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Protocol:
Microplate test for growth on PAHs

(Johnsen et al. 2002. Detection of microbial growth on PAHs in microtiter plates by using the respiration indicator WST-1, Applied and Environmental Microbiology, vol. 68 pp 2683-2689. Free down-load: http://aem.asm.org/content/vol68/issue6/METHODS).

Principle:
Cells are grown in microtitre plates for 10 days or four weeks with various three- or four-ring PAHs as the only source of energy and carbon. Cells that do not mineralize the PAHs become metabolically inactive, whereas cells able to mineralize PAHs will proliferate. After incubation, the metabolic status of the cells is assayed with the cell proliferation reagent WST-1. WST-1 in itself is not sensitive enough to demonstrate growth on PAHs so the metabolism of the cells is boosted by addition of easily degradable carbon along with WST-1, to measure the potential respiration. Wells with no growth will remain respiration negative during the 2 to 6 hour incubation with WST-1, whereas wells where growth has taken place will be respiration positive.

Reagents needed:
- Hexane.
- PAHs dissolved in hexane (5 mg/ml).
- Microtiter plates (flatbottom) with lids.
- Extraction buffer (tetrasodium pyrophosphate, 2.0 mM, pH 7.0).
- Phosphate minimal medium.
- WST-1 Cell Proliferation Reagent (Roche Biochemicals, Mannheim, Germany).
- Electron donor solution consisting of glucose, succinate and pyruvate (17 mM each) dissolved in 40 mM tris buffer, adjust to pH 6.5 after the addition of glucose, succinate and pyruvate.

Preparation of plates:
1. Dissolve PAHs in hexane (5 mg/ml). The solubility of anthracene in hexane is low. Shake the anthracene solution (5 mg/ml) over night to make a saturated solution.
2. Add 20 µl PAH-solution to each well (except anthracene) and evaporate the solvent for at least 1 h in the flow-hood (to ensure that all hexane is evaporated). Use four replicates for each PAH for each strain tested. Use hexane as a negative contol (no carbon source). Preferably, different PAHs should be added to different microplates to avoid cross-contamination through the gas-phase. For anthracene: add 40 µl of a saturated solution to each well and evaporate the hexane, then add another 40 µl of the saturated solution to each well and evaporate the hexane.

If pure strains are to be tested:
1. Add 200 µl of phosphate minimal medium (pH 6.8) to each well. Fill empty wells with medium or sterile water to reduce water loss.
2. Grow inoculum on the phosphate minimal medium with an appropriate carbon source.
3. Wash inoculum twice and dilute to OD 0.3

4. Inoculate each well with 10 µl inoculum. Put the lid on the microplate and wrap the microplate in a plastic bag.
5. Incubate for 10 days at room temperature (20-25 °C) in a fume hood.
6. Test the plates for growth as described below.
7. Compare to wells with hexane only (one-tailed t-test).

When testing pure cultures, do not use wells at the edges of the microplates as there might be some minor edge-effects. Mycobacteria often have high absorbances (0.050-0.150) in the hexane controls. Absorbance changes in positive wells are typically in the range 0.600-2.000 after 90 min. incubation with WST-1 and electron donors.

MPN-enumeration of PAH-degraders in soil

1. Sieve the soil sample.
2. Determine the soils' water content by drying app. 10 g over night at 105°C.
3. Extract cells by shaking 10 g soil with 90 ml tetrasodium pyrophosphate·10 H_2O (2.0 mM, pH 7.0) for 5 min.
4. Make 4-fold dilution series in phosphate minimal medium (pH 6.8).
5. Add 6 x 200 µl of each dilution to microplates containing PAH (4-fold dilution, 6-row MPN, use 1:160 dilution of the soil (= 1:16 dilution of the extract) as the lowest dilution).
6. Place a piece of wet filterpaper on the top of the microplate lid to prevent drying out of the wells.
7. Wrap the plates in plastic bags and incubate in a fume hood at room temperature (20-25 °C) for four weeks (phenanthrene, anthracene, fluoranthene and pyrene).
8. Test the plates for growth as described below.
9. Determine the MPN per g soil (dry weight) using a MPN-calculator as described below.

For the MPN-assay, the absorbance changes for positive phenanthrene wells are typically in the range of 0.200-1.500; positive fluoranthene wells 0.040-0.300; and positive pyrene wells 0.070-1.000. The threshold value for positive wells is 0.030. Edge-effects in the MPN assay are not a problem as the wells are only scored positive or negative. The pyrophosphate concentration has been increased from 1.2 mM to 2.0 mM.

Testing the plates for growth on the PAHs:

1. Prepare an electron donor solution consisting of glucose, succinate and pyruvate (17 mM each) dissolved in 40 mM tris buffer and adjust the pH to 6.5 (after addition of glucose, succinate and pyruvate). Filter sterilize through 0.2 µm filter and store the solution frozen in small aliquots.
2. Add 50 µl of the electron donor solution and 10 µl cell proliferation reagent WST-1 (obtained from Roche) to all wells.
3. Measure the absorbance (A450 nm with a refence wavelenght at A630 nm) of the wells (time zero).
4. Incubate at 22-25 °C on a shakertable at 300 rpm (250 rpm is not enough! Increase slowly from 0 to 300 rpm to avoid splash over between wells).
5. For pure cultures: read the absorbances after 90 min. If absorbances are low, incubate for another hour and read absorbances again.
6. For soil MPN: read the absorbances after 5 hours. If absorbances are low, incubate for another 1-2 hours and read absorbances again.
7. Subtract absorbances at time zero from all readings.
8. Compare to wells with hexane only.

Note:
Use of a reference wavelenght: The absorbance of each well is measured at 450 nm and at 630 nm. WST-1 has absorbance max at 450 nm, so at 450 nm you measure specific absorbance from reduced WST-1 as well as turbidity from PAH-crystals, cells, soil particles, finger prints etc. WST-1 has little absorbance at 630 nm, so at this wavelenght you only measure turbidity from PAH-crystals, cells, soil particles etc. By subtracting the A630 (reference wavelenght) from A450 (done automatically by the ELISA-reader) you eliminate most of the "noise" from crystals, soil particles etc.
pH: The pH is 6.8 in the medium and 6.5 in the electron donor solution because tetrazolium salts are most easily reduced at pH<7.

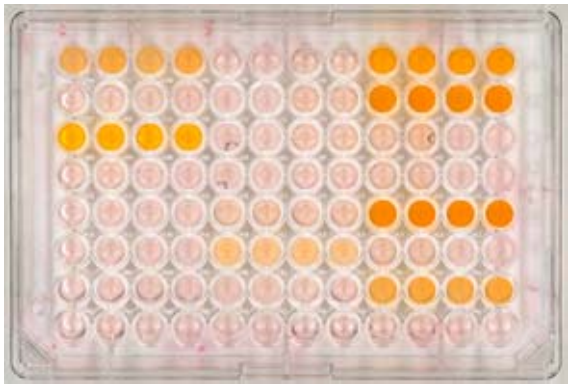

Fig 1. 24 strains were tested for growth on phenanthrene.

Fig. 2 MPN-estimation of the pyrene-degrader population-sizes in two soil samples.

Determination of MPN-estimates

Determine the MPN-estimate by using a DOS MPN-calculator (Klee, A. J. 1993. A computer program for the determination of most probable number and its confidence limits. J. Microbiol. Meth. 18:91-98, http://microtitermethods.dmu.dk/) or a Windows MPN-calculator (http://members.ync.net/mcuriale/mpn/index.html).

Results from the first row (1:160 dilution of the soil) must for some soils be discarded because bacterial growth is reduced by the presence of high concentrations of humic acids, toxic compounds (e.g. cyanide and heavy metals) and antagonistic organisms. This increases the detection limit from 80 cells/g to 320 cells/g.

Case 1. One or more dilutions show all wells positive. This is the ideal situation. Select the highest dilutions that give positive results in all wells (even if a lower dilution gives negative results) and the following higher dilutions (ex. a and b). Negative wells at the lowest dilutions should be considered false negatives due to depletion of substrate or accumulation of toxic metabolites.

Case 2. No dilutions show all wells positive. Select the 3 lowest dilutions (ex. c). If there are positive results in higher unselected dilutions, add those higher-dilution positive results to the results for the highest selected dilution (ex. d).

Example	Dilution factor and amount of original sample in each well (g)								Combination of positives
	160 $1.25 \cdot 10^{-3}$	640 $3.13 \cdot 10^{-4}$	2560 $7.81 \cdot 10^{-5}$	10240 $1.95 \cdot 10^{-5}$	40960 $4.88 \cdot 10^{-6}$	163840 $1.22 \cdot 10^{-6}$	655360 $3.05 \cdot 10^{-7}$	2621440 $7.63 \cdot 10^{-8}$	
	Number of positive wells out of 6								
a	6	6	6	2	0	0	0	0	6-6-6-2-0-0-0-0
b	0	2	4	6	6	5	1	0	x-x-x-6-6-5-1-0
c	0	0	1	0	0	0	0	0	0-0-1-x-x-x-x-x
d	4	4	1	1	0	0	0	0	4-4-2-x-x-x-x-x

These criteria are based on recommendations from Bacteriological Analytical Manual Online 2001, Appendix 2 Most Probable Number from Serial Dilutions, Center for Food Safety & Applied Nutrition, U.S. Department of Health and Human Services and U.S. Food & Drug Administration (http://www.cfsan.fda.gov/~ebam/bam-a2.html).

Statistical evaluation of MPN results

Cochran, W. G. 1950. Estimation of bacterial density by means of the 'most probable number'. Biometrics. 6:105-116.

Niemelä, S., 1983. Most probable number methods. In: Statistical evaluation of results from quantitative microbiological examinations, Nordic Committee on Food Analysis, Report No 1, 2nd edition, p. 25-28.

a is the dilution ratio and n is the number of replicates at each dilution i.e. the number of rows. The standard error (S.E.) of logMPN is given by:

$$S.E. = 0.58\sqrt{\frac{\log a}{n}} \qquad \text{for } a \geq 10$$

$$S.E. = 0.55\sqrt{\frac{\log a}{n}} \qquad \text{for } a < 10$$

A test value referring to the normal distribution is calculated to test whether two MPN estimates are significantly different.

$$Z = \frac{\log MPN_1 - \log MPN_2}{0{,}58\sqrt{\frac{\log a_1}{n_1} + \frac{\log a_2}{n_2}}}$$

where MPN_1 is the larger of the the two MPN-estimates

Example:
The numbers of phenanthrene degraders and pyrene degraders in a soil sample were estimated using the WST-1 microtiter plate method (4-fold dilution series, 6 row MPN).

The MPN estimates with 95% confidence limits are:

MPN_1 (phenanthrene)	6400 [1100-20000]
MPN_2 (pyrene)	4700 [550-14000]

$n = 6$, $a = 4$

$$S.E. = 0.55\sqrt{\frac{\log 4}{6}} = 0.174$$

$$Z = \frac{\log 6400 - \log 4700}{0{,}58\sqrt{\frac{\log 4}{6} + \frac{\log 4}{6}}} = 0.52$$

The calculated Z-value (0.52) is smaller than the critical Z-value (1.96), so the two MPN-estimates are not significantly different at the 95% level.

Phosphate minimal medium

Modified from Johnsen et al., 2000 (Appl. Environ. Microbiol. 66:3487-3491) by inclusion of Ni and Se in the trace elements solution.

Buffer stock solution

35 g/L	$Na_2HPO_4 \cdot 2\ H_2O$
4 g/L	KH_2PO4

Adjust pH to 6.8 and autoclave.

Mineral stock solution

10 g/L	$(NH_4)_2SO_4$
2 g/L	$MgCl_2 \cdot 6\ H_2O$
1 g/L	$Ca(NO_3)_2 \cdot 4\ H_2O$

Autoclave.

Trace elements stock solution

800 mg/L	Na_2-EDTA
300 mg/L	$FeCl_2$
10 mg/L	$MnCl_2 \cdot 4\ H_2O$
4 mg/L	$CoCl_2 \cdot 6\ H_2O$
1 mg/L	$CuSO_4$
3 mg/L	$Na_2MoO_4 \cdot 2\ H_2O$
2 mg/L	$ZnCl_2$
0.5 mg/L	LiCl
0.5 mg/L	$SnCl_2 \cdot 2\ H_2O$
1 mg/L	H_3BO_3
2 mg/L	KBr
2 mg/L	KI
0.5 mg/L	$BaCl_2$
4 mg/L	$NiCl_2, 6H_2O$
2 mg/ L	$Na_2SeO_3, 5H_2O$

Autoclave

Prepare the medium by mixing:

Buffer stock solution	25 ml
Mineral stock solution	25 ml
Trace elements stock solution	5 ml
Sterile water	945 ml