Royal Netherlands Institute for Sea Research

A stable isotope assay for determining microbial degradation rates of plastics in the marine environment

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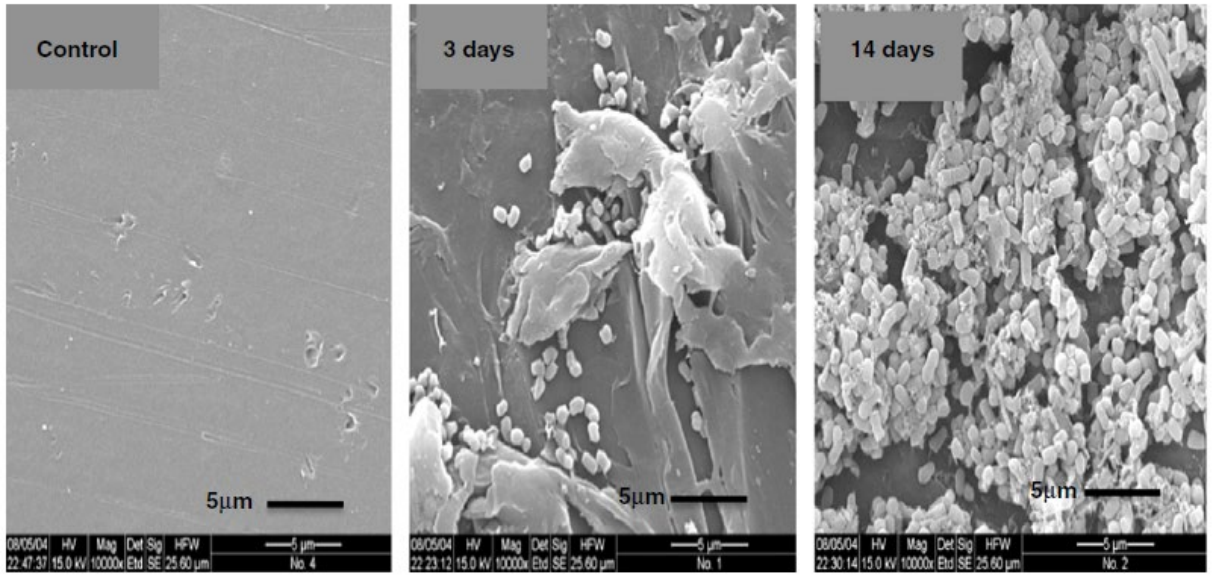
Utrecht University

Why do we think plastic can be degraded by microbes?

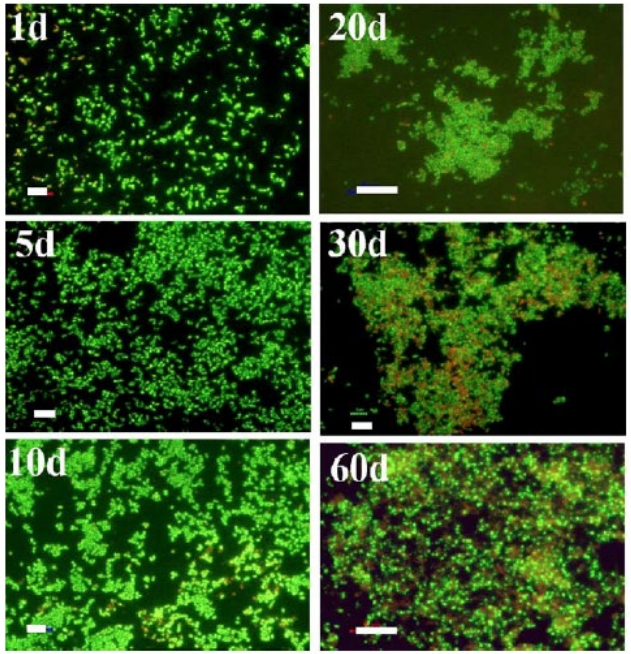
- Plastics are colonized by a diverse mirobial community
- Visualization of microbe-shaped pits in plastic surfaces → Deformations potentialy due to microbes
- *Rhodococcus ruber* found to survive on PE, degradation potential shown by gene analysis
- PETase from *Ideonella sakaiensis* identified and improved by site-directed mutagenesis
- Plastics similar to other types of complex organic matter



Biofilm development while PE is the sole carbon source (Sivan 2011)

Current state of degradation testing methods needs improvement of accuracy

- Gravimetrical (Difference in weight too small to be measured accurately)
- Oxygen consumption during incubation (Indirect method)
- Biofilm development (Not specific enough, opportunists?)
- Chemical changes of plastic (Indirect method)



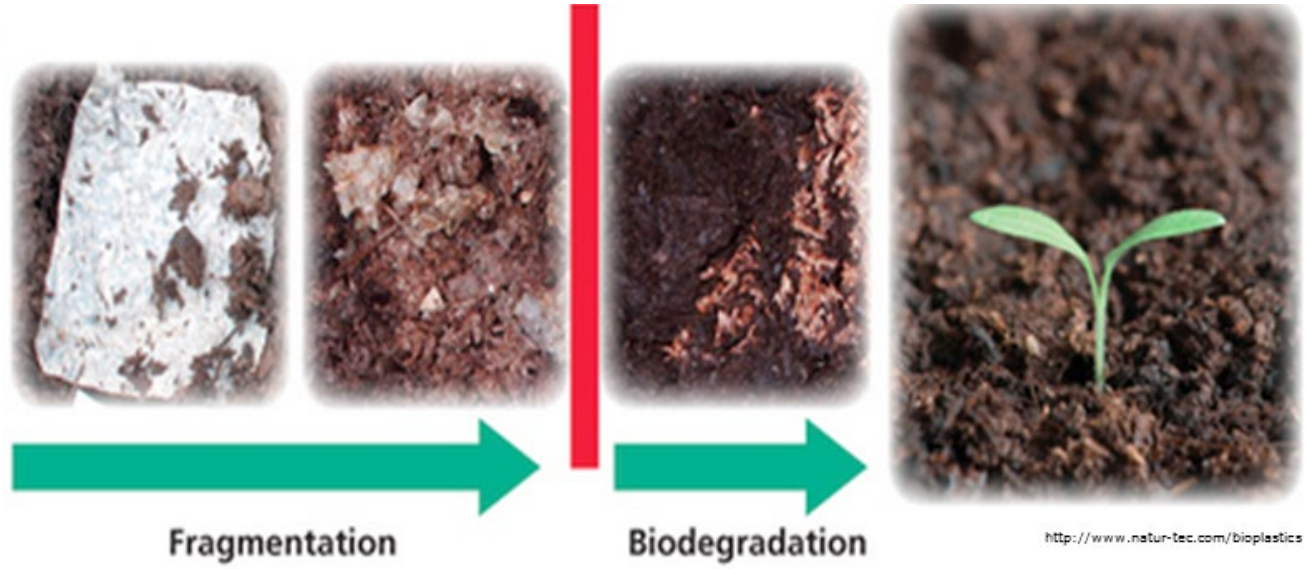
Life/dead stain of biofilm of *R. Ruber* on PE over time (Sivan 2006)

Biodegradation is a potential plastic sink in the marine environment

- Plastic polymers not bioavailable due to size, need to break down (physical and biological) in smaller carbon compounds (shorter carbon chains like monomers) for uptake in cell.

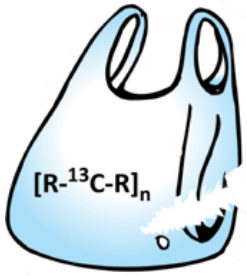
- Biodegradation (metabolization) defined as as full mineralization.
  - Catabolism of carbon compounds (oxidation) results in energy (ATP), H_2O and CO_2: Plastic Polymers → Smaller compounds → H_2O + CO_2 (+CH_4)

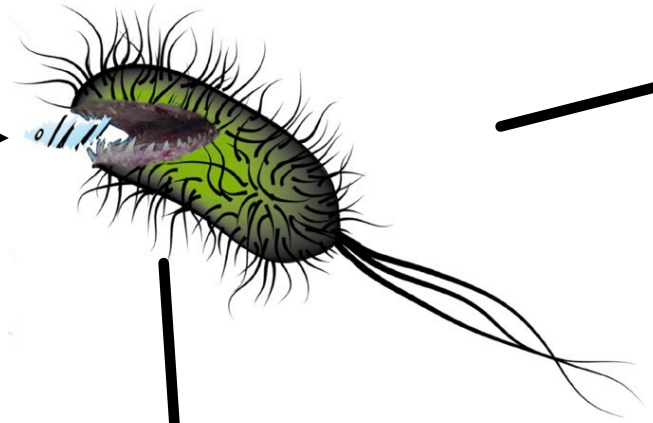
- Carbon compounds expected to support cell growth by anabolism.



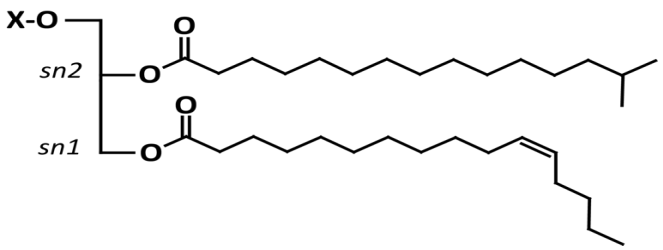
Isotopically labelled plastic – A potentially more accurate and direct method for testing plastic degradation

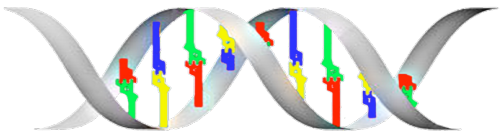
- PE that is comprised of ^{13}C for 99% is the sole carbon source for the microbial culture.
- ^{13}C can be traced in biomarkers and degradation products and can only come from the C-13 labelled polymer





Diagnostic biomarkers

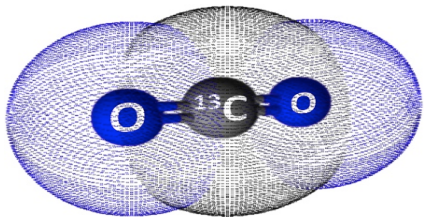




→ identity/abundance key microbes
→ identity degradation pathways

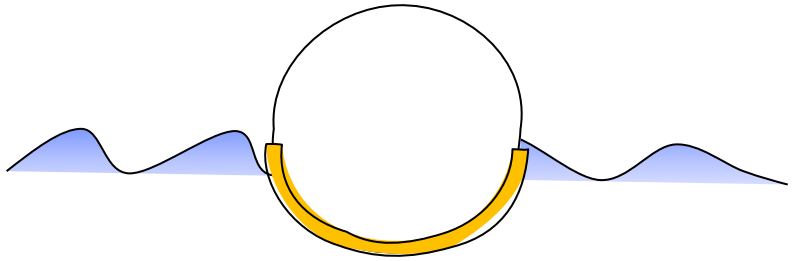
$\delta^{13}C$ values are used to calculate ^{13}C and ^{12}C atom%, changes of $\delta^{13}C$ values in comparison to the background can be used to determine 13C-excess production

Degradation products

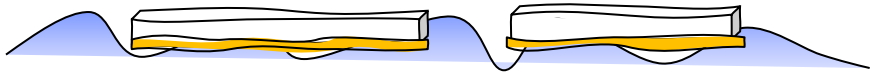


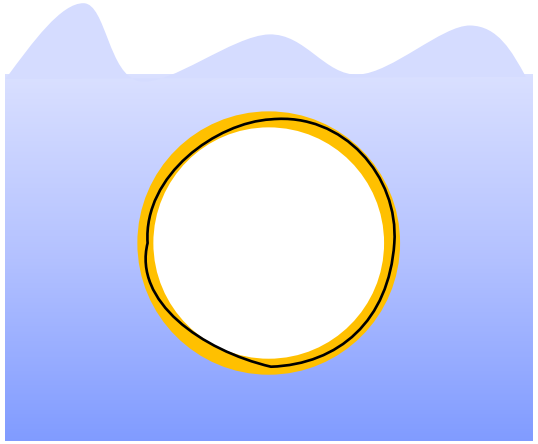
→ plastic degradation rates

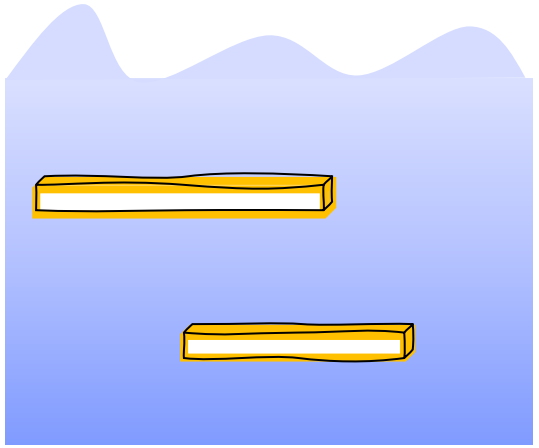
Why make sure the plastic is immerged? We tested two bottle set-ups to see if this would indeed make a difference.

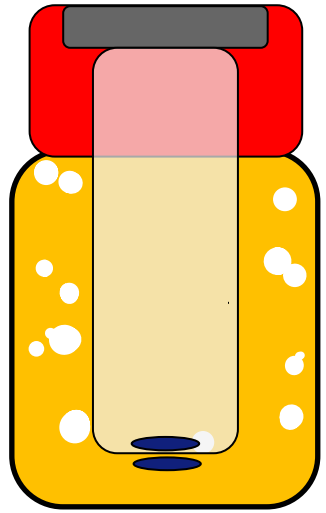


Biofilm might only grow on the wetted part.
Not possible to compare floating and sinking plastics, plus biofilm development could influenced by particle shape.
Get optimal, maximum degradation rate by full immersion.

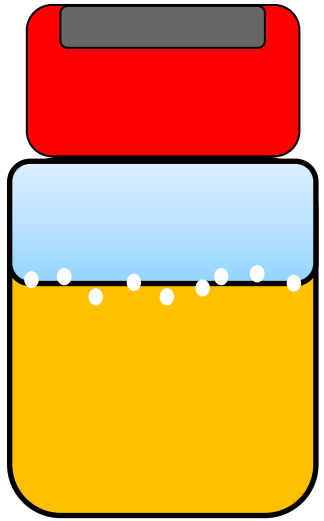








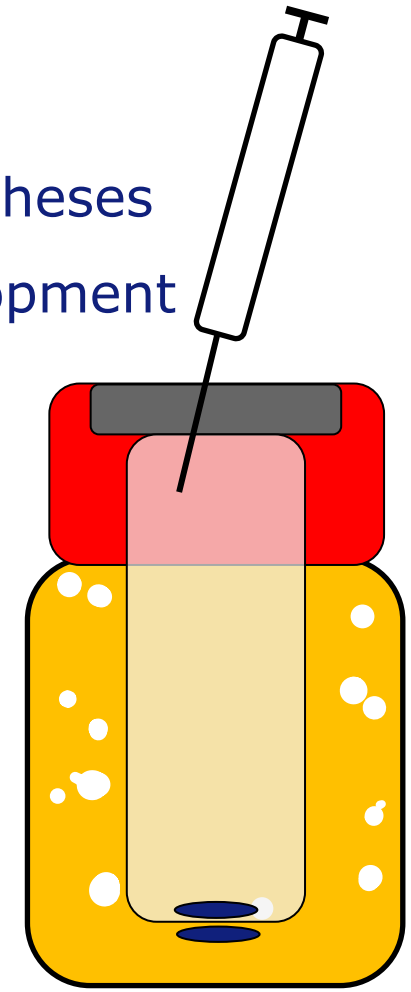
Headspace in see-through plastic tube, rest of bottle completely filled with liquid. Plankton mesh at the bottom to allow gas-exchange and magnetic stirrer bars to remove biofilm on membrane.



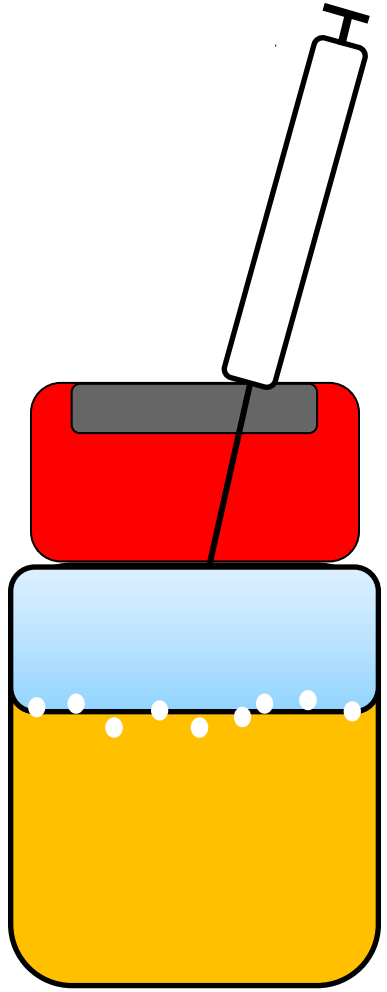
Same headspace – liquid ratio but without tube

Development of new bottle type (The Johannbottle) with our workshop

- Problems solved:
  - Immersion of plastic to test biofilm hypotheses
  - Create headspace to measure CO_2 development



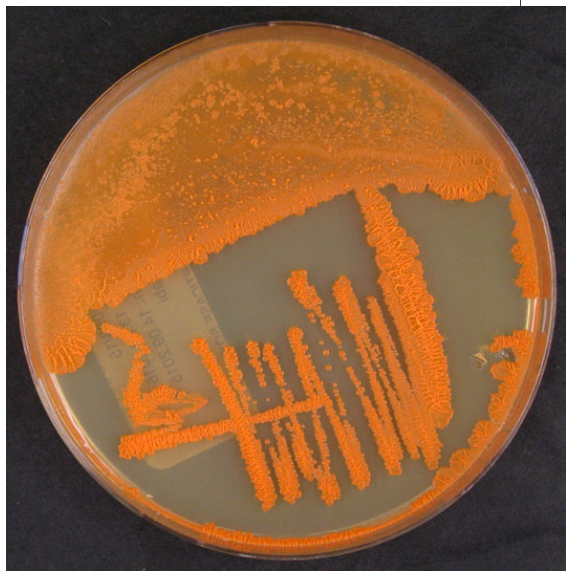
Johannbottle



Standard bottle

Rhodococcus Ruber

- Known terrestrial plastic degrader
- Yellow-orange in culture
- Ideal candidate for proof-of-concept study:
  - Demonstrate stable isotopes can be used to study microbial plastic degradation
  - Test biofilm-hypotheses



https://bacdive.dsmz.de/strain/10927

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DOI 10.1007/s00253-004-1584-8

APPLIED MICROBIAL AND CELL PHYSIOLOGY

I. Gilan (Orr) · Y. Hadar · A. Sivan

Colonization, biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*

Received: 21 August 2003 / Revised: 27 January 2004 / Accepted: 30 January 2004 / Published online: 19 February 2004

Abstract A two-
isolation of a stra
utilized polyethyle
culture, C208 fo
surface and degra
polyolefin within
adhesion to hydro
test both showed
C208 was higher
were obtained fro

Appl Microbiol Biotechnol (2006) 72: 346–352
DOI 10.1007/s00253-005-0259-4

APPLIED MICROBIAL AND CELL PHYSIOLOGY

A. Sivan · M. Szanto · V. Pavlov

Biofilm development of the polyethylene-degrading bacterium *Rhodococcus ruber*

Received: 18 August 2005 / Revised: 9 November 2005 / Accepted: 9 November 2005 / Published online: 14

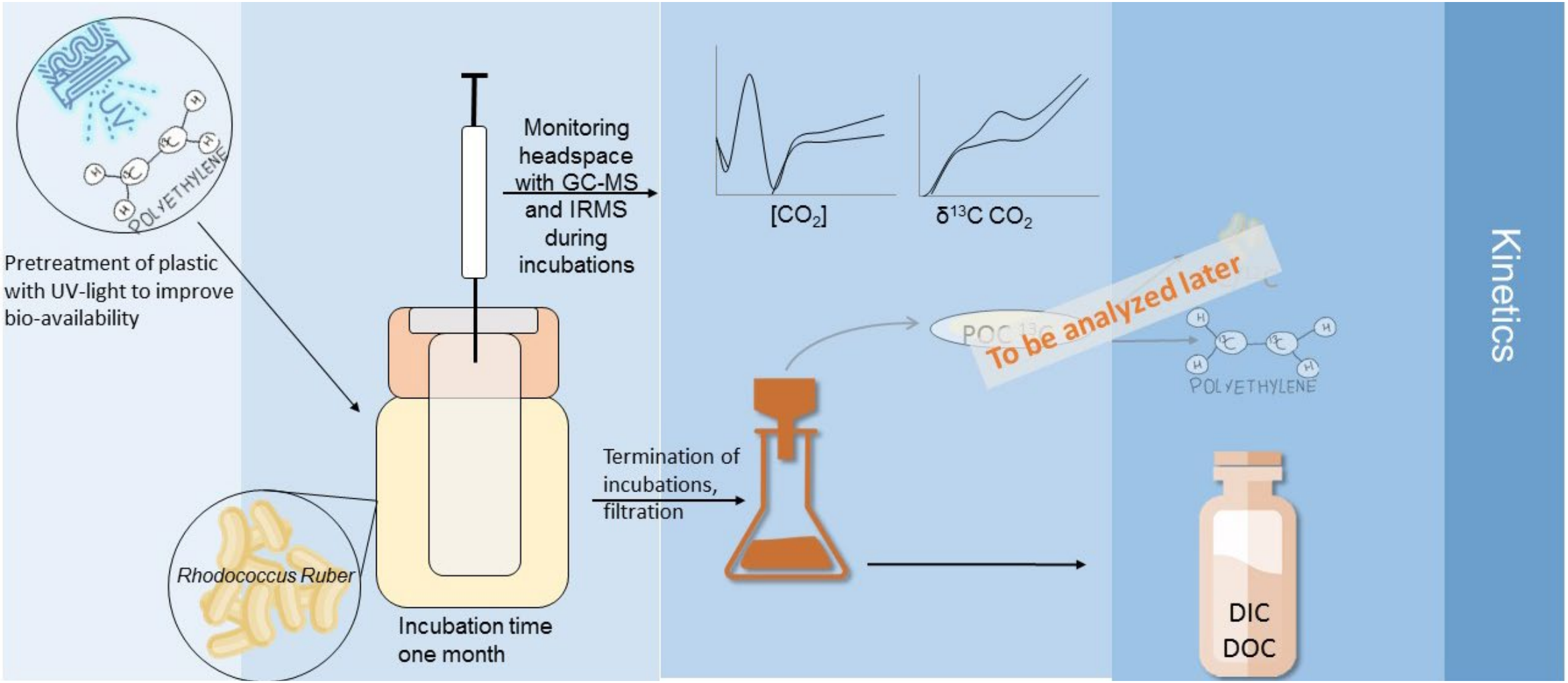
Abstract We have recently isolated a biofilm-producing strain (C208) of *Rhodococcus ruber* that degraded polyethylene at a rate of 0.86% per week (r^2=0.98). Strain C208 adheres to polyethylene immediately upon exposure to the polyolefin. This initial biofilm differentiates (in a stepwise process that lasts about 20 h) into cell-aggregation-forming microcolonies. Further organization yields "mushroom-like" three-dimensional structures on the mature biofilm. The ratio between the population densities of the biofilm and the planktonic C208 cells after 10 days of incubation was about 60:1, indicating a high preference for the biofilm mode of growth. Analysis of extracellular polymeric substances (EPS) in the biofilm of C208 revealed that the polysaccharides level was up to 2.5 folds higher than that of the protein. The biofilm showed a high viability even after 60 days of incubation, apparently due to polyethylene biodegradation.

extracellular polysaccharide produc
Nielsen 2003). Once an initial biofi
cell communication (i.e. quorum s
signalling molecules (bacterial phe
ther modification and development o
and Molin 2002).

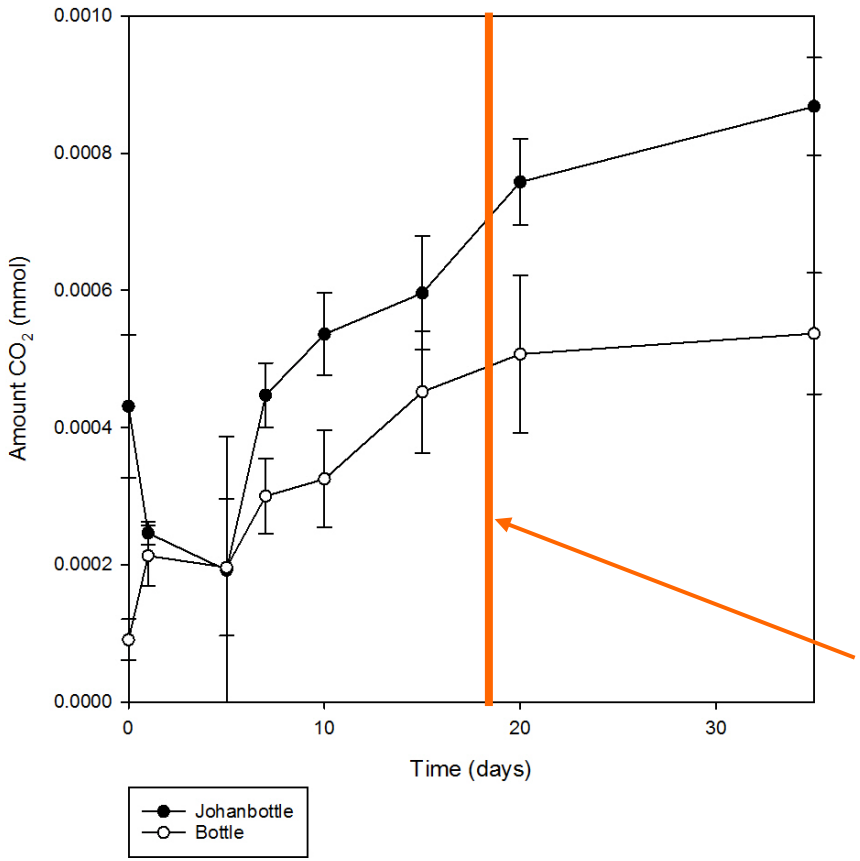
Although microbial biofilms oft
rious effects in natural, clinical or i
they may be useful in biodegrada
materials, such as plastics. Nonbioc
is accumulating in the environmen
tons/year (Orhan and Buyukgungo
considered a grave environmenta
Pruter 1987; Thompson et al. 2004

We recently isolated a strain
(designated C208) that utilized polyc
and energy source and which degra
(Or) et al. 2004). This strain wa

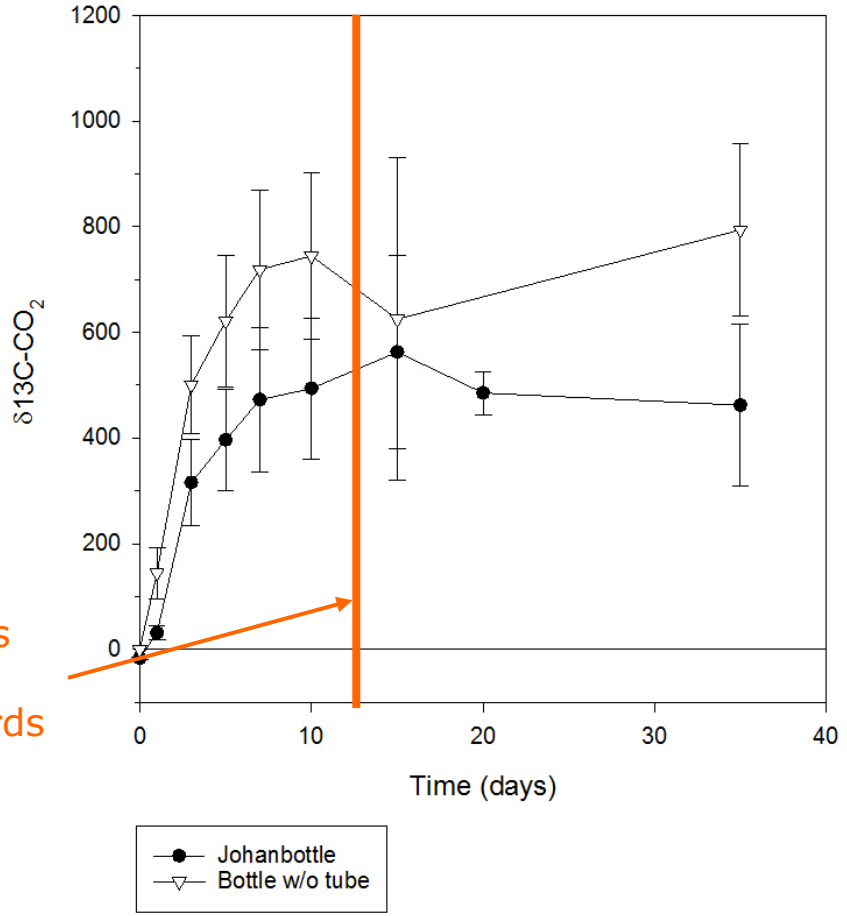
Experimental set-up



Both [CO_2] and δ13C-CO2 in headspace increase over time



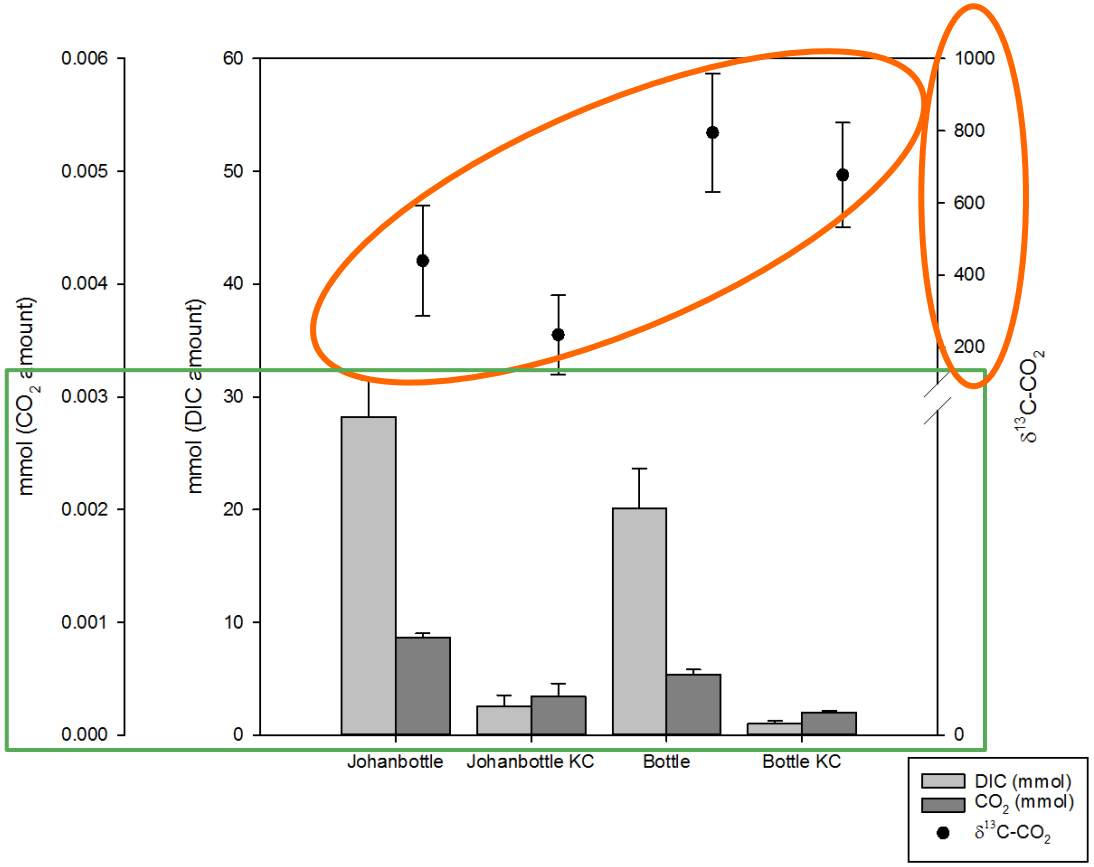
Production slows down from this timepoint onwards



- Both bottle types show production of CO_2 and 13C-CO_2 over time, seemingly approaching an asymptote.
- Methane during incubations below detection limit
- Killed-controls (not shown) produce CO_2 at much lower concentrations
- No significant difference between bottle types

Since PE is the only labelled carbon source, this shows plastic is being mineralized

Final values for DIC, [CO_2], $\delta^{13}C$-CO_2, pH and DOC after terminating incubations

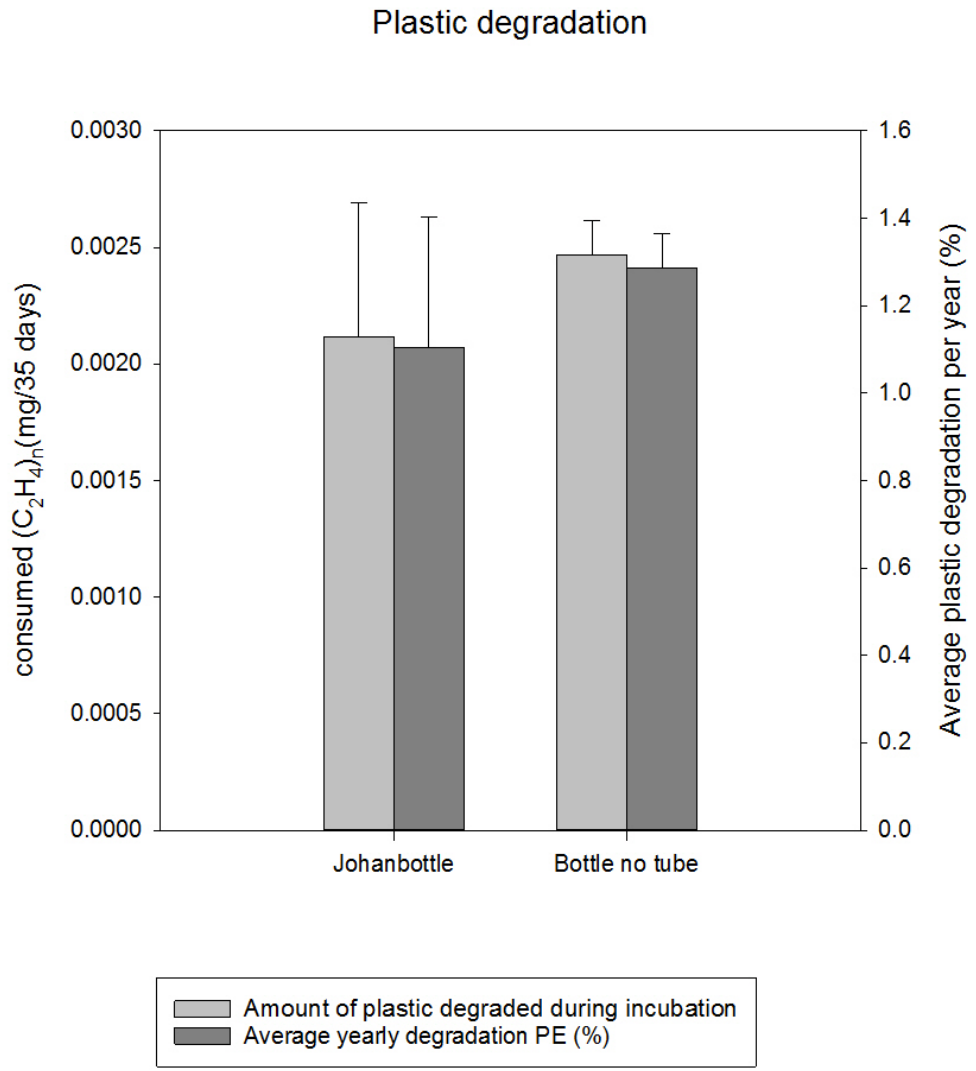


Headspaces highly enriched in ^{13}C. Life experiment more than killed-control

More CO_2 in DIC than in headspace. Total carbonate system needed to determine amount of plastic degraded

- End point total carbonate system and $\delta^{13}C$-CO_2 values allow us to calculate the net absolute change during the experiment and to calculate excess production of C13-CO_2 (labelled mineralization product)

Plastic degradation rates



- Since labelled Pe is the only carbon source and the only source of C-13, the amount of PE mineralized can be calculated from the excess ^{13}C – CO_2 production
- By assuming 1 mole of CO_2 is formed from mineralization of ½ a mole of PE, we can calculate the amount of CO_2 catabolized.
- From there we can extrapolate how much polymer could be catabolized in a year.

Conclusion

- Labelled plastic can be used to track mineralization of plastic
  - Linear range needed to calculate rates more accurately → shorter incubations, more data points during exponential growth needed.
  - More data needed to know full degradation and close carbon balance (biomass dry weight and stable isotope incorporation in biomass)

- Different bottles not significantly different

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Beached plastic on St. Eustatius

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