**Exoenzyme Assay Protocol (for use with fluorogenic substrates)**

As applied in Edwards, B. R., C. M. Reddy, R. Camilli, C. A. Carmichael, K. Longnecker, and B. A. S. Van Mooy. 2011. Rapid microbial respiration of oil from the Deepwater Horizon spill in offshore surface waters of the Gulf of Mexico. *Environmental Research Letters* **6**.

**Protocol history:** Created by B.R.E., October 2013  
Minor revisions by J.R.C., April 2014

**Good references:**

Hoppe, Hans-Georg. 1993. Use of Fluorogenic Model Substrates for Extracellular Enzyme Activity (EEA) Measurement of Bacteria. In Kemp, P. F., J. J. Cole, B. F. Sherr and E. B. Sherr (eds) *Handbook of Methods in Aquatic Microbial Ecology*. CRC Press, Boca Raton, Florida, 423-431.

Roberts, I. M. 1985. Hydrolysis of 4-Methylumbelliferyl Butyrate - a Convenient and Sensitive Fluorescent Assay for Lipase Activity. Lipids 20(4): 243-247.

**Supplies and reagents needed:**

1. Multi-well plate reader with appropriate ex/em filter pairs:
   1. For 4-MUF: 364/445 nm (filters at 360/465, both 35 nm bandwidth, worked for JRC)
   2. For MCA: 380/440 (filters at 380/430, both 35 nm bandwidth, worked for JRC)
2. 96-well plates suitable for fluorescence assay
3. Multichannel pipettors: 10 uL x 8-channel for prepping plates; 200 uL x 12-channel for adding samples
4. 10 mL serological pipettes
5. 1000 uL single-channel pipettor
6. 200 uL single-channel pipettor
7. Microcent tubes for alquotting and freezing standards
8. Incubator or other way of keeping plate at *in situ* temp and in the dark during incubation
9. Polystyrene reagent reservoirs
10. Balance for weighing out substrates **(\*\* you may need to do this before getting underway if this is for a cruise)**
11. Substrates (from Sigma or other source; these are what we usually use but see references for other options):
    1. MUF-butyrate lipase activity
    2. MUF-a-D-glucopyranoside a-D-glucosidase activity
    3. L-Leucine-4-methylcoumarinyl-7-amide (Leu-MCA) aminopeptidase activity
    4. MUF-PO4 phosphatase activity
12. “Pure” fluorochromes (needed for standards)
    1. 4-methylumbelliferone (4-MUF)
    2. 4-methylcoumarinyl-7-amide (MCA)
13. Milli-Q water
14. DMSO

**Protocol:**

1. Prepare substrates (2.5mM)
   1. MUF-butyrate 6.2mg in 10mL DMSO
   2. MUF-glu 5.3mg in 10mL DMSO
   3. MUF-PO4 6.4mg in 10mL water
   4. MCA-leucine 8.1mg in 10mL water
2. Prepare MUF and MCA standards (5mM) [I remember that these are hard to dissolve so I placed the falcon tubes in a beaker of water on a hot plate)
   1. MUF 12.37 mg in 12.5mL water
   2. MCA 10.9mg in 12.5mL DMSO
   3. Aliquot 250ul standard into cryovials (or mini-centrifuge tubes, whatever is available)
   4. Freeze in the dark until you are ready to use
3. Add 10mL substrate solution to a plastic reservoir. Aliquot 2ul of substrate into each well of one column of a 96 well plate using 8 channel pipette. Do this three times so you have triplicates for each substrate. You can freeze these plates or use them immediately.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 2ul MUF-but | 2ul MUF-but | 2ul MUF-but | 2ul MUF-glu | 2ul MUF-glu | 2ul MUF-glu | 2ul MUF-PO4 | 2ul MUF-PO4 | 2ul MUF-PO4 | 2ul MCA-Leu | 2ul MCA-Leu | 2ul MCA-Leu |
| B |  |  |  |  |  |  |  |  |  |  |  |  |
| C |  |  |  |  |  |  |  |  |  |  |  |  |
| D |  |  |  |  |  |  |  |  |  |  |  |  |
| E |  |  |  |  |  |  |  |  |  |  |  |  |
| F |  |  |  |  |  |  |  |  |  |  |  |  |
| G |  |  |  |  |  |  |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |  |  |  |  |  |

1. Run a standard curve plate for MUF and MCA daily. This will allow you to convert from fluorescence to umol substrate/L.
   1. Thaw 250ul aliquot of standard.
   2. Add 200ul to 20mL SW in a reagent reservoir.
   3. Add 200ul of this secondary standard from the reagent reservoir to first column in plate.
   4. Add 100ul of SW to columns 2-12
   5. Transfer 100ul of standard in first column to the 2nd column. Pipette to mix and then transfer 100um of solution in 2nd column to the 3rd column and so forth.
   6. Repeat for the other fluorochrome using row B of the plate
2. Actually running the enzyme assays.
   1. Pour a sufficient amount of your various sample (e.g., from different depths of a CTD cast) into a reagent reservoir
   2. Use 12 channel pipette to add 198ul of sample to each well in a row.
   3. Take at least 5 readings over 24 hours. I like to do t=0 t=1 hour t=2 hours and about every 6 hours thereafter.
   4. Incubate in the dark at in situ temp between readings (I usually incubate in the thermomixer)