- is more selective for oxidation by electron-transfer reaction and more powerful for the decomposition of contaminants at neutral pH (Mezyk et al. 2011; Zhang et al. 2015). Indeed, when peroxymonosulfate (PMS) is activated by catalysts it decomposes generating SO42-, which degrades pollutants. Thus, several catalysts have been explored for PMS oxidation and some attracted great interest due to its remarkable separation and catalysis, such as spinel ferrites of MFe2O4 (M = Fe, Mn, Co, Ni, and Cu), (Tan et al. 2017). Another mechanism putatively accounting for the decrease of paracetamol detection in the experiments with TGM (including in the non inoculated controls) is the possible formation of protein-paracetamol complexes. It has been shown that paracetamol and serum proteins form complexes (e.g. Daneshgar et al. 2009) and the TGM has beef extracts in its composition."

16. Please include statistical analysis comparing inoculated and un-inoculated controls. E.g., in section 3.2.2, when it is stated that there is a 10% difference been inoculated and un-inoculated reactors, is this difference statistically significant? If this difference is not statistical, then modify the text that states a "small degradation caused by biological activity".
Authors answer: Done.
The following sentence was added to the end of section 2.4:
- "One-Way ANOVA (Single Factor) tests using Excel Data Analysis Tools were performed to evaluate if differences between the inoculated test cultures and the respective non inoculated negative controls were significant for 5% error (α = 0.05)."
...
The following information was added to table 1:
- "* = paracetamol removal is significantly different (one way ANOVA for 5% error) from the observed in the respective negative control."
...
Corrections were made in section 3.2.1, in the sentences starting by:
- "Even so, in all these cases and for both sampling days, the differences in paracetamol removal averages between the inoculated and non-inoculated tests were relatively low..."
...
- "In the inoculated TGM medium under aerobic conditions with moderate

	oxygenation...” ... The following sentence was removed from section 3.2.1: - “The discrepancy between these aerobic tests using TGM and the anaerobic tests with the same medium discussed above is that in aerobic conditions the removal of paracetamol observed after 72 h is not higher than it was after 24 h (Table 1).” ... Corrections were made in section 3.2.2, in the sentences starting by: - “In what concerns the degradation of paracetamol, the results...” ... - “The maximum removal of paracetamol achieved was...” ... The following sentences were added to section 3.2.2: - “However, despite small, those differences were significant (for 5% error) after 14 days incubation for all paracetamol concentrations tested.” ... - “Nevertheless, this small difference achieved after 14 days of incubation was significant (for 5% error).” ... Corrections were made in section 3.2.3, in the sentences starting by: - “Moreover, in these conditions...” ... - “The partial removals of paracetamol achieved when...” ... - “After 24 h of incubation only 3±3%...” ... Corrections were made in section 3.2.4, in the sentences starting by: - “After 24 h incubation just 11±10%...” ... - “The low paracetamol removal observed in that test...” 17. It would be helpful if the subtitles also indicated the redox/electron acceptor tested. Authors answer: To avoid having long subtitles this information was added in the text. The following sentence was added to section 3.2.1: - “The main electron acceptor in TGM is oxygen in the tests under aerobic conditions, while oxidized forms of inorganics such as nitrate, sulphate, iron (III) and manganese (IV), as well as amino acids and glucose are the potential electron acceptors in the tests under anaerobic conditions.” ... The following sentence was added to section 3.2.2: - “This medium is optimized to cultivate SRB under anaerobic conditions, thus the main electron acceptor in its composition is sulphate.” ... The following sentence was added to section 3.2.3: - “This medium, exclusively composed by inorganic compounds, was used to test paracetamol biodegradation when this drug was the only carbon source available. The putative main electron acceptors of MSM are oxygen, in the tests under aerobic conditions, and nitrate sulphate and manganese (IV), in the tests under anaerobic conditions.” ... The following sentence was added to section 3.2.4: - “It was tested just under aerobic conditions, in which the main electron accepter is oxygen.” 18. The last sentence of section 3.2.2 is entirely too long and should be broken up for readability. Authors answer: Done. The last sentence of section 3.2.2 was substituted by this paragraph: - “These to some extent higher removals of paracetamol observed in the tests inoculated with the SRB enriched consortium compared to the respective non inoculated negative controls may indicate the existence of mechanisms associated with biological activity contributing to paracetamol removal. However, it remains unknown whether paracetamol was degraded via metabolic pathways, or if it reacted with compounds released by the active microbial communities. Despite this, it seems

that the SRB present in this consortium are not directly involved in the removal because it occurred at the same extent both in medium with lactate (with high SRB activity) and without lactate (with low SRB activity). Thus, probably other microorganisms than SRB, eventually less abundant in the inoculum, may have been directly or indirectly responsible for the observed paracetamol removal."

19. Table 1 indicates "No sludge or autoclaved sludge", but these two options are not equivalent. Table 1 should indicate which was which.
The following information was added to table 1:
- "c = negative controls carried out with autoclaved sludge."

20. Section 3.3, bottom of page 13: The drug is not "catalyzed". Reactions are catalyzed. Rephrase.
Authors answer: Done.
The word "catalyzed" was substituted by the word "metabolized" and corrections were made in the respective sentence of section 3.3, which now stands like this:
- "The occurrence of 4-aminophenol in all inoculated cultures and not in the negative controls (without sludge as inoculum) supports the major biodegradation pathway of paracetamol proposed for microbes by other authors: this drug is metabolized to produce 4-aminophenol, which is converted to hydroquinone through replacement of the amino group by a hydroxyl group, being then this aromatic compound the precursor of several carboxylic acids (2-hexenoic acid, succinic acid, malonic acid, oxalic acid and finally formic acid) (Hu et al. 2013; Wu et al. 2012; Zhang et al. 2013)."

21. Table 2 should indicate units.
Authors answer: Done.
The following information was added to table 1:
- "OTU abundances (%)"

22. Figure 4 would be more useful if the peaks of interest were labeled somehow. This figure could optionally be moved to supplemental given its lack of complexity.
Authors answer: The peaks of interest in figure 4 were labeled and we would like to maintain this figure as figure 4 in the paper.

23. How were replicated cultures handled during sequencing? Was sequencing conducted for all replicates? Was the data pooled? Replication is not displayed in Table 2. Was the identification of the phylotypes listed in the title reproducible across replicates? This is a critical point to address.
Authors answer: Sequencing was conducted just for one of the three replicates. No samples, neither sequencing data was pooled. This point is now addressed.
The following sentences were added to section 3.5:
- "The three replicate cultures of this test revealed identical behaviors for the concentrations of the analyzed compounds along the experiment. Thus, it was considered that the microbial dynamics studied through massive sequencing of 16S rRNA genes present in just one of the replicates along the incubation time would provide enough robust evidences to identify taxa putatively involved in the degradation of paracetamol and/or its metabolites."

24. Was sequencing conducted to separately measure Bacteria and Archaea? Or just Bacteria?
Authors answer: Sequencing and respective bioinformatic processing was focused on bacteria.
The following sentence was added to section 3.5:
- "The study was focused on bacteria using primers for the 16S rRNA gene region V1-3."

25. Dynamic trends in microbial community composition would best be visualized in a histogram rather than a table. Better yet, replicates could be displayed via principle component analysis.
Authors answer: We have created a histogram displaying the dynamic trends in microbial community composition for the 25 most abundant bacteria; however, we consider that it thus not improves the visualization and interpretation of results. Therefore, we would prefer to maintain Table 2.
Since sequencing was carried out in just one of the three replicates, we didn't try to

	display the results via principle component analysis.
Additional Information:	
Question	**Response**
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Have you read the 'Letter from the Editor-in-Chief' and the full journal author instructions, located at the journal homepage **www.springer.com/11270?**	Yes
Has this material (or data) been literally or substantially submitted (or will it be) for simultaneous consideration in another publication in English or another language? **If Yes, do not continue with the submission.**	No
Has this material (or data) been literally or substantially published elsewhere in English or another language? **If Yes, do not continue with the submission.**	No
Once the article is submitted for review, no changes in authorship, the order of authors, or designation of the corresponding author will be permitted. Have all authors been actively involved in making a substantial scholarly contribution to the design and completion of this research, interpretation of data and conclusions, assisted in drafting and revising the manuscript, and read and approved this submission? (YES/NO). If NO, please do not submit your manuscript. Please review the authorship guidelines on the journal's homepage for guidance.	Yes
Does the article report existing science applied to a local situation?	No
If yes, how is this research significant to furthering worldwide knowledge on this	-

topic	
Is the article of local, national or international value? (Choose one.)	International
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Putative role of *Flavobacterium*, *Dokdonella* and *Methylophilus* strains in paracetamol biodegradation

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ABSTRACT

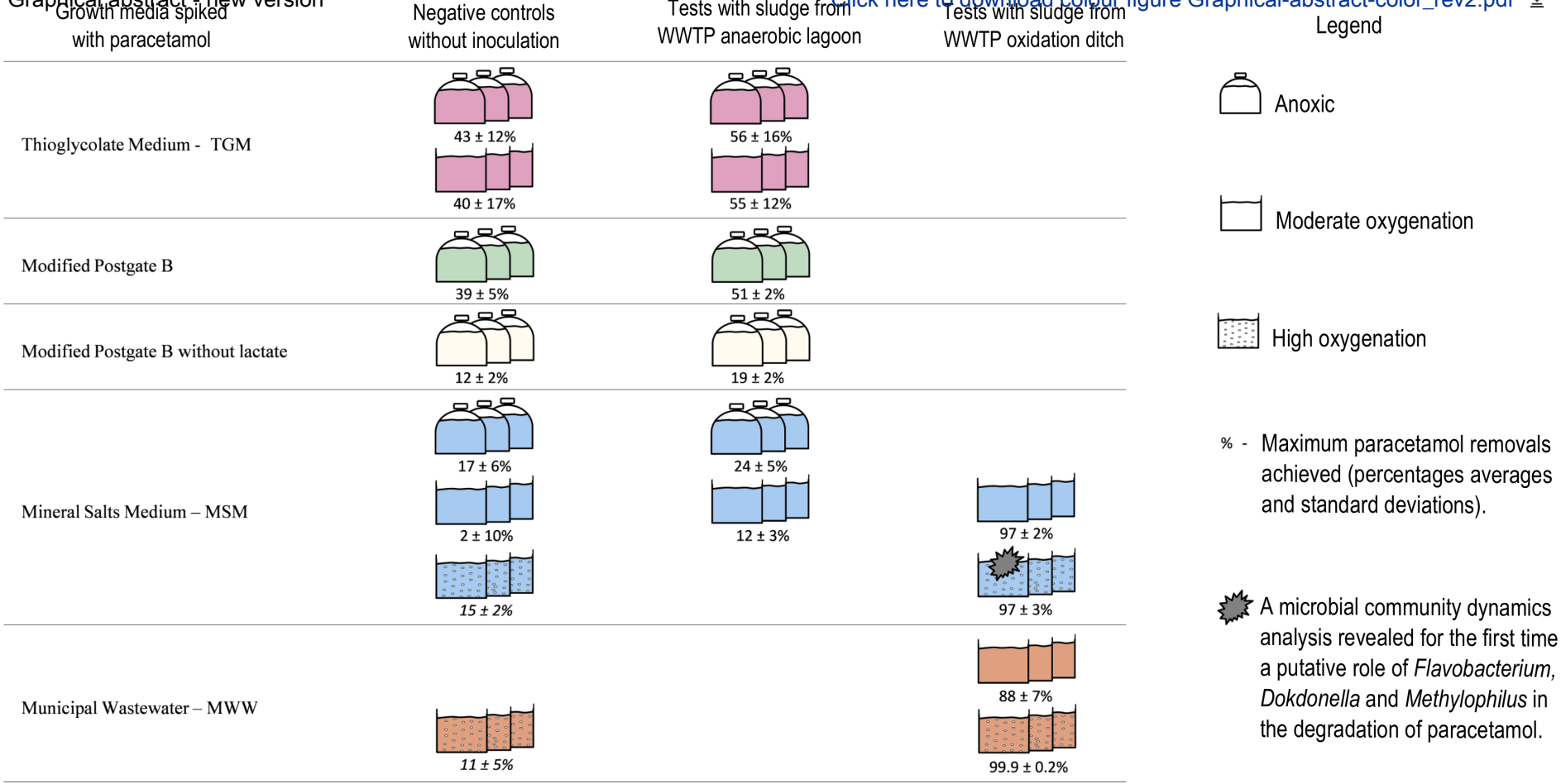
Paracetamol, the most widely and globally used analgesic and antipyretic, is easily accumulated in aquatic environments. In the present study, the biodegradation of paracetamol in different media (one for general growth, one specific for sulphate reducing bacteria, a mineral salts medium and municipal wastewater) inoculated with two types of sludge (from anaerobic lagoon and from oxidation ditch) under different oxygenic conditions (anoxic; moderate oxygenation in open flasks and high oxygenation by aeration) was investigated. In addition, bacteria with relative abundances increasing simultaneously with paracetamol degradation, when this drug was the only carbon source, thus with a putative role in its degradation, were identified using 16S rRNA gene sequences. The results show that aerobic microorganisms had a major role in the degradation of paracetamol, with 50 mg/L totally removed from municipal wastewater after two days incubation with aeration, and that the metabolites 4-aminophenol and hydroquinone plus one compound not identified in this work were produced in the process. The identification of bacteria with a role in the degradation of paracetamol revealed a strain from genus *Pseudomonas* with the highest final relative abundance of 21.2%, confirming previous works reporting strains of this genus as paracetamol decomposers. Besides, genera *Flavobacterium*, *Dokdonella* and *Methylophilus* were also in evidence, with initial relative abundances of 1.66%, 1.48% and 0.00% (not detected) in the inoculum and 6.91%, 3.80% and 3.83% after incubation, respectively. Therefore, a putative role of these genera in paracetamol biodegradation is suggested for the first time.

Keywords: Acetaminophen; 4-aminophenol; bioremediation; hydroquinone; pharmaceutical compounds

Highlights

- The IC_{50} of paracetamol for the growth of bacteria from municipal WWTP sludge was estimated.
- Biodegradation of paracetamol was tested in different media and oxygenic conditions.
- *Flavobacterium*, *Dokdonella* and *Methylophilus* are for first time associated to paracetamol degradation.
- The putative role of these genera in paracetamol degradation is discussed.

Graphical abstract - new version



Putative role of *Flavobacterium*, *Dokdonella* and *Methylophilus* strains in paracetamol biodegradation

ABSTRACT

Paracetamol, the most widely and globally used analgesic and antipyretic, is easily accumulated in aquatic environments. In the present study, the biodegradation of paracetamol in different media (one for general growth, one specific for sulphate reducing bacteria, a mineral salts medium and municipal wastewater) inoculated with two types of sludge (from anaerobic lagoon and from oxidation ditch) under different oxygenic conditions (anoxic; moderate oxygenation in open flasks and high oxygenation by aeration) was investigated. In addition, bacteria with relative abundances increasing simultaneously with paracetamol degradation, when this drug was the only carbon source, thus with a putative role in its degradation, were identified using 16S rRNA gene sequences. The results show that aerobic microorganisms had a major role in the degradation of paracetamol, with 50 mg/L totally removed from municipal wastewater after two days incubation with aeration, and that the metabolites 4-aminophenol and hydroquinone plus one compound not identified in this work were produced in the process. The identification of bacteria with a role in the degradation of paracetamol revealed a strain from genus *Pseudomonas* with the highest final relative abundance of 21.2%, confirming previous works reporting strains of this genus as paracetamol decomposers. Besides, genera *Flavobacterium*, *Dokdonella* and *Methylophilus* were also in evidence, with initial relative abundances of 1.66%, 1.48% and 0.00% (not detected) in the inoculum and 6.91%, 3.80% and 3.83% after incubation, respectively. Therefore, a putative role of these genera in paracetamol biodegradation is suggested for the first time.

Keywords: Acetaminophen; 4-aminophenol; bioremediation; hydroquinone; pharmaceutical compounds

1 INTRODUCTION

Emerging pollutants are not covered by existing water quality regulations but they are considered potential threats to environmental ecosystems, human health and safety, especially because their large-scale production and use results in their release into the environment usually via sewage and wastewater treatment plant (WWTP) discharges (Deblonde et al. 2011). The biodegradation and behavior of these compounds in aqueous systems are largely unknown, leading to simplified assumptions in the estimation of their environmental risk. Additionally, they may have an impact on wastewater treatment processes by increasing or decreasing sewage or industrial contaminant removal efficiency (Deblonde et al. 2011).

Paracetamol (N-(4-hydroxyphenyl)acetamide), or acetaminophen, is the most consumed analgesic. For example, in France 3303.08 tonnes were consumed in year 2008 (Vulliet and Cren-Olivé, 2011). It is considered an emerging pollutant due to its widespread global use and to the fact that it is readily accumulated in aquatic ecosystems with adverse effects (De Gusseme et al. 2011; Wu et al. 2012; Mbokou et al. 2016). Though paracetamol appears to be well removed during sewage treatment when compared to other pharmaceuticals, its concentrations detected in surface waters ranges from 110 to 10000 ng/L (Wilkinson et al. 2017). A warning sign of the global importance of this pollutant is the detection of paracetamol in the open sea waters of the western Mediterranean in concentration ranges from 0.468 to 1.70 ng/L (Brumovský et al. 2017). In fact, the worldwide presence of this drug in the WWTP effluents reminds the need of implementing treatment processes more efficient for its removal, even if in general the higher concentrations of paracetamol detected in raw sewage than in treated wastewaters indicate high removal rates. For example, concentrations up to 180 mg/L have been reported in WWTP influents while in the effluents the concentrations reported were up to 0.305 mg/L in systems with aerobic tanks and up to 13 mg/L in wetlands (*e.g.* Roberts and Thomas, 2006; Papageorgiou et al. 2016; Vymazal et al. 2017). In Portugal, the highest concentration of paracetamol recorded in wastewater effluent was 32 µg/L (Pereira et al. 2016). Moreover, paracetamol´s main metabolite from animals, paracetamol glucuronide, also enters the environment via human excretion and has been detected in WWTP effluents at levels of up to 462 µg/L (Santos et al. 2013). Paracetamol glucuronide has been detected in Portuguese surface waters at concentrations of up to 3.57 µg/L (Santos et al. 2013).

Although paracetamol stability decreases in acidic or alkaline conditions due to be slowly degraded via a base- or acid-catalyzed hydrolysis of the amide bond into acetic acid and 4-aminophenol, stability studies in purified water at room temperature demonstrated that after incubation for more than one month this drug was completely stable due to the high energy needed to overcome the barrier for the cleavage of its amide bond (Karaman et al. 2016). In addition, being 4-aminophenol a photo and thermal sensitive compound, like other aromatic amines, the results obtained by Khan et al (2006) showed the occurrence of its autoxidation after 7 days but at low rates: 0.8%, 26.6%, 20% and 13% at 50, 100, 150 and 200 mg/L, respectively. In any case, the authors of both works concluded that biodegradation was more effective. Indeed, several studies have demonstrated that microorganisms play a major role in the environmental degradation of paracetamol and during the past decades research related to the biodegradation of this drug has allowed the identification and characterization of metabolic intermediates involved in the catabolic pathways of aerobic bacteria (Wu et al. 2012).

When paracetamol is used in excess, it can cause liver failure and necrosis due to N-Acetyl-p-benzoquinone imine (NAPQI), a highly toxic paracetamol´s metabolite formed by enzymatic oxidation in the liver (Bessems and Vermeulen 2001). About 30% and 55% of administered paracetamol is excreted in urine as conjugates paracetamol sulfate and paracetamol glucuronide, respectively (Thomas 1993). Moreover, a glutathione conjugate (1,4-Michael adduct) of NAPQI, the corresponding cysteine conjugate and

mercapturic acid breakdown products have also been found in urine after ingestion of paracetamol (Prescott 1980). NAPQI is known to be fairly unstable, however, in aqueous solution it readily hydrolyzes into 1,4-benzoquinone, which is another toxic metabolite (Dahlin and Nelson 1982; Snyder 2000; Bedner and Maccrehan 2006). On the other hand, 4-aminophenol (the hydrolytic product of paracetamol) has highly genotoxic and mutagenic effects (Majeska and Holden 1995; Yoshida et al. 1998). The potential toxic effects of paracetamol in aquatic systems, mediated by its reactive oxygen species, have been reported. For example, Antunes et al. (2013) studied the effects of paracetamol exposure on physiological traits of bivalves and the results showed a significant increase in all oxidative stress biomarkers, evidencing the onset of deleterious effects.

The detection of paracetamol and its metabolites in surface water suggests that the degree of human use and the disposal in sewage overwhelms their effective removal by conventional sewage treatment (Peake et al. 2016). However, there is a lack of information about the environmental behavior of the human metabolites of paracetamol and respective transformation products resulting from their physicochemical and microbial removal in sewage treatment facilities (Peake et al. 2016). Yet, given that these metabolites can be persistent and some are even more toxic than the parent compound, it is essential to determine their fate in biological systems (Wu et al. 2012; Marchlewicz et al. 2015; Peake et al. 2016; Torun et al. 2015; Mbokou et al. 2016) and, hence, to search for new and effective ways to degrade or remove them from wastewater and resulting treated effluents. In this regard, biodegradation may represent a low-cost effective solution (Wu et al. 2012), though several advanced oxidation processes have also been extensively studied for the degradation of paracetamol (e.g. Vogna et al. 2002; Andreozzi et al. 2003; Fatta-Kassinos et al. 2011; Moctezuma et al. 2012; Villota et al. 2016).

In the beginning of the century little was known about microbial degradation of paracetamol (Pieper and Reineke, 2000). Since then, however, some advances have been made in the knowledge of this subject. When biodegradation is complete (mineralization), the substances are converted to inorganic compounds such as water, carbon dioxide, ammonium and nitrate, whereas partial breakdown results in the transformation of pharmaceuticals into other metabolites such as the aromatic compounds 4-aminophenol and hydroquinone (and its oxidized form 1,4-benzoquinone) and several carboxylic acids and nitrogen-containing straight chain compounds (Wu et al. 2012).

Several microorganisms capable of using paracetamol as a carbon and energy source have been isolated and metabolic pathways for the biodegradation of this drug have been proposed. De Gusseme et al. (2011) demonstrated that a membrane bioreactor (MBR) inoculated with an enriched nitrifying bacterial culture was efficient in removing continuously 99.9% paracetamol from a synthetic WWTP effluent spiked with 100 μg/L of this drug. Moreover, in the same work two paracetamol degrading strains identified as *Delftia tsuruhatensis* and *Pseudomonas aeruginosa* were isolated from the MBR biomass and during incubation of these isolates hydroquinone was formed and considered a potential transformation product. Furthermore, Hu et al. (2012) have demonstrated the removal of paracetamol by aerobic granules developed in a

sequencing batch reactor. Then Zhang et al. (2013) have isolated from these microbial aggregates three bacterial strains of the genera *Stenotrophomonas* and *Pseudomonas* capable of using paracetamol as their sole carbon, nitrogen and energy sources and proposed metabolic pathways for the degradation of this drug, with 4-aminophenol and hydroquinone as first metabolic products. More recently, Karaman et al. (2016) demonstrated that paracetamol in activated sludge underwent biodegradation within less than one month and reported that *P. aeruginosa* was the responsible for the biodegradation of paracetamol to 4-aminophenol and hydroquinone. It is worth to note that the identification of bacteria of the genus *Pseudomonas* is common in these various works. In fact *Pseudomonas* is also known for its ability to degrade other aromatic compounds of environmental concern (Neumann et al. 2004; Cámara et al. 2009).

Thus, to our knowledge all the microbial catabolic pathways known to be involved in paracetamol biodegradation were studied in a small number of aerobic organisms; therefore, it is possible that unidentified mechanisms with a role in paracetamol biodegradation exist in other organisms. The present study focuses on the characterization of bacterial communities with capacity to biodegrade paracetamol, taking into account the well-known advantages of consortia over pure cultures (Mukred et al. 2008), aiming the identification of new genera with a putative role in the mineralization of this drug. For that purpose bacterial communities obtained from sludge of two types of wastewater treatment plants processes (anaerobic lagoon and oxidation ditch) were tested using media with different nutritional characteristics: multipurpose ThioGlycolate Medium (TGM) generally used for cultivation of anaerobes, and aerobes, Mineral Salts Medium (MSM), modified Postgate B medium specific for sulphate reducing bacteria (SRB) and raw Municipal Wastewater (MWW), in different oxygenic conditions: anaerobic (sealed flasks), moderate oxygenation (open flasks without aeration) and high oxygenation (open flasks with aeration) according to Figure 1 and Table 1, in order to test the enriched bacterial communities broad from such variety of tested conditions.

2 MATERIALS AND METHODS

2.1 Chemicals

The following reagents were purchased from Panreac (Barcelona, Spain): sodium chloride (NaCl), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), calcium chloride ($CaCl_2$), zinc sulfate ($ZnSO_4$), cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$), manganese(II) sulfate ($MnSO_4$), ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)), monopotassium phosphate (KH_2PO_4), ammonium chloride (NH_4Cl), sodium sulfate (Na_2SO_4), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), sodium lactate ($C_3H_5NaO_3$), ammonium hydroxide (NH_4OH). Ethylenediamine tetraacetic acid (EDTA), potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) both AnalaR NORMAPUR® were obtained from VWR Prolabo Chemicals (Leuven, Belgium). Yeast extract was acquired from HiMedia Laboratories (Mumbai, India) and thioglycolic acid was purchased from Merck (Darmstadt, Germany). All the solutions of

paracetamol (99% purity), hydroquinone (99% purity), 4-aminophenol (97% purity), 4-nitrophenol (99% purity) were purchased from Sigma-Aldrich (Deisenhofer, Germany). Acetonitrile (ACN) and methanol, both HPLC grade, and phosphoric acid (85% purity) were supplied by VWR Prolabo Chemicals (Fontenay-sous-Bois, France) and formic acid (HCOOH) high purity grade was obtained from Amresco (Solon, USA). Phosphate buffer solution of pH = 4.88 was prepared by dissolving 4.5 g KH_2PO_4 and 0.0412 g $K_2HPO_4.3H_2O$ in 500 mL of ultra pure water, using phosphoric acid (85%) to adjust pH if necessary.

2.2 Inocula source and preparation

Sludges from two Portuguese WWTPs with different processes were used as inocula sources. Sludge collected in the Faro East WWTP's lagoon system without aeration (anaerobic sludge) was used for a first set of experiments under anaerobic and moderate aerobic conditions, while sludge from the Faro Northwest WWTP's oxidation ditch with aeration (aerobic sludge) was used in a second set of experiments in which highly aerobic conditions were tested (Table 1).

To enrich the inocula from the lagoon system, 2 g of sludge sediment were inoculated on each of two separate 250ml culture flasks containing 200 mL of TGM medium. In one flask 10 mL of paraffin-oil was added to prevent gas diffusion and the flask was sealed with a butyl rubber stopper and an aluminum crimp seal to avoid air intake in order to create anaerobic condition. In the other flask, cotton was used to serve as lid, thus allowing the maintenance of aerobic conditions. To enrich the inoculum from the oxidation ditch, 2 mL of water with sludge was inoculated in just one flask with 200 mL TGM medium with cotton as lid. The anaerobic flask was kept without shaking and the two aerobic flasks were placed in an orbital shaker at a speed of 150 rpm; all were maintained at room temperature and grown for 24 hours. The anaerobic enriched culture was used to inoculate all tests under anaerobic conditions. The aerobic enriched cultures from each type of sludge were used to inoculate the respective tests under moderate and highly aerobic conditions.

In the inocula enrichments, initial and final optical densities were measured at 600 nm (OD_{600}) as absorbance using a Hach-Lange spectrophotometer DR-2800 (Sköndal, Sweden). The initial OD_{600} values of enrichment media immediately after inoculation with the anaerobic lagoon and the oxidation ditch sludges were similar: 0.286 and 0.264, respectively. Thus, the also similar OD_{600} values measured after the 24 hours of incubation at room temperature in all enrichment conditions (anaerobic lagoon sludge/anaerobic growth = 0.838; anaerobic lagoon sludge/aerobic growth = 0.831; oxidation ditch sludge/aerobic growth = 0.833) roughly indicates similar bacterial growths.

A SRB consortium enriched under anaerobic conditions for 7 days in Postgate B medium from the sludge sample collected in the anaerobic lagoon system of Faro East WWTP was used to test the paracetamol biodegradation ability of that particular anaerobic community. The initial and the final OD_{600} values in the SRB inoculum enrichment were 0.165 and 0.657, respectively.

Previous steps of sludge washing were carried out to eliminate dissolved carbon compounds that could serve as carbon and energy sources for bacterial growth because in some experiments (with MSM and with

the modified Postgate B without lactate) paracetamol was expected to be the only carbon source. With that purpose, the enriched inocula coming from the anaerobic and aerobic sludges were centrifuged at 2500 × *g* for 10 min at room temperature, the supernatant was discarded and the pellets re-suspended using the MSM. This procedure was repeated twice before inoculation of test cultures.

2.3 IC_{50} of paracetamol for bacterial growth

The half maximal inhibitory concentration (IC_{50}) for bacterial growth, the concentration of paracetamol that causes a 50% drop in bacteria growth, was estimated from duplicate batch cultures in TGM under aerobic and under anaerobic conditions with different paracetamol concentrations (4, 6, 8, 10 and 12 g/L) and without paracetamol (as a reference for normal bacterial growth) during an incubation period of 24 h at room temperature. Aerobic sludge was used as an inoculum for the aerobic cultures and anaerobic sludge for the anaerobic cultures.

The bacterial growth for IC_{50} determination was analyzed by measuring the OD_{600} using a Hach-Lange spectrophotometer DR-2800 (Sköndal, Sweden). Based on the OD_{600} values, the percentage of bacterial viability (OD_{600} in a culture with a drug concentration over the OD_{600} in the culture without drug) was calculated for the different tested paracetamol concentrations. IC_{50} values were estimated using GraphPad Prism 6 Software published by GraphPad Software, Inc. using dose response curve log (inhibitor) *vs.* normalized response-variable slope analyses. For both anaerobic and aerobic assays, bacterial viability percentage (%) *versus* logarithm of paracetamol concentration (g/L) were plotted and best fitting sigmoidal curves were used to calculate IC_{50} values.

2.4 Biodegradation of paracetamol

Anaerobic and aerobic cultures without aeration as well as aerated cultures were evaluated in order to mimic the conditions in WWTPs lagoon systems and in WWTPs with oxidation ditch.

The cultures were carried out in triplicated batches with 10% (v/v) sludge enriched inoculum on five different media spiked with paracetamol: (i) TGM, an universal complex medium; (ii) MSM, a medium without carbon compounds composed of 1 g/L K_2HPO_4, 1 g/L KH_2PO_4, 20 mg/L NaCl, 0.41 g/L $MgCl_2.6H_2O$, 0.04 g/L $CaCl_2$, 1.53 mg/L $ZnSO_4$, 0.78 g/L $CoCl_2.6H_2O$, 0.74 mg/L $MnSO_4$, 20 mg/L EDTA and 0.64 mg/L $(NH_4)_6Mo_7O_{24}.4H_2O$); (iii) a modified Postgate B medium used for SRB composed of 0.5 g/L KH_2PO_4, 1 g/L NH_4Cl, 1 g/L Na_2SO_4, 1 g/L yeast extract, 0.1 g/L ascorbic acid, 0.1 g/L thioglycolic acid, 2 g/L $MgSO_4.7H_2O$ and 7.75 g/L of the carbon source $C_3H_5O_3Na$ – sodium lactate; (iv) the modified Postgate B medium without the carbon source; and (v) raw MWW, a sample collected at the entry of the Faro Northwest WWTP.

All media, except the raw MWW, were autoclaved (120ºC for 45 min) and cooled to room temperature before the addition of paracetamol and inoculation. Paracetamol was added using a PES syringe filter of 0.2 µm pore size from VWR (Leuven, Belgium) to make the tested drug concentrations: between 10 mg/L and

100 mg/L, which are in the range of values reported in European WWTP influents (*e.g.* Roberts et al. 2006; Papageorgiou et al. 2016; Vymazal et al. 2017), as well as a much higher concentration (10000 mg/L), which is above the IC_{50} values estimated in this work for bacterial growth.

Negative controls (without sludge inoculum) were performed with all tested media to evaluate the eventual degradation of these compounds in ways other than the biological route, or to check eventual interactions of the drug with the medium. Moreover, to evaluate the possible adsorption of paracetamol on the sludge used as inoculum, independent assays were performed with autoclaved (120ºC for 45 min) sludge in MSM and MWW media with 50 mg/L of this drug.

All the assays were carried out in triplicated in the dark (to avoid photodegradation) at room temperature and at atmospheric pressure. To create anaerobic conditions, liquid paraffin was added to cultures and the batch flasks were sealed with butyl rubber stoppers and aluminum crimp seals. The liquid paraffin prevents gas diffusion through the medium surface and the flask sealed with a butyl rubber stopper and an aluminum crimp seal avoids air intake into the flask; thus the oxygen initially present is rapidly consumed and anaerobic conditions are created. In the anaerobic tests resazurin was added as an indicator to confirm the absence of oxygen. To maintain aerobic conditions with moderate oxygenation, the cultures were incubated in open batch flasks without stirring or shaking. To achieve aerobic conditions with high oxygenation levels, cultures were carried out in open batches with aeration (110 mL/min airflow).

The averages and standard deviations of dissolved oxygen (DO) percent saturation in the various tests under aerobic conditions were calculated using measurements made in the respective replicate cultures with a portable CD650 meter (Eutech Instruments) after 24 hours of incubation since inoculation. For moderate oxygenation conditions in open flasks without aeration, the DO percent saturation was: 9.3±1.8% in the tests with TGM, 31.1±2.3% in the tests with MSM and 27.5±2.2% in the tests with MWW (24 hours after both first media were inoculated with the anaerobic lagoon sludge and the third medium inoculated with the oxidation ditch sludge). For high oxygenation conditions in aerated flaks, the DO percent saturation was: 94.8±1.6% in the tests with MSM and 95.5±1.3% in the tests with MWW (24 hours after both these media were inoculated with the oxidation ditch sludge).

The experiments in TGM, MSM and MWW were incubated during 72 h. The experiments in modified Postgate B were maintained during 14 days, the time to assure high SRB activity and total reduction of the sulphate in the growth media, according to the experience of the research group with these bacteria, namely for acid mine drainage bioremediation purposes (Costa and Duarte, 2005; Da Costa et al. 2013; Vitor et al. 2015). Aiming to evaluate the SRB activity, the cultures in modified Postgate B and in modified Postgate B without lactate were monitored for pH and redox potential (E_h) using a GLP 21 pH meter, Crison (Barcelona, Spain) and for the sulphate concentration through molecular UV/Visible spectroscopy at 450 nm using a Hach-Lange™ DR 2800 spectrophotometer (Sköndal, Sweden) and the sulfaVer4 method from Hach-Lange (Düsseldorf, Germany).

One-Way ANOVA (Single Factor) tests using Excel Data Analysis Tools were performed to evaluate if differences between the inoculated test cultures and the respective non inoculated negative controls were significant for 5% error ($\alpha = 0.05$).

2.5 Effect of paracetamol on chemical oxygen demand (COD) degradation

COD is often used to measure organic matter, allowing an indirect quantification of the amount of oxidizable compounds in wastewaters, treated effluents and receiving waters

The COD (mg O_2/L) was analyzed in assays with MWW medium in the presence and absence of paracetamol to evaluate the influence of this drug on the degradation of the organic matter present in wastewater. COD was also determined in the MSM medium assays with paracetamol (i) inoculated with aerobic sludge and (ii) without inoculum, to evaluate the variation of COD caused by adding this drug and by its removal. Samples collected immediately after preparation of these assays and after 72 h incubation were used for COD analysis.

Measurements were carried out using cuvette tests for the dichromate method with LCK 514 kit, purchased from Hach-Lange (Düsseldorf, Germany). Tubes with pre-determined amounts of potassium dichromate, sulphuric acid and silver sulphate as catalyst were homogenized and mixed with 2 mL of samples. The samples were digested in an AccuBlock™ Digital Dry Bath, Labnet (Massachusetts, USA) at 148 ºC during 120 min. Samples were allowed to cool to room temperature and COD values were measured at 605 nm using a Hach-Lange™ DR 2800 spectrophotometer (Sköndal, Sweden).

2.6 HPLC analysis

Immediately after collecting the samples, they were filtered with 0.2 µm polypropylene (PP) syringe filters from VWR (Leuven, Belgium) and then stored overnight at 4 ºC before chromatographic analysis.

Paracetamol and respective metabolic products were analyzed using a modular Advanced Scientific Instrument KNAUER HPLC system with a Smartline UV detector 2600 Smartline Manager 5000 (Berlin, Germany). The output signal was monitored and integrated using ClarityChrom® software. The compounds were separated using a reversed phase Xbridge-C18 column (250 × 4.6 mm, 5 µm particle size) - Hybrid technology connected to a Guard Column Xbridge-C18 column (4.6 × 200 mm, 5 µm particle size), both purchased from Waters Corporation (Milford, MA, USA).

2.6.1 Quantification of paracetamol

To analyse the biodegradation of paracetamol, HPLC analysis was performed with a fast isocratic method using a mobile phase consisted of acetonitrile:water (25:75, v/v) adjusted to pH 3.74 with orthophosphoric acid (85%) using a flow rate of 1.0 mL/min and a run time of 5 minutes with the column maintained at room temperature. The injection volume was 20 µL and a wavelength of 244 nm was used for detection.

Exclusive standard calibration curves were constructed for each experiment to determine the concentrations of paracetamol in the corresponding samples. The concentration ranges of paracetamol standards, prepared with the respective medium were: TGM medium: 5 to 100 mg/L and 50 to 2500 mg/L, depending on the paracetamol concentration tested; MSM medium: 5 to 1000 mg/L; Modified Postgate B medium: 1 to 110 mg/L; MWW assays: 5 to 250 mg/L. The limits of detection (LOD) were determined by the analysis of standards with known concentrations to establish the minimum level at which the analyte peak could be reliably detected by visual evaluation, as described in the harmonized tripartite guideline (ICH, 1996). LODs of 1, 0.5, 0.15 and 0.2 mg/L were estimated for TGM, MSM, modified Postgate B and MWW, respectively.

2.6.2 Identification of paracetamol metabolic products

Samples from the experiments in which high degradation of paracetamol was observed when using the fast isocratic HPLC method were run with a longer method that allows a good separation, and therefore identification, of products known to be generated from the degradation of this drug. This analysis was performed with the mobile phase potassium-phosphate-buffer (pH 4.88):methanol, which was optimized by Calinescu et al. (2012), and the following gradient program with respective mobile phase ratios (v/v): 1st ramp from 0 to 8 min with 80:20 to 50:50 (v/v); 1st stationary step from 8 to11 min with 50:50 (v/v); 2nd ramp from 11 to 12 min with 50:50 to 80:20 (v/v); 2nd stationary step from 12 to 15 min with 80:20 (v/v). The injection volume was 20 µL, the flow rate was set at 0.8 mL/min, the column was maintained at room temperature and detection was performed at 244 nm.

2.7 Characterization of paracetamol degrading bacterial communities

Microbial communities were studied along the experiment in one culture of the three replicates with MSM, in which paracetamol (50 mg/L) was the only carbon compound added to the medium, aiming to identify microorganisms with a putative role in the degradation of this drug. As the three replicate cultures were inoculated exactly with the same microbial community (source: enriched culture from WWTP's oxidation ditch sludge; quantity: 10% (v/v)) and were maintained under exactly the same conditions (aeration: a shared air pump; temperature and light: flasks maintained side by side), it was assumed that studying the microbial dynamics through massive sequencing of 16S rRNA genes on just one of the cultures would provide enough robust evidences to identify taxa putatively involved in the degradation of paracetamol and/or its metabolites.

For that purpose, DNA was extracted from culture samples with the PowerSoil® DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), which uses bead beating and silica spin filter technology for extraction. The concentration and quality of eluted DNA was determined using a NanoDrop spectrophotometer Thermo Scientific 3300 and DNA samples were sent to the company DNASense ApS

(Aalborg, Denmark) for large-scale biodiversity analysis through massive parallel sequencing of 16S rRNA genes according to the following procedures.

2.7.1 16S rRNA amplicon library preparation

The procedure for bacterial 16S ribosomal ribonucleic acid (rRNA) amplicon sequencing targeting the V1-3 variable regions is based on Caporaso et al. (2012) using primers adapted from the Human Gut Consortium (Ward et al. 2012). Ten nanograms of extracted DNA was used as template and the polymerase chain reaction (PCR) (25 μL) contained deoxynucleotide (dNTPs) (400 nM of each), $MgSO_4$ (1.5 mM), Platinum® Taq DNA polymerase HF (2 mU), 1X Platinum® High Fidelity buffer (Thermo Fisher Scientific, USA), and barcoded library adaptors (400 nM) containing V1-3 specific primers: 27F AGAGTTTGATCCTGGCTCAG and 534R ATTACCGCGGCTGCTGG. PCR settings: Initial denaturation at 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 56 °C for 30 s, 72 °C for 60 s and final elongation at 72 °C for 5 min.

All PCR reactions were run in duplicate and pooled. The amplicon libraries were purified using the Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA) with the following exceptions: the sample/bead solution ratio was 5/4, and the purified DNA was eluted in 33 μL nuclease-free water. Library concentration was measured with Quant-iT™ HS DNA Assay (Thermo Fisher Scientific, USA) and quality validated with a Tapestation 2200, using D1K ScreenTapes (Agilent, USA). Based on library concentrations and calculated amplicon sizes, the samples were pooled in equimolar concentrations and diluted to 4 nM.

2.7.2 DNA sequencing

The samples were paired end sequenced (2 x 301bp) on a MiSeq (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina) following the standard guidelines for preparing and loading samples on the MiSeq. 10% Phix control library was spiked in to overcome low complexity issue often observed with amplicon samples.

2.7.3 16S rRNA amplicon bioinformatic processing (bacteria V1-3)

Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 (Bolger et al. 2014) with the settings SLIDINGWINDOW:5:3 and MINLEN:275. The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (Magoc and Salzberg, 2011) with the settings -m 25 -M 200. The merged reads were dereplicated and formatted for use in the UPARSE workflow (Edgar, 2013). The dereplicated reads were clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. Operational taxonomic unit (OTU) abundances were estimated using the usearch v. 7.0.1090usearch_global command with -id 0.97. Taxonomy was assigned using the RDP classifier (Wang et al. 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso et al. 2010), using the

MiDAS database v.1.20 (McIlroy et al. 2015). The results were analyzed in R (R Core Team, 2015) through the Rstudio IDE using the ampvis package v.1.24.0 (Albertsen et al. 2015).

The raw massive sequencing data from 16S rRNA gene amplicons generated in this work was archived in the NCBI Sequence Read Archive (SRA) database under SRA study SRP125056 and BioProject PRJNA418409. The OTU sequences were published with GenBank accession numbers MG554745 to MG557554.

3 RESULTS AND DISCUSSION

3.1 IC_{50} of paracetamol for bacterial growth

The studies started by the determination of paracetamol IC_{50} for bacterial growth, which is the concentration of this drug causing a drop of 50% in bacterial viability (defined as the percentage of the OD_{600} measured in a culture with a certain paracetamol concentration in relation to the OD_{600} in the control culture without the drug). Estimates were based on measurements in cultures with the general growth medium TGM containing different concentrations of paracetamol, under anaerobic and aerobic conditions.

The bacterial viability as a function of the logarithm of paracetamol concentration fits reasonably for both conditions a sigmoidal curve (Figure 2). Those functions were similar; therefore, the IC_{50} values for both conditions are also similar: 6.20 and 6.162 g/L, respectively for anaerobic and aerobic conditions. These IC_{50} values are much higher than the concentrations usually reported in raw untreated wastewaters of less than 0.2 g/L (*e.g.* Roberts et al. 2006; Papageorgiou et al. 2016; Vymazal et al. 2017). Thus, problems in WWTPs caused by paracetamol affecting the bacterial communities that degrade the organic matter are not expected and have not yet, to our knowledge, been reported.

3.2 Biodegradation of paracetamol

The results obtained clearly indicate that the major routes for the biodegradation of this drug in WWTPs involve aerobic organisms; some already reported previously and others identified in this work for the first time, as discussed further below. However, some slow biodegradation seems to occur under anaerobic conditions, which was not more evident due to the short time of the assays.

The overall results obtained in the tests performed to evaluate paracetamol biodegradation under diverse experimental conditions are summarized in Table 1.

3.2.1 TGM Medium

The TGM medium is a complex and non selective medium for cultivation of anaerobes, aerobes and microaerophiles, thus it allows the growth of a wide diversity of bacteria. The main electron acceptor in TGM is oxygen in the tests under aerobic conditions, while oxidized forms of inorganics such as nitrate,

sulphate, iron (III) and manganese (IV), as well as amino acids and glucose are the potential electron acceptors in the tests under anaerobic conditions. This medium was chosen to test one paracetamol concentration (10000 mg/L) above the IC_{50} values of this drug estimated in this work for bacterial growth (6204 and 6162 mg/L) and lower concentrations (10, 50 and 80 mg/L) closer to values reported in wastewaters. Anaerobic and moderate aerobic (open flasks without aeration) were tested with TGM using the inoculum enriched from the WWTP lagoon system sludge.

In the inoculated TGM cultures under anaerobic conditions with the lower tested paracetamol concentrations of 10, 50 and 80 mg/L, drug removals of 24%, 22% and 12% (respectively) were observed after 24 h and removals around twice these values (56%, 45% and 33%) were achieved after 72 h (Table 1). With the same conditions but in the presence of 10000 mg/L paracetamol, a removal efficiency of 22±14% was attained after 24 h assay, but no major changes were observed after 72 h (26±12%). According to the plotted IC_{50} curves (Figure 2) the bacterial viability (%) for 10000 mg/L paracetamol is low (20% to 30%). Therefore, probably this high concentration inhibited the growth of bacteria degrading this drug. Even so, in all these cases and for both sampling days, the differences in paracetamol removal averages between the inoculated and non-inoculated tests were relatively low (< 15%) and not significant (for 5% error), suggesting that biodegradation had a limited role in the removal of this drug.

In the inoculated TGM medium under aerobic conditions with moderate oxygenation (open flasks without aeration), the differences in paracetamol removal between the inoculated and non-inoculated tests were also low (< 20%) and not significant (for 5% error), thus suggesting a limited role of biological activity in the drug removal also in this case.

In summary, in the experiments with TGM media, three days were necessary to achieve paracetamol removals around 55% under anaerobic conditions while in moderate aerobic conditions this level of removal was achieved after only one day, and in both these conditions a small difference was observed between the inoculated tests and the non inoculated negative controls. Paracetamol resistance to biodegradation and biotransformation under anaerobic conditions was already described (*e.g.* Musson et al. 2010). Moreover, these authors also reported that measureable losses from solution may occur through abiotic mechanisms as described in BIOWIN, an anaerobic degradation prediction model developed by the US Environmental Protection Agency (Musson et al. 2010).

3.2.2 Modified Postgate B

This medium is optimized to cultivate SRB under anaerobic conditions, thus the main electron acceptor in its composition is sulphate. For all tested paracetamol concentrations (10, 80 and 100 mg/L) in cultures using modified Postgate B medium the pH remained within the interval of 6.5 to 7.5, which is in the optimum range for SRB. Indeed, the parameters analyzed to monitor these bacteria indicated high activity in the inoculated tests: the redox potential (E_h) evolved to values between -350 and -400 mV after 2 weeks incubation under anaerobic conditions and the sulphate removal in this period was equal or higher than the

72% observed in the control culture without the drug. These results demonstrate that the inoculated SRB consortium enriched from the Faro East WWTP's lagoon system sludge was resistant to all concentrations of paracetamol tested. In what concerns the degradation of paracetamol, the results were slightly different from those observed in the tests with TGM. The maximum removal of paracetamol achieved was in the same range (51±2%) and the differences on the removal averages of this drug between the inoculated cultures and the respective non inoculated negative controls were also small (less than 15%). However, despite small, those differences were significant (for 5% error) after 14 days incubation for all paracetamol concentrations tested.

In the assay performed using the same SRB inoculum in modified Postgate B medium without lactate and spiked with 100 mg/L of paracetamol, the pH also remained neutral (7.0±0.2) in all cultures and the redox potential (E_h) in the inoculated tests also evolved to values between -350 and -400 mV, which are optimal conditions for SRB. However, the concentration of sulphate only decreased 25%, indicating low SRB activity. In any case, the values of paracetamol removal achieved were still low (19±2%) and only slightly higher than in the non inoculated negative controls (12±2%). Nevertheless, this small difference achieved after 14 days of incubation was significant (for 5% error).

These to some extent higher removals of paracetamol observed in the tests inoculated with the SRB enriched consortium compared to the respective non inoculated negative controls may indicate the existence of mechanisms associated with biological activity contributing to paracetamol removal. However, it remains unknown whether paracetamol was degraded via metabolic pathways, or if it reacted with compounds released by the active microbial communities. Despite this, it seems that the SRB present in this consortium are not directly involved in the removal because it occurred at the same extent both in medium with lactate (with high SRB activity) and without lactate (with low SRB activity). Thus, probably other microorganisms than SRB, eventually less abundant in the inoculum, may have been directly or indirectly responsible for the observed paracetamol removal.

3.2.3 MSM medium

This medium, exclusively composed by inorganic compounds, was used to test paracetamol biodegradation when this drug was the only carbon source available. The putative main electron acceptors of MSM are oxygen, in the tests under aerobic conditions, and nitrate sulphate and manganese (IV), in the tests under anaerobic conditions. Experiments with MSM were first performed using sludge from the WWTP lagoon system as inoculum for the anaerobic and moderate aerobic conditions. In these tests, minor (< 25%) or no degradation of paracetamol was observed either after 24 or 72 h of incubation with the three concentrations of paracetamol tested (10, 50, and 80 mg/L). Moreover, in these conditions the non significant (for 5% error) and small differences (<10%) in paracetamol removal obtained between the negative controls and the inoculated cultures, reinforce the idea that there was no degradation, or just small degradation, due to biological activity, as observed in the experiments with TGM.

The partial removals of paracetamol achieved when the WWTP lagoon sludge was used as inoculum in TGM and MSM under anaerobic and moderate aerobic conditions and the negligible contribution of the inoculum in these removals suggest that the conditions of growth and/or the nature of the inoculum probably hampered the biodegradation of the total amount of the drug in the tested cultures. Therefore, trying to improve paracetamol biodegradation, further experiments were carried out in highly oxygenated (aerated) aerobic conditions and using as inoculum sludge from a WWTP's oxidation ditch. One concentration of paracetamol (50 mg/L) was tested in aerated MSM inoculated with this sludge. After 24 h of incubation only 3±3% of the drug was removed, which was not significantly different (for 5% error) from the 1±1% removal observed in the negative control. However, after 72 h of incubation a removal of 97±2% was achieved, while practically no removal (3±8%) was still observed in the non-inoculated negative controls, indicating that the major cause of paracetamol removal was due to inoculation with sludge. An additional test with autoclaved sludge in MSM was performed to evaluate the possible adsorption of paracetamol to sludge. The results revealed just a small drug removal (15±2%), reinforcing the idea that the major cause of paracetamol removal using MSM medium in highly oxygenated conditions was the biological activity of aerobic microorganisms.

3.2.4 MWW Media

MWW was used in order to mimic the real conditions in WWTPs. It was tested just under aerobic conditions, in which the main electron accepter is oxygen. Cultures with 50 mg/L paracetamol were tested under moderate aerobic conditions and highly oxygenated aerobic conditions (as described above) using sludge from the WWTP oxidation ditch as inoculum. After 24 h incubation just 11±10% paracetamol was removed from MWW without aeration and 70±10% was removed with aeration, whereas after 72 h the removal was nearly complete in both cases: 88±7% and 99.9±0.2%, respectively (Table 1). In this experiment, negative controls without sludge were not carried out because the MWW is itself a source of microbial inoculum, but a test with both autoclaved MWW and sludge was performed. The low paracetamol removal observed in that test (6±2% and 11±5% for 24 h and 72 h incubation, respectively) is significantly different from that achieved in the tests with active sludge, which indicates that biological degradation was the main mechanism involved in paracetamol removal using MWW medium under highly oxygenated conditions, as happened in the tests with highly oxygenated MSM described above.

3.2.5 General analysis of biodegradation experiments

The results suggest that paracetamol removal in the assays under anaerobic and moderate aerobic conditions using sludge from the Faro East WWTP lagoon system was mainly due to other causes than microbial activity: removal was always below 60% and was, in general, similar to, or weakly higher than, the obtained in the respective negative controls (without microbial inoculum). Moreover, the results of the non inoculated tests show higher removals for TGM (removals up to 43±12%) and modified Postgate B medium

(removals up to 39±5%) than for the simpler MSM medium (removals below 17±6%), suggesting that some components in the most complex media were able to chemically react with paracetamol, contributing for its removal. One non biological mechanism that can account for the observed losses of paracetamol in all tested media is its oxidation by SO_4^{2-} ions. It is known that compared with hydroxyl radical ($^{\bullet}OH$), SO_4^{2-} is more selective for oxidation by electron-transfer reaction and more powerful for the decomposition of contaminants at neutral pH (Mezyk et al. 2011; Zhang et al. 2015). Indeed, when peroxymonosulfate (PMS) is activated by catalysts it decomposes generating SO_4^{2-}, which degrades pollutants. Thus, several catalysts have been explored for PMS oxidation and some attracted great interest due to its remarkable separation and catalysis, such as spinel ferrites of MFe_2O_4 (M = Fe, Mn, Co, Ni, and Cu), (Tan et al. 2017). Another mechanism putatively accounting for the decrease of paracetamol detection in the experiments with TGM (including in the non inoculated controls) is the possible formation of protein-paracetamol complexes. It has been shown that paracetamol and serum proteins form complexes (*e.g.* Daneshgar et al. 2009) and the TGM has beef extracts in its composition.

On other hand, the results from the assays under moderate aerobic and highly aerobic conditions inoculated with sludge from the Faro North WWTP oxidation ditch indicate that in this case aerobic microbial activity played a major role in the removal of paracetamol: (i) the final removal of paracetamol was always higher than 85% in the inoculated tests while in the respective negative controls (without sludge inoculum or with autoclaved sludge) it was small (< 20%) and (ii) within tests with the same media (MSM or MWW) the removal of paracetamol was faster under highly aerobic conditions (aerated cultures) than in the moderate aerobic conditions (non-aerated open cultures). Thus, in these assays paracetamol concentrations were also studied in samples collected after 48 h of incubation for a better analysis of its degradation over time. With that it was possible to verify that in the inoculated MWW under aeration, paracetamol removal was already complete (99.1±0.4%) after 48 h, which did not happen in the non aerated MWW cultures, neither in both the aerated and non aerated MSM cultures (Figure 3). That may be attributed to three causes: (i) MWW was itself an additional source of microorganisms involved in the biodegradation of paracetamol; (ii) MWW contained compounds, not present in MSM, which play a role in biological pathways contributing to the biodegradation of this drug and (iii) MWW contained compounds, also not present in MSM, which participate in chemical reactions contributing to a non-biological removal of the drug.

Though the biomass growth was not analyzed during the biodegradation tests, it was evaluated by optical density measurements at the beginning and at the end of the inocula enrichment in TGM (a rich medium for anaerobic and aerobic microorganisms) and during that period roughly similar growths were observed in the different redox conditions (as described in section 2.2). Taking that into account, together with the fact that roughly similar biomasses were inoculated in all tests, it can be assumed that at least for the tests with TGM the bacterial growth under anaerobic conditions was not a limiting factor. However, when MWW was used, the lower paracetamol degradation rates observed under low oxygenic conditions (27.5±2.2% DO) compared with those observed under high oxygenic conditions (95.0±1.3% DO) were probably the result of

slower biomass growth rates in the former compared with the second. This can be considered expected due to the fact that in high oxygenic conditions the complex organic molecules present in MWW are faster oxidized and transformed into simpler carbon sources suitable for bacteria.

3.3 Identification of paracetamol metabolic products

Due to the fact that the paracetamol secondary metabolites can be persistent and more toxic than the drug itself it is essential to verify their fate in biological systems (Wu et al. 2012; Marchlewicz et al. 2015; Peake et al. 2016; Torun et al. 2015; Mbokou et al. 2016). The major route for biodegradation of paracetamol in microbes was proposed to yield 4-aminophenol and hydroquinone as main intermediates before ring fission and subsequent total mineralization (Wu et al. 2012; Zhang et al. 2013). Moreover, these two metabolites, as well as 1,4-benzoquinone (the oxidized form of hydroquinone), 4-nitrophenol and NAPQI (N-acetyl-benzoquinone imine) have been reported as intermediates/oxidation products in non biological advanced oxidation processes (Bedner et al. 2006; Moctezuma et al. 2012; Postigo and Richardson, 2014).

In this experiment, standards of 4-aminophenol, hydroquinone and 4-nitrophenol were chosen to be used as references for the identification of intermediate products of paracetamol degradation by HPLC analysis. Samples from the experiments with MSM and MWW spiked with paracetamol using sludge from the WWTP oxidation ditch as inoculum revealed high paracetamol removal when the HPLC analysis was conducted using the fast method in isocratic conditions. Therefore, these samples were re-analyzed using an HPLC gradient method that allows a good separation of the metabolites listed above, usually associated to paracetamol degradation (both HPLC methods are described in materials and methods). Two peaks with retention times (RT) corresponding to 4-aminophenol and hydroquinone standards, and another with a RT not matching any of the tested standards (herein referred to as unknown) were detected in samples from the tests in MWW and MSM inoculated with sludge (Figure 4). The two peaks corresponding to the paracetamol secondary metabolites 4-aminophenol and hydroquinone, as well as the unknown peak, emerged only in samples from cultures in which the concentration of paracetamol decreased. Moreover, they were neither detected at the beginning of the experiment nor in samples from the respective negative controls (without inoculum). This clearly indicates that biological activity is related to the appearance of these metabolites and that they are products from the degradation of paracetamol.

The peak corresponding to 4-aminophenol (RT = 4.1 min) and the unknown peak (RT = 8.8 min) were detected in all sampling days (Figure 5), whereas the peak corresponding to hydroquinone (RT = 5.3 min) was only occasionally detected with relatively small peak areas: an area of 40.7±3.0 mAUs was detected just in samples from tests with MWW inoculated with aerobic sludge and spiked with paracetamol collected after two days incubation under moderately oxygenated conditions. The occurrence of 4-aminophenol in all inoculated cultures and not in the negative controls (without sludge as inoculum) supports the major biodegradation pathway of paracetamol proposed for microbes by other authors: this drug is metabolized to produce 4-aminophenol, which is converted to hydroquinone through replacement of the amino group by a

hydroxyl group, being then this aromatic compound the precursor of several carboxylic acids (2-hexenoic acid, succinic acid, malonic acid, oxalic acid and finally formic acid) (Hu et al. 2013; Wu et al. 2012; Zhang et al. 2013). The rare detection of hydroquinone in these experiments can be due to its fast degradation into those simpler molecules, or because its main production occurred in a later phase of the culture through the biodegradation of 4-aminophenol.

3.4 Effect of paracetamol on COD degradation

COD is an important water quality parameter providing an index to determine the effect an effluent will have on the receiving water body. It is therefore a major reference in the control of wastewater discharges. For example in Portugal the general COD emission limit value for wastewater discharges is 150 mg O_2/L (Decree-Law nº 236/98 of 1 August) and the limit for discharges from urban WWTP is 125 mg O_2/L (Decree-Law nº 152/97 of 19 June).

In this study the effect of paracetamol on the removal of COD was also addressed. COD was measured in the biodegradation experiments with MWW and MSM using the sludge from the WWTP oxidation ditch as inoculum in the beginning of incubation and after 72 h (Figure 6).

The COD for MWW inoculated with sludge was 940 mg O_2/L, while after addition of 50 mg/L paracetamol was 1095 mg O_2/L. In MSM inoculated with sludge and with 50 mg/L paracetamol COD was 503 mg O_2/L, while in the absence of sludge it was 154 mg O_2/L. This led to the following observations: (i) the tested wastewater had a COD of approximately 590 mg O_2/L, (ii) the sludge inoculum contributed to an increase of approximately 350 mg O_2/L of COD, and (iii) a paracetamol concentration of 50 mg/L generates approximately a COD value of 155 mg O_2/L. After 72 h of incubation the COD values dropped to about 20% of the initial values in both media (MWW and MSM) inoculated with sludge, independently of the presence or absence of 50 mg/L paracetamol, which suggests that the degradation of organic matter achieved with aerobic sludge was not affected by the presence of paracetamol up to this concentration (50 mg/L). This was expected as the IC_{50} values of this drug for bacterial growth, estimated in this work, are around 6 g/L. In contrast, for MSM with 50 mg/L of paracetamol but without sludge inoculum, the COD values after 72 h remained equal to their initial values, confirming the results which showed no removal of paracetamol in this negative control. The final COD values in the tests with MWW varied between 116 and 290 mg O_2/L, with averages around 200 mg O_2/L, which is slightly above the regulatory limit of 125 mg O_2/L for discharges from urban WWTP (Decree-Law nº 152/97 of 19 June). This can be justified the presence of slow degradable organic compounds from the MWW still in solution after 72 h incubation, together with the intermediate metabolites generated from paracetamol degradation, such as the 4-aminophenol and the unknown compound detected in cultures.

3.5 Bacterial communities degrading paracetamol

Aiming to identify bacteria able to degrade paracetamol, thus with potential for biotechnological applications to improve the removal of this drug from wastewaters, microbial communities were studied on samples from the experiment with aerated cultures of inoculated MSM spiked with 50 mg/L paracetamol, in which high removal rates of this drug were observed. The three replicate cultures of this test revealed identical behaviors for the concentrations of the analyzed compounds along the experiment. Thus, it was considered that the microbial dynamics studied through massive sequencing of 16S rRNA genes present in just one of the replicates along the incubation time would provide enough robust evidences to identify taxa putatively involved in the degradation of paracetamol and/or its metabolites. The study was focused on bacteria using primers for the 16S rRNA gene region V1-3. Samples for microbial community analysis were collected from the WWTP oxidation ditch sludge used as inoculum and from cultures with 1, 2, 3 and 6 days of incubation after inoculation.

DNA sequencing of 16S rRNA amplicons targeting the V1-V3 variable regions was used for taxonomic classifications and to count the numbers of 16S rRNA genes in the samples, which were then used to estimate the relative abundances of bacteria. The number of reads analyzed on each sample (from 66927 to 106904) allowed following the evolution of main taxa from the inoculum towards new dynamic equilibriums (probably with mutualistic relationships) in a medium with paracetamol as the only source of energy (Table 2; Online Resources 1 and 2). This evolution in the diversity seems to reflect shifts in the bacterial population caused by a trend towards the extinction of strains not capable of degrading paracetamol or the intermediates of its degradation and the survival of strains putatively with that capacity.

Within the 25 most abundant bacterial groups, the gradual decreases in percentages of 16S rRNA gene amplicons from genera *QEDR3BF09*, *CYCU-0281*, *Azospira*, *Comamonas*, *MK04* and *Arcobacter* over the incubation time of the experiment suggest that the representatives of these genera in this population were not able to use paracetamol, or the products of their degradation as sources of energy.

On the other hand, the percentages of 16s rRNA gene amplicons classified in the genera *PHOS-HE31*, *Uliginosibacterium*, *188up*, *PHOS-HE28*, *Hyphomicrobium*, *SBR1029*, *Candidatus Odyssella*, *Opitutus* and in two groups from families *Comamonadaceae* and *PHOS-HE51* not classified by genus, showed a rising trend followed by decline throughout the experiment. These amplicons may be from bacteria that have used as nutrients the products generated by the degradation of other microorganisms in the culture which became perishable as the conditions changed ceasing to be viable for them.

Finally, the continuing raise of 16S rRNA gene amplicons relative abundances from some taxonomic groups along the whole experiment (genera *Pseudomonas*, *Flavobacterium*, *Dokdonella*, *Dechloromonas*, *Methylophilus*, *Achromobacter*, *Acidovorax*, *Lautropia* and a group of uncultured bacteria from family *Cytophagaceae*) indicates the presence of bacterial strains from these groups probably capable of metabolizing paracetamol and/or its degradation intermediates to obtain energy. Among these, the genera *Pseudomonas*, *Flavobacterium*, *Dokdonella* and *Methylophilus* were in evidence with final abundances of 21.2%, 6.91%, 3.8% and 3.83%, respectively.

Within the genus *Pseudomonas*, OTU_7 (GenBank accession MG554751) stands out with an increase from the inoculum to the sixth day from 0.04% to 20.63%, while for the other seven OTUs of this genus there were no increases or increases were less than 0.3% (Online Resource 1). For OTU_7 the highest increases were from the second to the third day, *i.e.* when the largest decrease in paracetamol concentration occurred (Figure 3: MSM + sludge + air) and between the third and the sixth day, *i.e.* when there was no paracetamol but intermediate products of its degradation were available, such as those analyzed in this work: 4-aminophenol and an unidentified product (Figure 5: MSM + sludge + air). This suggests that, most probably, the bacteria corresponding to OTU _7 had an important contribution both in the first phase of paracetamol biodegradation and in the subsequent phase of biodegradation of the products generated in the first phase. These results together with other results previously reported (Khan et al. 2006; De Gusseme et al. 2011; Zhang et al. 2013; Karaman et al. 2016) reinforce the idea that some *Pseudomonas* strains have the ability to metabolize paracetamol and the intermediate of its degradation 4-aminophenol as the sole source of energy. With the RDP classifier (Wang et al. 2007) the classifications were just up to the genus (Table 2), so for OTUs putatively corresponding to bacteria with a role in the degradation of paracetamol an attempt was made to classify the species by alignment with BLASTN (Zhang et al. 2000) in the database rRNA_typestrains/prokaryotic_16S_ribosomal_RNA. However, it was not possible to point out a more probable species to which OTU_7 corresponded because its alignment revealed sequences of 8 different species of *Pseudomonas* with more than 99% coverage (query cover) and 99% similarity (identities) (*P. umsongensis, P. helmanticensis, P. taiwanensis, P. vancouverensis, P. graminis, P. entomofila, P. monteilii, P. lutea*), as well as a series of sequences of other species of this genus fully aligned and with similarities of 98%. Curiously, among these there was no sequence of *P. aeruginosa*, a species identified by other authors as having the ability to degrade paracetamol (De Gusseme et al. 2011; Karaman et al. 2016). Thus, the results here presented suggest that others species of this genus may have also that ability.

In the genus *Flavobacterium*, 47 OTUs were identified, but only one (OTU_20; GenBank accession MG554764) was noted for percentage increase over the experiment, while in the genus *Methylophilus* only one OTU was identified (OTU_23; GenBank accession MG554767). In these cases, the highest increases in the percentage occurred after the third day, *i.e.* when paracetamol was not available but only metabolites of its degradation. Trends in decreasing concentrations of 4-aminophenol and the unidentified product observed from the second to the third day of incubation (Figure 5: MSM + sludge + air) indicate the beginning of a phase of the culture in which these products began to be degraded due to the proliferation of bacteria capable of metabolizing them.

This is not the first time that microbial populations exposed to a pharmaceutical led to shifts towards an evident selection of *Flavobacterium* species. For example, in cultures acclimated with the antibiotic enrofloxacin a selection of the genera *Flavobacterium* was observed with its relative abundance increasing 20.8% (Alexandrino et al. 2017). In fact, by the end of the 70's it had already been described the isolation of a *Flavobacterium sp*. capable of degrading the herbicide asulam and its structure analogous sulphanilamide

in a synthetic medium with no other carbon sources added (Walker, 1978). Then, the isolation of plasmids carrying genes for degradation of aromatic compounds (Chaudhry and Huang, 1988) and the purification of enzymes capable of hydrolyzing aromatic compounds (Van Berkel and Van Den Tweel, 1991) from *Flavobacterium* strains, achieved in the subsequent years, clearly showed the potential biotechnological application of those strains for the bioremediation of recalcitrant pollutants. The isolation of *Flavobacterium* strains biodegrading nylon oligomers (Kinoshita et al. 1981) is another evidence of the capacity of these microorganisms to use synthetic recalcitrant compounds as the sole source of carbon. The capacity of biodegrading nylon oligomers was also identified on *Pseudomonas* strains (Kanagawa et al. 1993), which is a clue for the possibility of common metabolic pathways in these two taxonomic groups. A *Flavobacterium sp.* able to use 4-nitrophenol as a carbon and energy source has already been isolated (Raymond and Alexander, 1971) and this compound could also be used as the sole carbon and energy source by a strain of *P. aeruginosa* (Zheng et al. 2009), a specie with strains able to biodegrade paracetamol and 4-aminophenol as described above. Therefore, based on the results here presented it seems reasonable to propose that probably the *Flavobacterium* strains in the culture corresponding to OUT_20 were capable of biodegrading 4-aminophenol. The alignment of OTU_20 in the NCBI database revealed a *Flavobacterium luvivivi* sequence with 100% coverage and 99% similarity and sequences of several species of this genus with similarities of less than 95%.

An association between *Methylophilus* and successful biodegradation of pharmaceuticals using next-generation sequencing of 16S rRNA genes over time, besides the work here presented, was also described recently by Kim et al. (2017), though not for paracetamol. Evidences that *Methylophilus* can grow on methylated amines (mono-, di- and trimethylamine) have been reported (Large and Haywood, 1981). However this genus is known as a group of methanol-utilizing bacteria (Jenkins et al. 1987) and methanotrophs have been studied for their potential to be used directly in bioremediation due to the methane monooxygenase enzyme(s) they possess, which have broad substrate specificity and have been shown to co-oxidise aromatic pollutants (De Marco et al. 2004). For example, a *Methylophilus sp.* isolated from a humic lake degraded phenol and humic matter (Hutalle-Schmelzer et al. 2010). These evidences together with the results of this work support the hypothesis that the *Methylophilus* bacteria corresponding to OTU_23 were also able to metabolize products resulting from the degradation of paracetamol, either the aromatic compounds generated in the first steps of the degradation of this drug, or the simpler compounds subsequently generated. The alignment of OTU_23 in the NCBI database revealed sequences from several *Methylophilus* species with coverages and similarities of less than 97% and two sequences with 98% coverage and 99% similarity, one from *M. methylotrophus* and one from *M. leisingeri*.

In the genus *Dokdonella* 5 OTUs were identified, but also in this case only one (OTU_10; GenBank accession MG554754) stood out in the evolution of relative abundances. The percentage of amplicons in this OTU increased mainly in the first two days of incubation, then remained almost the same from the second to the third day and after six days it increased again. Though the genus *Dokdonella* has not yet been

associated with pharmaceutical degradation it has been detected in alkane degrading cultures (Alonso-Gutiérrez et al. 2009) and a *Dokdonella* potentially degrading polycyclic aromatic hydrocarbons was detected by Bacosa and Inoue (2015). Moreover, *Dokdonella* has been previously isolated from denitrifying environments in the presence of O_2 (Sun et al. 2009) and was one of the predominant microorganisms in a study with activated sludge at 2% O_2 concentration (without inhibitory effects on N_2O biodegradation), in which it was suggested that heterotrophic denitrification was the most likely mechanism of N_2O removal (Figueroa-González et al. 2016). It may therefore be hypothesized that the *Dokdonella* corresponding to OTU_10 may have used the nitrate generated after the release of the amine group from 4-aminophenol (by substitution by a hydroxyl group) as an electron receptor to obtain energy by oxidation of hydroquinone (the aromatic organic compound generated by the amine group release of 4-aminophenol) and/or by oxidation of the simpler carbon compounds generated in the degradation of hydroquinone by other microorganisms (such as *Pseudomonas*). The alignment of OTU_10 in the NCBI database revealed a *Dokdonella immobilis* sequence with 100% coverage and 94% similarity and sequences of several species of different genera with similarities of 92% or less.

4 CONCLUSIONS

Estimated paracetamol IC_{50} values for bacterial growth under anaerobic and aerobic conditions are similar (6.204 and 6.162 g/L, respectively) and much higher than the concentrations of this drug usually detected in WWTP influents and effluents.

In what concerns the biodegradation of paracetamol, the results indicate that aerobic microorganisms had a major role in the degradation of this drug. In municipal wastewater inoculated with sludge from a WWTP´s oxidation ditch paracetamol (50 mg/L) was completely removed after two days incubation in batches with aeration and after three days in open batches without aeration. The metabolites 4-aminophenol and hydroquinone, two known intermediates of paracetamol degradation, plus one compound not identified in this work were produced in the cultures during paracetamol degradation. Moreover, it was observed that 50 mg/L of paracetamol generates a COD of about 155 mg O_2/L and that such a concentration of this drug does not affect the degradation of COD in municipal wastewater with activated sludge based treatments.

Regarding the identification of bacteria with a role in the degradation of paracetamol, this study corroborates previous works reported in literature showing the ability of species from *Pseudomonas* genus to use this drug and intermediates of its degradation as sources of energy and hypothesizes for the first time that species from genera *Flavobacterium*, *Dokdonella* and *Methylophilus* may have the capacity to degrade the metabolites produced from paracetamol's degradation.

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TABLES

Table 1

Conditions			24 hours incubation [a]			72 hours incubation [b]		
Growth media	Oxygenic	Paracetamol concentrations (mg/L)	No sludge *or autoclaved sludge* [c] (negative control)	Sludge from Faro East WWTP anaerobic lagoon	Sludge from Faro Northwest WWTP oxidation ditch	No sludge *or autoclaved sludge* [c] (negative control)	Sludge from Faro East WWTP anaerobic lagoon	Sludge from Faro Northwest WWTP oxidation ditch
TGM	Anaerobic	10 50 80 10000	17 ± 3% 21 ± 5% 2 ± 5% 13 ± 4%	24 ± 9% 22 ± 13% 12 ± 8% 22 ± 14%	NT	43 ± 12% 37 ± 10% 25 ± 9% 11 ± 10%	56 ± 16% 45 ± 20% 33 ± 9% 26 ± 12%	NT
	Aerobic without aeration	10 50 80 10000	40 ± 17% 22 ± 11% 27 ± 6% 22 ± 11%	55 ± 12% 25 ± 2% 33 ± 7% 25 ± 3%	NT	31 ± 8% 25 ± 14% 8 ± 13% 20 ± 11%	45 ± 8% 44 ± 5% 16 ± 5% 22 ± 1%	NT
Modified Postgate B	Anaerobic	10 80 100	5 ± 3% 32 ± 10 % 17 ± 3%	8 ± 2% 44 ± 2% 31 ± 4%*	NT	7 ± 2% 39 ± 5 % 23 ± 2%	18 ± 2%* 51 ± 2%* 33 ± 6%*	NT
Modified Postgate B without lactate	Anaerobic	100	7 ± 3%	10 ± 4%	NT	12 ± 2%	19 ± 2%*	NT
MSM	Anaerobic	10 50 80	0.8 ± 8% NR NR	6 ± 10% NR 8 ± 13%	NT	NR 14 ± 6% 17 ± 6%	6 ± 10% 24 ± 5% 21 ± 4%	NT
	Aerobic without aeration	10 50 80	2 ± 10% NR NR	10 ± 9% 3 ± 4% NR	NT NR NT	2 ± 10% 3 ± 3% 0.4 ± 11%	10 ± 9% 12 ± 3%* 9 ± 5%	NT 97 ± 2%* NT
	Aerobic with aeration	50	1 ± 1% *NR* [c]	NT	3 ± 3%	3 ± 8% *15 ± 2%* [c]	NT	97 ± 3%*
MWW	Aerobic without aeration	50	NT	NT	11 ± 10%	NT	NT	88 ± 7%
	Aerobic with aeration	50	*6 ± 2%* [c]	NT	70 ± 10%*	*11 ± 5%* [c]	NT	99.9 ± 0.2%*

a, b = 7 and 14 days, respectively, for tests with modified Postgate B medium; c = negative controls carried out with autoclaved sludge.
* = paracetamol removal is significantly different (one way ANOVA for 5% error) from the observed in the respective negative control.
NR = no removal; NT = not tested.

Table 2

Phylum	Family	Genus	OTU abundances (%)				
			Inoculum – sludge	MSM Day 1	MSM Day 2	MSM Day 3	MSM Day 6
Proteobacteria	*Pseudomonadaceae*	*Pseudomonas*	**0,11**	**0,06**	**0,10**	**7,86**	**21,2**
Bacteroidetes	*Flavobacteriaceae*	*Flavobacterium*	**1,66**	**2,52**	**2,57**	**3,67**	**6,91**
Proteobacteria	*Comamonadaceae*		**2,86**	**5,28**	**4,68**	**3,55**	**2,68**
Bacteroidetes	*Chitinophagaceae*	*PHOS-HE31*	**2,16**	**3,31**	**4,10**	**3,37**	**2,35**
Bacteroidetes	*PHOS-HE51*		**2,25**	**2,63**	**3,62**	**3,91**	**2,07**
Proteobacteria	*Xanthomonadaceae*	*Dokdonella*	**1,48**	**2,20**	**3,13**	**3,29**	**3,80**
Bacteroidetes	*Saprospiraceae*	*QEDR3BF09*	**6,06**	**2,34**	**1,81**	**1,02**	**0,87**
Bacteroidetes	*Saprospiraceae*	*CYCU-0281*	**3,36**	**2,81**	**2,71**	**1,15**	**1,03**
Proteobacteria	*Rhodocyclaceae*	*Azospira*	**6,03**	**1,26**	**1,29**	**0,81**	**1,52**
Proteobacteria	*Rhodocyclaceae*	*Uliginosibacterium*	**2,67**	**3,43**	**2,13**	**1,96**	**0,23**
Proteobacteria	*Comamonadaceae*	*188up*	**2,81**	**3,30**	**2,55**	**1,00**	**0,62**
Proteobacteria	*Comamonadaceae*	*Comamonas*	**0,44**	**0,12**	**0,15**	**0,10**	**0,20**
Bacteroidetes	*NS9 Marine group*	*PHOS-HE28*	**1,63**	**1,89**	**2,40**	**1,65**	**1,61**
Bacteroidetes	*Saprospiraceae*	*MK04*	**2,42**	**2,20**	**2,14**	**0,64**	**1,16**
Proteobacteria	*Rhodocyclaceae*	*Dechloromonas*	**0,92**	**1,35**	**0,97**	**2,15**	**1,94**
Proteobacteria	*Methylophilaceae*	*Methylophilus*	**0,00**	**0,08**	**0,34**	**0,43**	**3,83**
Proteobacteria	*Alcaligenaceae*	*Achromobacter*	**0,00**	**0,00**	**0,00**	**0,00**	**0,05**
Proteobacteria	*Hyphomicrobiaceae*	*Hyphomicrobium*	**0,40**	**2,10**	**2,07**	**1,00**	**1,63**
Proteobacteria	*Comamonadaceae*	*Acidovorax*	**0,75**	**1,18**	**1,05**	**1,18**	**1,74**
Proteobacteria	*Campylobacteraceae*	*Arcobacter*	**2,33**	**0,86**	**0,84**	**1,62**	**0,24**
Proteobacteria	*Burkholderiaceae*	*Lautropia*	**0,58**	**1,19**	**1,52**	**0,91**	**1,55**
Chloroflexi	*Anaerolineaceae*	*SBR1029*	**0,26**	**1,90**	**1,60**	**0,71**	**0,21**
Bacteroidetes	*Cytophagaceae*	*uncultured*	**0,28**	**0,79**	**0,97**	**1,18**	**1,73**
Proteobacteria	*Rickettsiales Incertae Sedis*	*Candidatus Odyssella*	**0,15**	**0,85**	**1,15**	**0,67**	**1,94**
Verrucomicrobia	*Opitutaceae*	*Opitutus*	**0,69**	**1,05**	**1,07**	**1,50**	**0,47**

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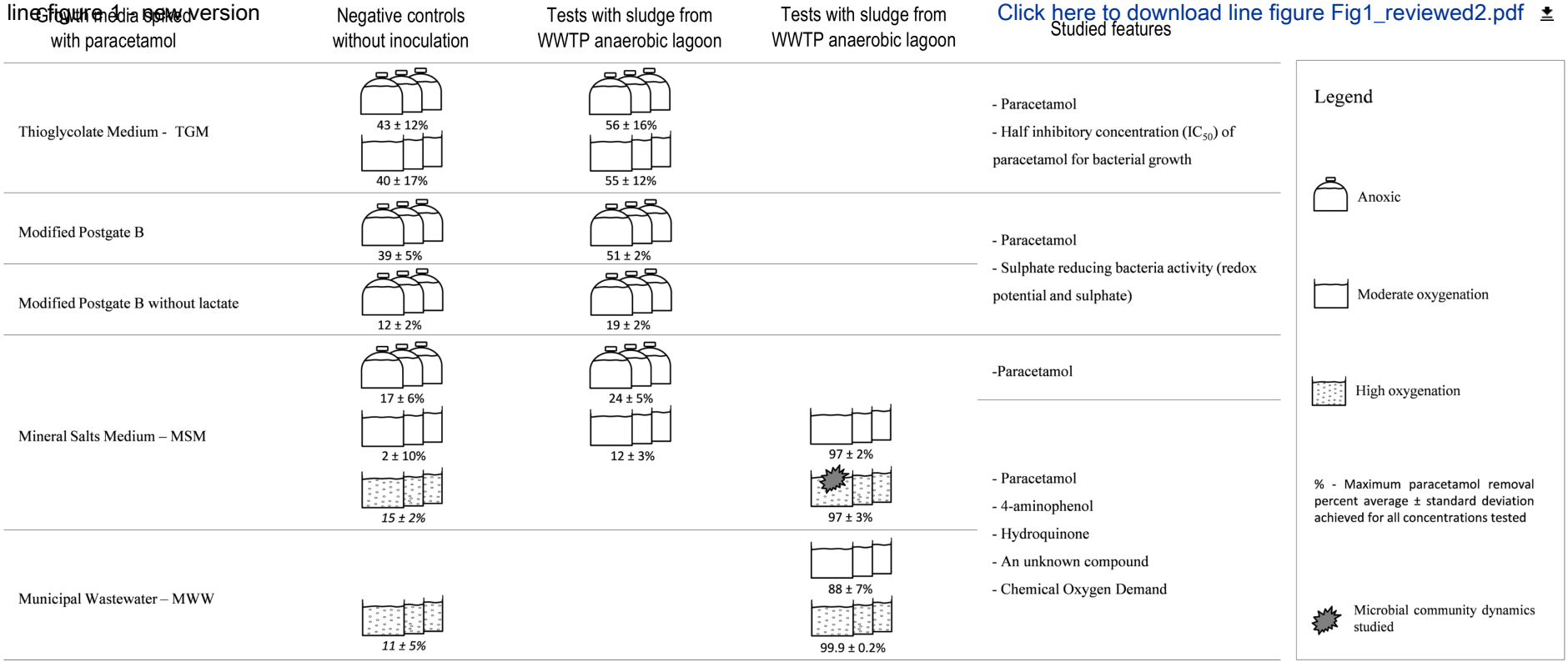

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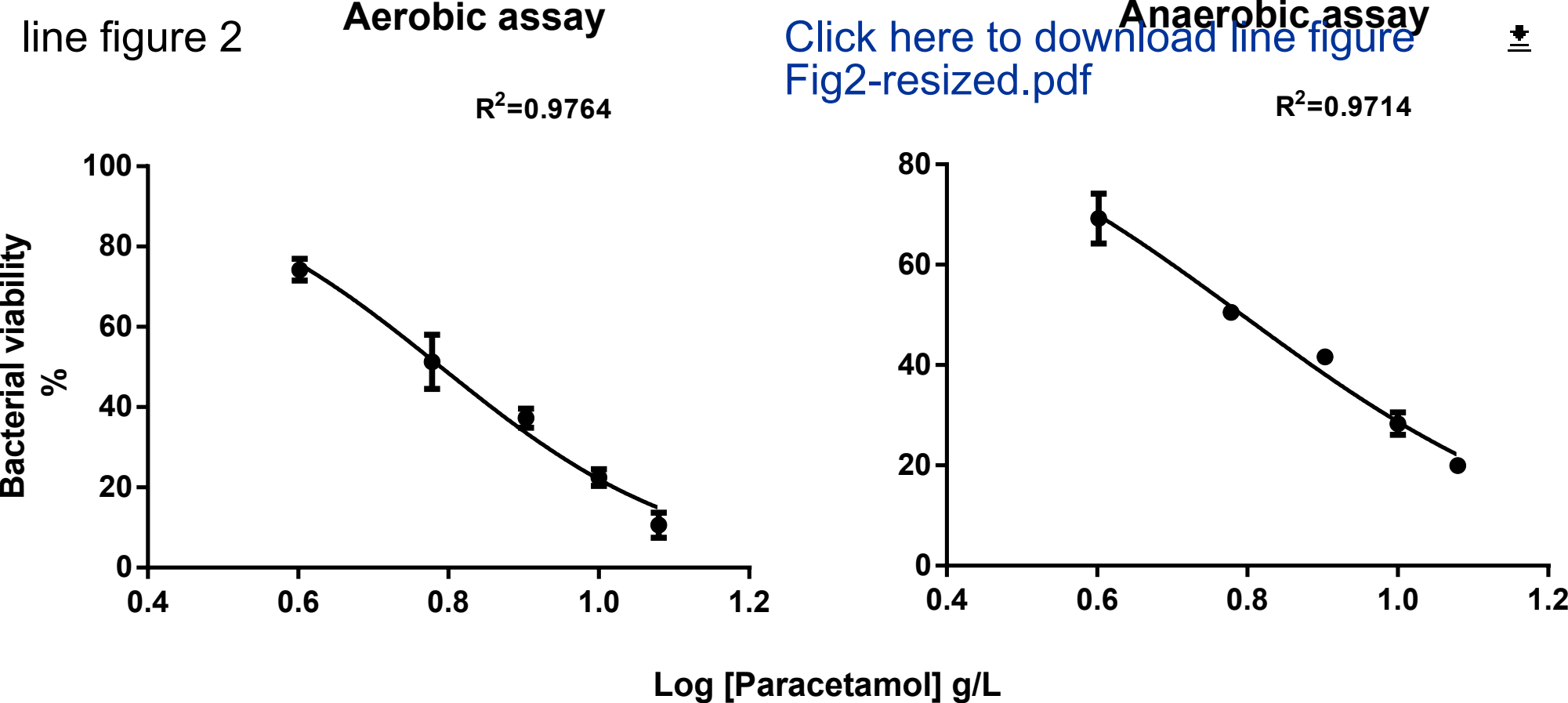

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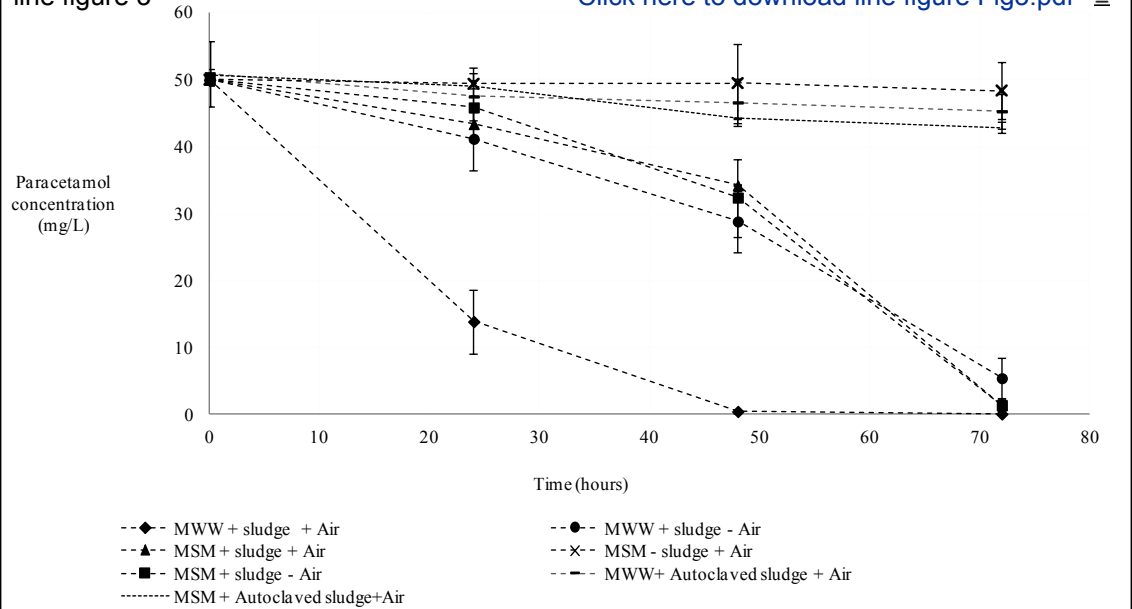

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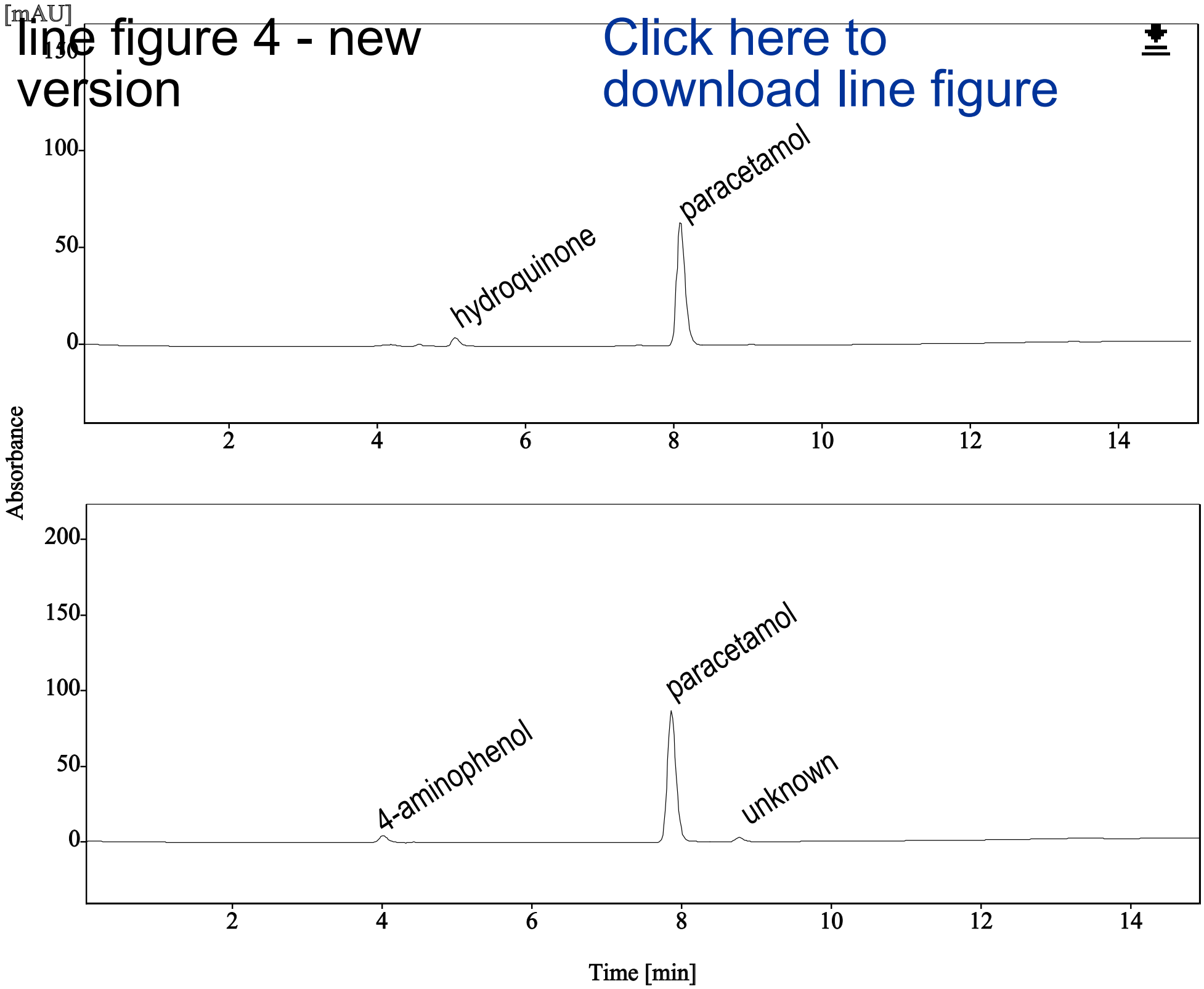

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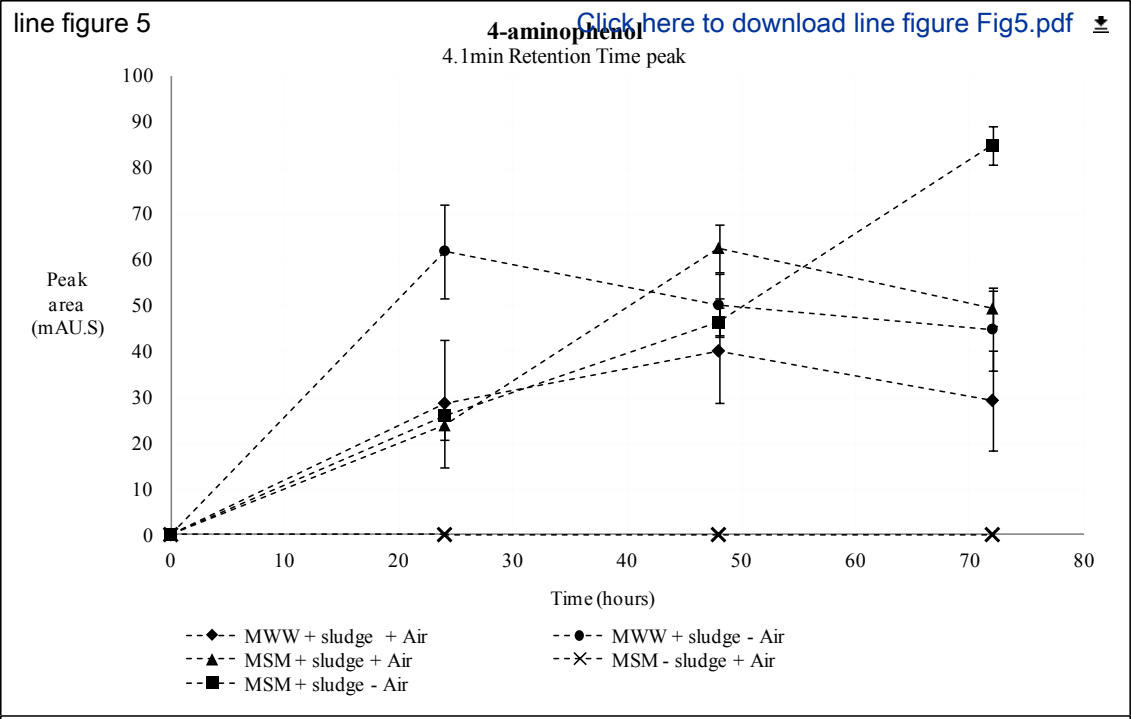

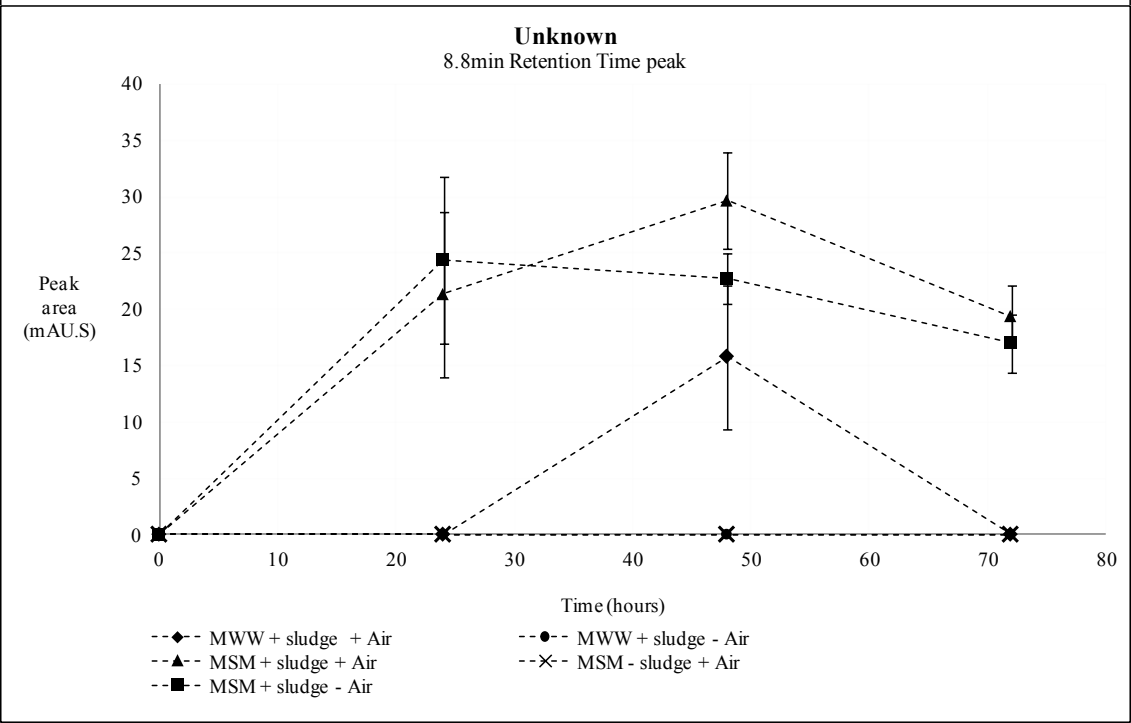

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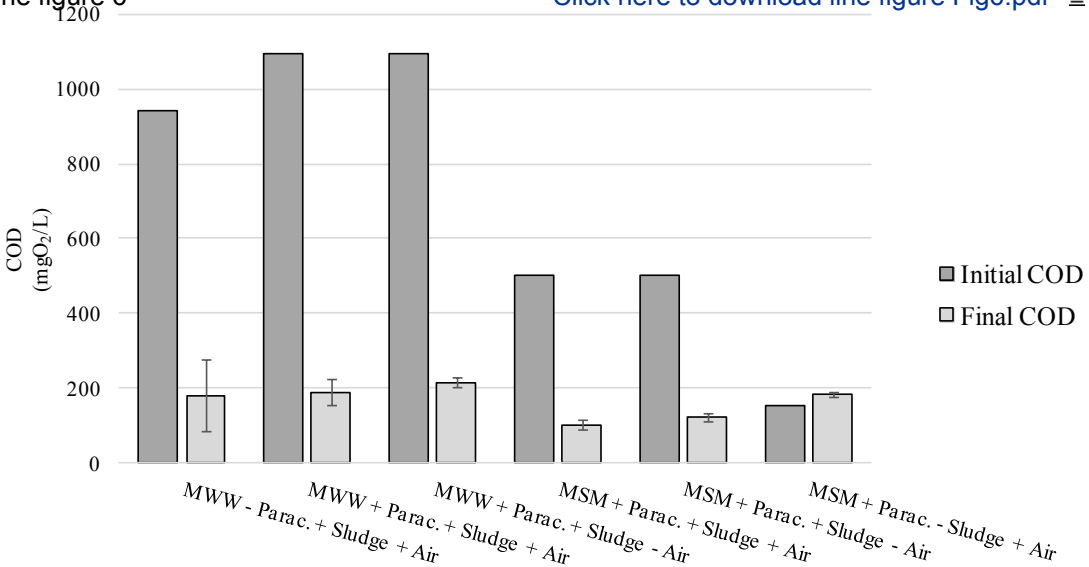

CAPTIONS

Fig. 1 Graphical representation of experiments carried out and features studied.

Fig. 2 Graphical representation of best fitting sigmoidal curves for bacterial viability (%) as a function of logarithm of paracetamol concentration (g/L), for aerobic and anaerobic assays, with values expressed as mean ± distance to individual data points (n=2). The percentage of bacterial viability is the optical density at 600 nm (OD_{600}) measured in a culture with a certain drug concentration divided by the OD_{600} in the control culture without drug, multiplied by 100. In some points the bars are smaller than the symbol, therefore not visible.

Fig. 3 Profiles of paracetamol concentration as a function of time in the biodegradation experiments with MSM and MWW using sludge from the WWTP oxidation ditch as inoculum. Inoculated cultures (+ sludge), negative control without inoculum (– sludge), aerated cultures (+ air) and cultures in open flasks but without aeration (– air). Values are expressed as the mean ± standard deviation (n=3). In some points the bars are smaller than the symbol, therefore not visible.

Fig. 4 Chromatograms obtained by HPLC exemplifying the identification of putative paracetamol biodegradation products in MWW (up) and MSM (down) inoculated with sludge from the WWTP oxidation ditch and containing 50 mg/L paracetamol, collected after two days incubation under aerobic conditions. The retention times are approximately: 4.1, 5.3, 8.0 and 8.8 min for 4-aminophenol, hydroquinone, paracetamol and an unknown product, respectively. Vertical axis = Area (mAUs); horizontal axis = Time (minutes).

Fig. 5 Profiles of 4-aminophenol (up) and one unknown metabolite (down), the two putative products from paracetamol biodegradation detected throughout the experiments with MSM and MWW using sludge from the WWTP oxidation ditch as inoculum. Inoculated cultures (+ sludge), negative control without inoculum (– sludge), aerated cultures (+ air) and open flasks cultures without aeration (– air). Values are expressed as the mean ± standard deviation (n=3). In some points the bars are smaller than the symbol, therefore not visible.

Fig. 6 COD values measured in the experiments with MWW and MSM using the sludge from the WWTP oxidation ditch as inoculum. Initial and final values were obtained just after inoculation and after 72 h of incubation, respectively. Medium with 50 mg/L paracetamol (+ Parac), medium without paracetamol (- Parac) inoculated cultures (+ sludge), negative control without inoculum (– sludge), aerated cultures (+ air), cultures in open flasks but without aeration (– air). Final values are expressed as the mean ± standard deviation (n=3).

Table 1. Percentages of paracetamol removal in each assay after 24 and 72 hours of incubation (7 and 14 days, respectively, for tests with modified Postgate B medium). Values are expressed as the Mean ± Standard Deviation (n=3)

Table 2. The 25 most abundant bacteria (in percent) in samples collected along the experiment with aerated cultures using MSM spiked with 50 mg/L paracetamol inoculated with 10% (v/v) sludge from the Faro

Northwest WWTP's oxidation ditch. Each bacterium has both a broad group name (Phylum) and more specific names (Genus and/or family)

Supplementary Electronic Material. Percentages of 16S rRNA amplicons per OTU for the 25 most abundant bacteria, taxonomic classifications assigned using the RDP classifier as described in Materials and methods

paracetamol biodegradation

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Percentages of 16S rRNA amplicons per OTU for the 25 most abundant bacteria					Taxonomic classifications assigned using the RDP classifier as described in materials and methods.						OTU (see GenBank accession numbers in Online Resource 2)
Inoculum - sludge from WWTP oxidation ditch	Culture in MSM - Day 1	Culture in MSM - Day 2	Culture in MSM - Day 3	Culture in MSM - Day 6	Kingdom	Phylum	Class	Order	Family	Genus	
0.00	0.00	0.02	0.08	0.05	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_144
0.01	0.00	0.00	0.46	0.21	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_1497
0.03	0.02	0.01	0.14	0.27	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_16
0.00	0.00	0.00	0.00	0.01	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_166
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_1694
0.01	0.01	0.01	0.01	0.00	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_1739
0.01	0.01	0.00	0.01	0.02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_67
0.04	**0.00**	**0.04**	**7.17**	**20.63**	**Bacteria**	**Proteobacteria**	**Gammaproteobacteria**	**Pseudomonadales**	**Pseudomonadaceae**	**Pseudomonas**	**OTU_7**
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1030

0.01	0.00	0.01	0.01	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1043
0.00	0.01	0.00	0.00	0.02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1081
0.00	0.00	0.00	0.01	0.58	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_112
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1162
0.00	0.00	0.00	0.00	0.03	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1203
0.23	0.25	0.19	0.22	0.15	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_130
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1322
0.00	0.00	0.00	0.00	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1376
0.01	0.01	0.01	0.01	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1378
0.00	0.00	0.00	0.00	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1419
0.01	0.02	0.02	0.02	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1506
0.00	0.00	0.00	0.00	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1507
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1540
0.00	0.00	0.00	0.01	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1636
0.00	0.00	0.00	0.01	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1672
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1687
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1755
0.01	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1788
0.00	0.00	0.00	0.01	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1845
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1924
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1992
0.17	**0.63**	**0.93**	**1.67**	**4.89**	**Bacteria**	**Bacteroidetes**	**Flavobacteriia**	**Flavobacteriales**	**Flavobacteriaceae**	**Flavobacterium**	**OTU_20**
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2141
0.00	0.00	0.01	0.01	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2179
0.00	0.00	0.00	0.01	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2185
0.26	0.41	0.35	0.38	0.25	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_220
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2383
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2388
0.00	0.00	0.00	0.00	0.02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2538
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2595
0.00	0.00	0.00	0.00	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2655
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2711
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2807

0.11	0.13	0.12	0.09	0.06	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_281
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_436
0.09	0.02	0.01	0.02	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_451
0.02	0.05	0.04	0.10	0.12	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_453
0.03	0.03	0.05	0.03	0.02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_525
0.02	0.02	0.02	0.01	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_562
0.61	0.82	0.73	0.94	0.62	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_60
0.01	0.02	0.02	0.02	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_602
0.02	0.02	0.02	0.03	0.02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_776
0.00	0.02	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_803
0.01	0.01	0.02	0.02	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_804
0.00	0.00	0.00	0.00	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_861
0.03	0.01	0.01	0.01	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_976
2.86	5.28	4.68	3.55	2.68	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		OTU_6
1.28	2.10	2.63	1.80	1.18	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	PHOS-HE31	OTU_15
0.46	0.74	0.94	1.04	0.86	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	PHOS-HE31	OTU_239
0.43	0.48	0.53	0.53	0.31	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	PHOS-HE31	OTU_82
2.25	2.63	3.62	3.91	2.07	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	PHOS-HE51		OTU_9
1.41	**2.11**	**3.01**	**3.16**	**3.67**	**Bacteria**	**Proteobacteria**	**Gammaproteobacteria**	**Xanthomonadales**	**Xanthomonadaceae**	**Dokdonella**	**OTU_10**
0.00	0.01	0.01	0.01	0.01	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dokdonella	OTU_1065
0.00	0.00	0.01	0.01	0.02	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dokdonella	OTU_1410
0.07	0.08	0.10	0.10	0.10	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dokdonella	OTU_245
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dokdonella	OTU_2759
4.97	1.70	1.25	0.58	0.57	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_11
0.01	0.01	0.01	0.06	0.02	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_1436
0.00	0.00	0.01	0.04	0.02	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_1580
0.41	0.17	0.13	0.07	0.05	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_173
0.53	0.23	0.24	0.06	0.05	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_182
0.09	0.09	0.10	0.03	0.03	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_288
0.02	0.09	0.06	0.06	0.06	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_318
0.03	0.05	0.03	0.12	0.09	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_336
0.29	0.26	0.24	0.19	0.16	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_105
0.28	0.24	0.22	0.04	0.13	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_114

0.01	0.00	0.01	0.00	0.00	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_1165
0.27	0.25	0.21	0.08	0.04	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_133
0.30	0.21	0.22	0.13	0.08	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_158
0.23	0.11	0.13	0.06	0.04	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_196
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_1966
0.69	0.64	0.74	0.23	0.29	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_41
0.68	0.58	0.48	0.14	0.23	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_72
0.02	0.01	0.00	0.01	0.00	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_828
0.59	0.51	0.44	0.26	0.05	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_90
0.00	0.02	0.02	0.10	0.02	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azospira	OTU_26
6.03	1.25	1.27	0.71	1.50	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azospira	OTU_29
0.03	0.00	0.00	0.01	0.00	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Uliginosibacterium	OTU_1154
0.00	0.00	0.00	0.01	0.01	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Uliginosibacterium	OTU_1337
2.63	3.43	2.13	1.94	0.22	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Uliginosibacterium	OTU_17
2.06	2.17	1.63	0.71	0.45	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	188up	OTU_32
0.75	1.13	0.92	0.29	0.17	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	188up	OTU_944
0.01	0.00	0.01	0.02	0.06	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_123
0.01	0.01	0.00	0.00	0.01	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_1379
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_2476
0.00	0.00	0.00	0.00	0.01	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_2666
0.40	0.09	0.11	0.05	0.04	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_308
0.01	0.01	0.01	0.01	0.07	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_33
0.02	0.01	0.02	0.01	0.01	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_551
0.34	0.27	0.35	0.30	0.18	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_117
0.00	0.00	0.00	0.01	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_1487
0.27	0.44	0.57	0.36	0.45	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_215
0.13	0.23	0.22	0.16	0.15	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_232
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_2349
0.11	0.13	0.13	0.11	0.09	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_241
0.16	0.13	0.21	0.14	0.11	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_478
0.35	0.34	0.54	0.38	0.43	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_73
0.27	0.34	0.37	0.18	0.20	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_94
2.42	2.20	2.14	0.64	1.16	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	MK04	OTU_13

0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	MK04	OTU_1889
0.17	0.20	0.09	0.22	0.37	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	OTU_202
0.04	0.06	0.07	0.03	0.04	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	OTU_357
0.59	0.76	0.59	0.44	0.80	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	OTU_36
0.01	0.01	0.02	0.01	0.04	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	OTU_749
0.11	0.32	0.20	1.45	0.70	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	OTU_80
0.00	**0.08**	**0.34**	**0.43**	**3.83**	**Bacteria**	**Proteobacteria**	**Betaproteobacteria**	**Methylophilales**	**Methylophilaceae**	**Methylophilus**	**OTU_23**
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	OTU_1102
0.00	0.00	0.00	0.00	0.02	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	OTU_207
0.00	0.00	0.00	0.00	0.03	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	OTU_35
0.02	0.30	0.32	0.21	0.25	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_107
0.10	0.27	0.24	0.05	0.08	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_129
0.05	0.08	0.06	0.05	0.02	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_256
0.03	0.08	0.09	0.04	0.04	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_305
0.02	0.08	0.07	0.01	0.03	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_313
0.14	1.20	1.18	0.57	1.05	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_37
0.02	0.03	0.03	0.02	0.08	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_418
0.02	0.05	0.06	0.02	0.05	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_532
0.00	0.00	0.01	0.01	0.02	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_819
0.00	0.01	0.01	0.01	0.00	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_901
0.61	1.13	1.00	1.12	1.69	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	OTU_332
0.14	0.05	0.06	0.06	0.05	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	OTU_744
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_1356
0.00	0.00	0.01	0.00	0.00	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_2145
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_2324
0.01	0.00	0.00	0.01	0.00	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_2723
0.21	0.06	0.07	0.08	0.03	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_341
0.04	0.01	0.01	0.07	0.01	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_482
1.93	0.73	0.72	1.14	0.19	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_51
0.09	0.02	0.01	0.30	0.01	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_520
0.05	0.03	0.01	0.02	0.01	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_569
0.01	0.01	0.02	0.01	0.00	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_1341
0.08	0.15	0.24	0.10	0.48	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_189

0.41	0.95	1.13	0.73	1.01	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_42
0.04	0.05	0.09	0.04	0.04	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_567
0.02	0.02	0.03	0.02	0.00	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_687
0.01	0.01	0.02	0.01	0.02	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_917
0.00	0.00	0.00	0.02	0.00	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	SBR1029	OTU_1511
0.00	0.00	0.00	0.00	0.00	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	SBR1029	OTU_2778
0.23	1.89	1.57	0.58	0.20	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	SBR1029	OTU_30
0.03	0.01	0.04	0.10	0.01	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	SBR1029	OTU_433
0.28	0.79	0.97	1.18	1.73	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	uncultured	OTU_28
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiales Incertae Sedis	Candidatus Odyssella	OTU_1491
0.00	0.00	0.00	0.00	0.01	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiales Incertae Sedis	Candidatus Odyssella	OTU_1762
0.15	0.85	1.15	0.67	1.93	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiales Incertae Sedis	Candidatus Odyssella	OTU_21
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiales Incertae Sedis	Candidatus Odyssella	OTU_2473
0.00	0.00	0.01	0.01	0.00	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_1283
0.03	0.00	0.01	0.01	0.01	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_1364
0.01	0.00	0.00	0.00	0.00	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_1890
0.00	0.00	0.00	0.00	0.00	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_2002
0.00	0.01	0.00	0.00	0.00	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_2052
0.17	0.14	0.08	0.22	0.05	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_283
0.07	0.11	0.12	0.14	0.03	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_297
0.03	0.07	0.08	0.12	0.03	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_376
0.01	0.01	0.02	0.03	0.01	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_676
0.37	0.71	0.74	0.96	0.34	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_84

Putative role of *Flavobacterium*, *Dokdonella* and *Methylophilus* strains in paracetamol biodegradation

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List of OTUs and respective GenBank Numbers (MG554745 to MG557554):

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