Comparison of three techniques for measuring biological parameters in lake sediment

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ABSTRACT

Three new techniques are described and applied to nine Lake Erie Shipek samples. The three techniques, radioactive thymidine incorporation into DNA, dehydrogenase reduction of resazuin and extraction and quantification of ATP measure three important, but distinctively different biochemical parameters. Analyses of the sediments reveal a close correlation between ATP content and DNA synthesis ($r = 0.96$, $p < 0.01$), ATP and dehydrogenase activity ($r = 0.91$, $p < 0.05$) and a non-significant correlation between DNA synthesis and dehydrogenase activity ($r = 0.71$). The sensitivity and reproducibility of these techniques make them suitable for large-scale monitoring operations.

INTRODUCTION

Microbial activity in sediments of the Great Lakes is considered important in nutrient cycling, transformation of metals, degradation and modification of organics and in hypolimnetic oxygen depletion. A recent IJC report (1976) cites microbial transformation and recycling of organics as a "critical research need". Due to increasing interest in these areas, a concerted effort is being made to understand and quantify the microbial activity in sediments.

The present understanding of biological activity in sediments comes from several kinds of studies. The most commonly measured parameter is heterotrophic bacterial number which is estimated by culture methods such as the Most Probable Number (MPN) or Membrane Filtration (MF) tests (Vanderpost and Dutka 1971) or direct counting of "live" bacteria can be made by fluorescent microscopy (Bell and Dutka 1972). Metabolic tests such as oxygen consumption or CO_2 evolution (Hartgrave 1969, Liu 1973), dehydrogenase activity or substrate utilization (including radioisotopically-labelled compounds) are also employed. Recently, ATP content has been used to determine biomass in sediments (Lee et al. 1971, Afghan et al. 1976).

We have developed three independent methods which determine dehydrogenase activity (by resazurin reduction), DNA

synthesis (tritiated thymidine incorporation) and ATP content. Each of the methods has been described (Afghan et al 1976, Liu 1976, Tobin and Anthony 1976) and are considered to be significant advances over comparable techniques. They were applied to nine Lake Erie sediments and a correlation of the results was made. The results and important features of each of the methods are described.

MATERIALS AND METHODS

Sediment Sampling and Subsampling

The sediment was sampled by Shipek sampler from the CCS Limnos between August 9-14, 1976. One kg composite samples were taken, stored in plastic bags at 4°C until brought to shore. Sediments were immediately subsampled after careful mixing into 3 portions of 250 ml each. These were distributed to the 3 laboratories for analysis. Dry weight determinations were performed in duplicate on 0.6 ml aliquots of the sediments.

ATP Analysis

ATP analyses were performed as recently described (Afghan *et al.* 1976) with the exception that 0.6 ml samples were analyzed. Analyses were performed in duplicate and the ATP content of each of these was analyzed in triplicate. Values are expressed as µg ATP (as $Na_2ATP \cdot 3H_2O$) per g sediment (dry wt).

DNA Synthesis Measurement

DNA synthesis was measured by the incorporation of tritiated thymidine into the microbial cells followed by selective extraction and digestion of the DNA and scintillation counting of the digest (Tobin and Anthony, 1976). The control used was a *m*-cresol inhibited reaction tube, incubated in the normal manner, although a zero-time incubation gave the same results. Complete details are given in Appendix "A".

Dehydrogenase Activity

The microbial dehydrogenase activity was determined by a new Resazurin technique (Liu, D., in preparation). Enzymatic reduction of resazurin is followed by spectrophotometric measurement at 610 nm (Liu 1976). Complete details are given in Appendix "B".

Chemical Analysis of Sediments

Analysis of particulate OC, P, N. was performed by Water Quality Laboratories by standard methods (Water Quality Branch 1974).

RESULTS

The nine Lake Erie Surface sediments (Table 1) were collected during a cruise of the S.S. Limnos in August, 1976. All samples were mainly silty clay except station number 8 which was sandy.

The biological parameters measured on each of the sediments are shown in Table 2. Stations 1 and 4 are the highest in DNA synthesis, dehydrogenase activity and ATP content. Station 8 (sandy) is the lowest in DNA synthesis and dehydrogenase activity, but third lowest in ATP content. The differences in values between sediments was found to be statistically significant for each of the parameters ($p<0.01$).

Station 1 is the location of transitory sediment deposition with low accumulation rates; 2 represents deposition conditions for clayey silts; 3, conditions for deposition for silty clay; 4 may be affected by contaminants being recirculated from a dredge disposal site near Cleveland; 5 and 6 represents depositional sites for silty materials; 7 has a low sedimentation rate because of its location in the path of a major sediment stream for particulates between the central and eastern basins; 8 is close to the influence of mercury-rich materials; 9 represents an accumulation site for silts (Thomas, Jaquet, Kemp and Lewis, 1976, J. Fish. Res. Bd. Canada 33, 385-403; P.G. Sly, pers. commun.).

To compare the three sets of values, the values were plotted one against another and the linear correlation coefficients (r) were determined. ATP content and DNA synthesis in sediments have a high degree of correlation; $r=0.96$ ($p<0.01$) (Figure 2). A lesser correlation was observed between ATP content and dehydrogenase activity; $r=0.91$ ($p<0.05$) (Figure 3). DNA synthesis and dehydrogenase activity were less highly correlated; $r=0.71$ (Figure 4), which was not significant at $p=0.05$. The sediments causing the lesser correlations of the dehydrogenase activity were at stations 4 and 5 which were higher in dehydrogenase activity and 1 and 7 which were lower than would be predicted by the relationship to the other parameters.

The results of each of the biochemical parameters was compared with the OC, N and P content of the sediments. The biochemical parameters correlated best with P content. Correlation coefficient analysis with P was performed on ATP content ($r=0.91$, $p<0.01$), DNA synthesis ($r=0.94$, $p<0.01$) and dehydrogenase activity ($r=0.70$, $0.01<p<0.05$). Correlation coefficients were not as good between the biochemical parameters and OC content ($0.01<p<0.05$) except with dehydrogenase activity ($r=0.78$, $p<0.01$). There was no significant correlation between N and these biochemical parameters nor with the C/N ratios which can be used as an indicator of allocthonous or autocthonous origins.

Each of the three techniques has undergone testing for reproducibility and reliability (Tobin & Anthony, in preparation, Afghan et al. 1976, Afghan, Ryan and Tobin, in preparation, Liu 1976, Liu, in preparation). Measurements for DNA synthesis had a coefficient of variability of 5.1% on sediment from station 4 (N=8) and 5.9% average on all the other sediments (N=2 to 4). The ATP analyses had an average coefficient of variation of 5.1% using 10 replicates on a sample containing 0.43 µg ATP/g sediment. The dehydrogenase activity had a coefficient of variability of 5.0%.

A series of experiments were performed with the enzymatic reactions to determine the optimum or at least favorable reaction temperature. Incubation at 4^{o} , 20^{o} and 30^{o}C for DNA synthesis was performed (Fig. 5). 20^{o}C appeared to be ideal for the reaction as incorporation was much higher than that at 4^{o} and the 30^{o} temperature, while giving a higher initial velocity, soon decreased to about the same level as obtained at 20^{o}C. Dehydrogenase activity at 1, 10,

20 and 30°C was also determined (Figure 6). Less difference was noted between the biological dehydrogenase activity (=total-chemical activity) at the various temperatures. Maximum activity occurred at 20°C with the 30°C reaction showing similar kinetics to those obtained with DNA synthesis (ie. a short period of high initial velocity which then reached a plateau similar to the 20°C reaction.)

Discussion

While sediment microbial activity is a very important Lake process, it is impossible to measure the activity in a way which relates to all the types of information desired. Methods have been developed, and will be continued to be developed for specific purposes. The ATP method is an important development in the determination of biomass. ATP is an ubiquitous component in living organisms and is rapidly degraded upon cell death or lysis. Problems with ATP determinations in sediments have been an important factor in their little usage thus far. The major problems have been solubilization of cations and humic and fulvic acids in the extractant which interferes with the bioluminescence reaction and adsorption of ATP on sediment particulates. These problems have been overcome by the use of phosphate buffer and $CHCl_3$ with homogenization in a Polytron homogenizer (Afghan et al. 1976) and an internal standard added to an aliquot of the extract to allow correction for interferences with the luciferin-luciferase reaction.

While the factor used to convert ATP content to "biomass" (wt. ATPX 250 = wt. organic carbon) is subject to considerable debate, the actual measurement of ATP is rather precise, highly sensitive and directly comparable to measurements made in water, sludges, etc. (Afghan et al. 1976).

Use of tritiated thymidine to label DNA of growing cells is a classic technique in cell biology (Cleaver 1967). It has received some use for labeling individual aquatic cells followed by autoradiography to determine specific growth rates (Brock 1967, 1971). Thomas et al. (1974) have described a method for measuring DNA synthesis in soil microorganisms by [^{3}H]dThyd incorporation followed by extraction of the DNA with hot acid and precipitation by cupric sulfate. It is known however that the acid extraction extensively hydrolyzes DNA (Munro and Fleck 1966) and makes it almost completely unprecipitable. In our hands, this method yielded only 7% of DNA (Tobin, unpublished) in the precipitate. The method described in detail here (Appendix A) uses an alkaline extraction at 37°C to solubilize DNA without hydrolysis, followed by cold acid precipitation and washing steps. Only at the last stage is the DNA hydrolyzed to nucleotides to liberate it from humic substances and separate it from any possible contaminants that could be labeled. This procedure gives quantitative recovery of DNA. For this reason, and because of low background (0-time incubation or inhibition of the reaction with 50 µl m-cresol of

ca.60-100 cpm, this method is uniquely suitable for determination of DNA synthesis in sediments.

The measurement of dehydrogenase activity is important because it represents major energy yielding reactions in the cell under anaerobic or aerobic conditions. The commonly-used triphenyltetrazolium chloride (TTC) method for measuring dehydrogenase activity in sediment is very time-consuming (24 hr. incubation) and inaccurate because of the numerous steps involved in the extraction of formazan from the reaction mixture. The resazurin-reduction method is fast, simple, precise and allows correction for chemical dye reduction (by subtraction of chemical production of reduced dye in the presence of m-cresol which prevents biological dehydrogenase activity).

It was considered important to determine whether the measurements made on the sediments were reasonable in relation to one another. While dehydrogenase activity per cell is variable, certain inferences could be made about ATP content and DNA synthesis. The maximal rate of DNA synthesis was 438 f moles dThyd /g/2h. An "average" bacterial cell contains about 1.7×10^{-14}g DNA consisting of 23.1%T, 26.3%C, 26.8%G and 23.8%A (data of E. coli.) This incorporation represents the complete replication of 2.1×10^{4} cells or 1.05×10^{4} cells /hr/g.

The ATP of this sample was 1.40 µg/g. E. coli has about 1.5×10^{-15}g ATP per cell (Karl 1975; also 1.5×10^{-15}g ATP/cell for

average marine bacteria, Hamilton and Holm-Hansen 1967) so this is the equivalent of 9×10^8 cells/g sediment.

The DNA synthesis in 1h then represents the replication of 0.0023% of the organisms detectable by ATP methods. If about 1 out of 300 of the microorganisms in sediment replicate (may be inferred from Clark, 1967), the average replication time would be 6 days, a not unlikely time under suboptimal conditions. While calculations such as these have several problematic assumptions, it is apparent that the DNA synthesis found is in the right order of magnitude in relation to the ATP content.

The relationship to the N, C, P measurements was somewhat surprising. Good correlations obtained between total P and each of the three biochemical parameters could suggest that P is a limiting factor in sediments. Indeed, the increase in biological activity of 3-5 orders of magnitude from the water column to surface sediment is accompanied by a similar total P increase. This finding may be very significant in view of recent evidence that apatite-PO_4 (a sparingly soluble mineral comprised of Ca^{2+}, PO_4^{3-} and associated anions) can be used as the sole source of PO_4^{3-} and may, in fact, influence the mobilization of the PO_4^{3-} (Smith, Wong and Mayfield, 1976). A study of P forms in the surficial sediments of Lake Erie (Williams, Jaquet and Thomas, 1976, J. Fish. Res. Bd. Canada 33, 313-349) shows that 88% of the total P is in the inorganic form and 63% of this fraction is apatite P.

It was noted in the dehydrogenase activity measurements, that at least two of the sediments had much higher (stations 4, 5) activity and two had much lower (stations 1, 7) activity than would be predicted from their relationship with ATP (Fig. 3) and DNA synthesis (Fig.4). The reasons for these anomalies were not apparent, but could lead to some interesting speculation. The most simple explanations would be increased or decreased amounts of dehydrogenase enzyme per cell because of genetic or environmental differences. An alternate explanation for the low values would be a particular inhibitory or toxic compound that selectively affects the dehydrogenase activity but not DNA synthesis activity. High dehydrogenase activity could be influenced by local substrate concentrations.

If the two enzymatic activities, DNA synthesis and dehydrogenase activity are considered, for a moment, as indicators of toxic or inhibitory substances and ATP as a measure of biomass, then any common decrease in DNA synthesis and dehydrogenase could be considered as an indicator of inhibitory compounds. Two such sediments displayed this characteristic (7 and 8) and none displayed the opposite (common higher activity). This could lead one to examine these sediments more carefully for potentially toxic compounds.

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Table 1. Location of Lake Erie Sediment Stations and Water Depths

Sediment Number	Location	Water Depth (m)
1	41°38'39"N 82°45'15"W	10
2	41°40'00"N 82°29'36"W	10
3	41°41'45"N 82°00'00"W	22
4	41°32'12"N 82°57'00"W	15
5	41°51'45"N 81°16'15"W	21
6	42°00'00"N 80°46'36"W	18
7	42°11'00"N 80°16'30"W	23
8	42°11'24"N 80°03'00"W	13
9	42°43'06"N 79°07'39"W	20

Table 2. Comparison of ATP content, DNA synthesis and Dehydrogenase activity and chemical constituents of each sediment sample.

Sediment	OC	N (% dry wt)	P	ATP (µg/g)	DNA Synthesis (f moles/g)	Dehydrogenase (µ mole/h/g)
1	3.70	0.300	0.104	1.40	434	3.19
2	3.61	0.229	0.061	0.46	161	1.56
3	3.19	0.306	0.066	0.64	260	2.21
4	3.46	0.293	0.074	1.19	309	5.18
5	2.96	0.451	0.060	0.36	114	2.26
6	1.94	0.131	0.050	0.19	113	1.07
7	1.99	0.131	0.054	0.44	159	0.11
8	0.85	0.393	0.043	0.27	102	0.02
9	1.48	0.226	0.054	0.24	159	0.45

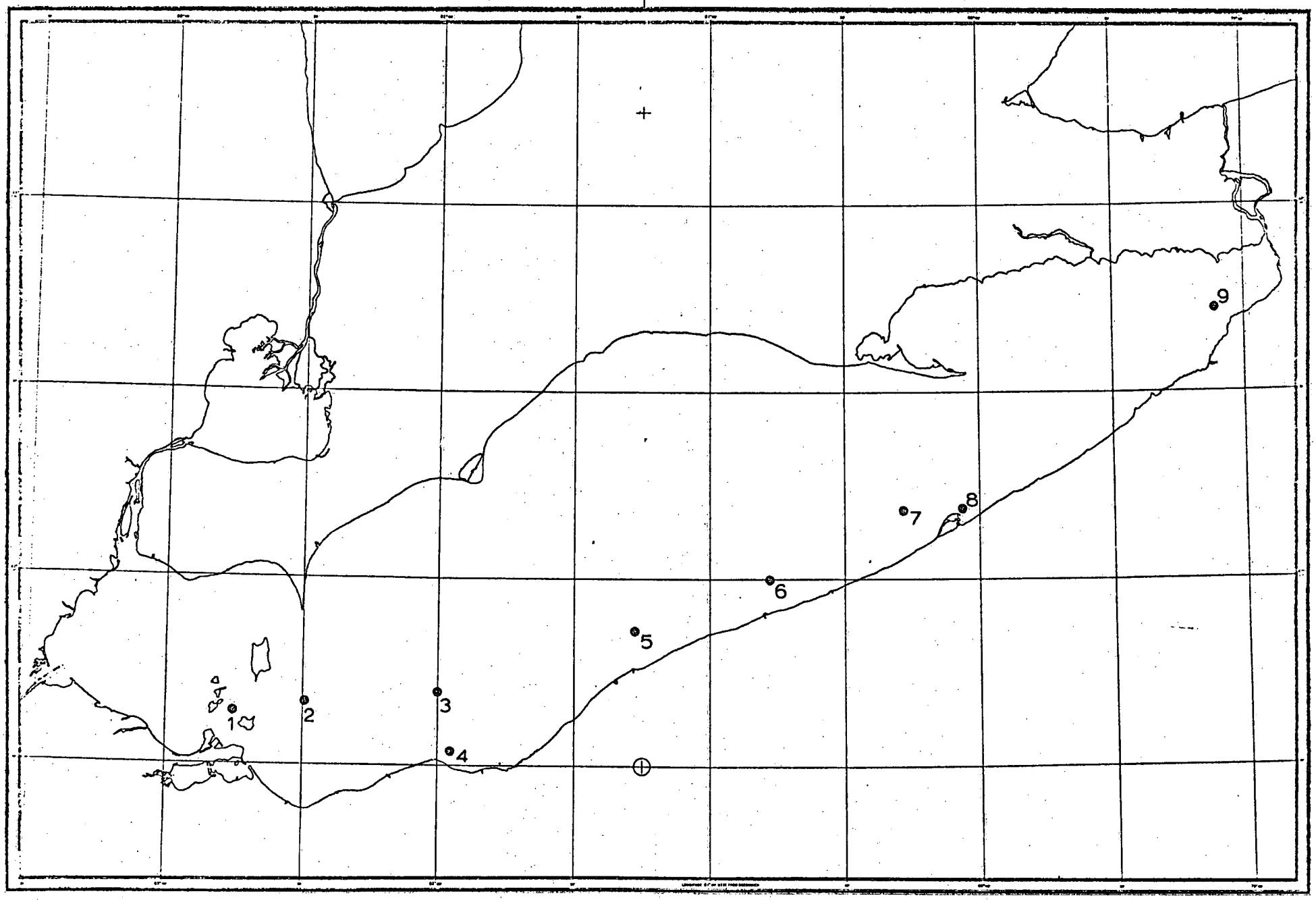

Figure 1. Lake Erie sediment station locations.

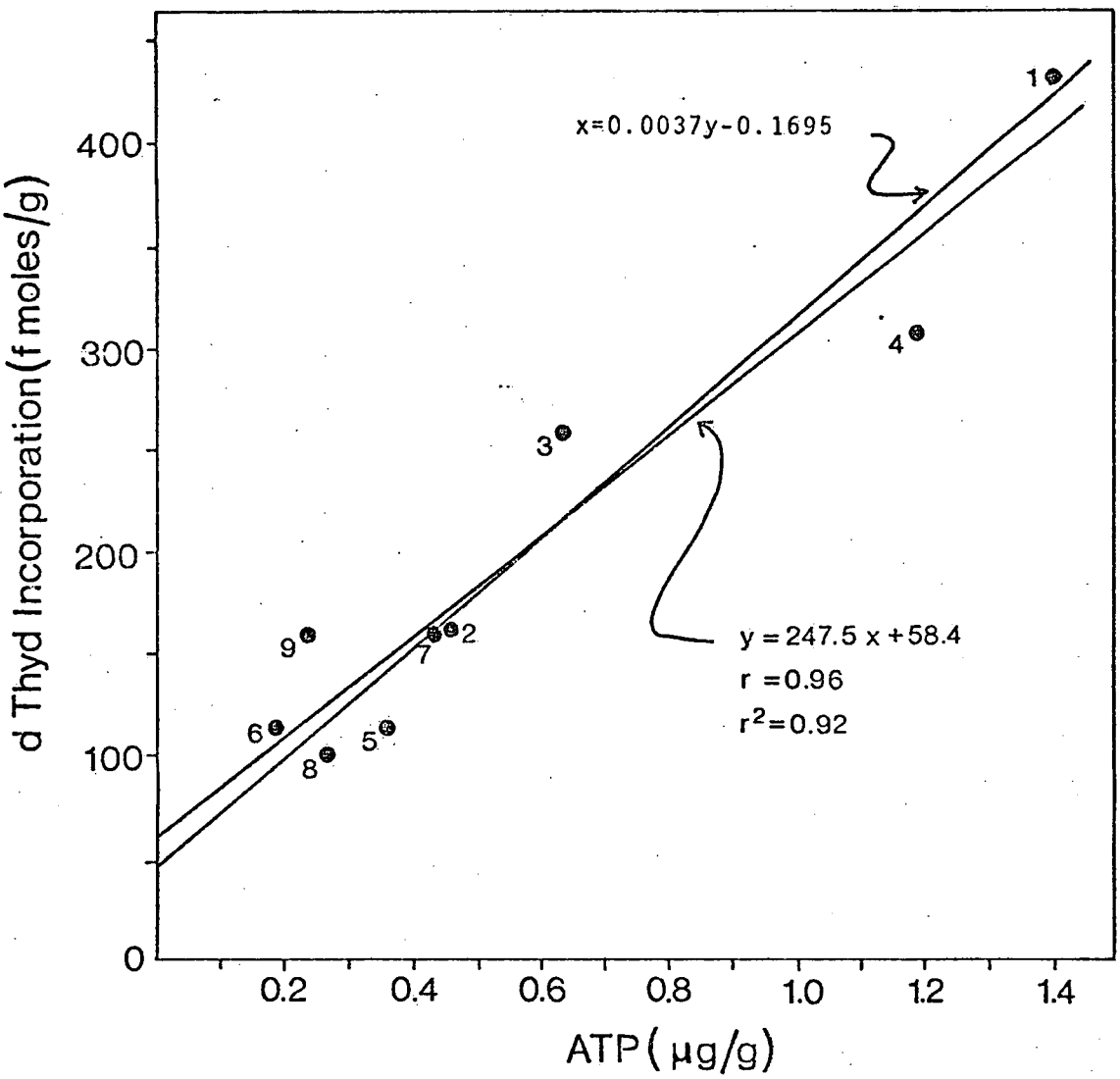

Figure 2. Correlation of DNA synthesis with ATP content at each sediment station. Numbers at each point signify the station number.

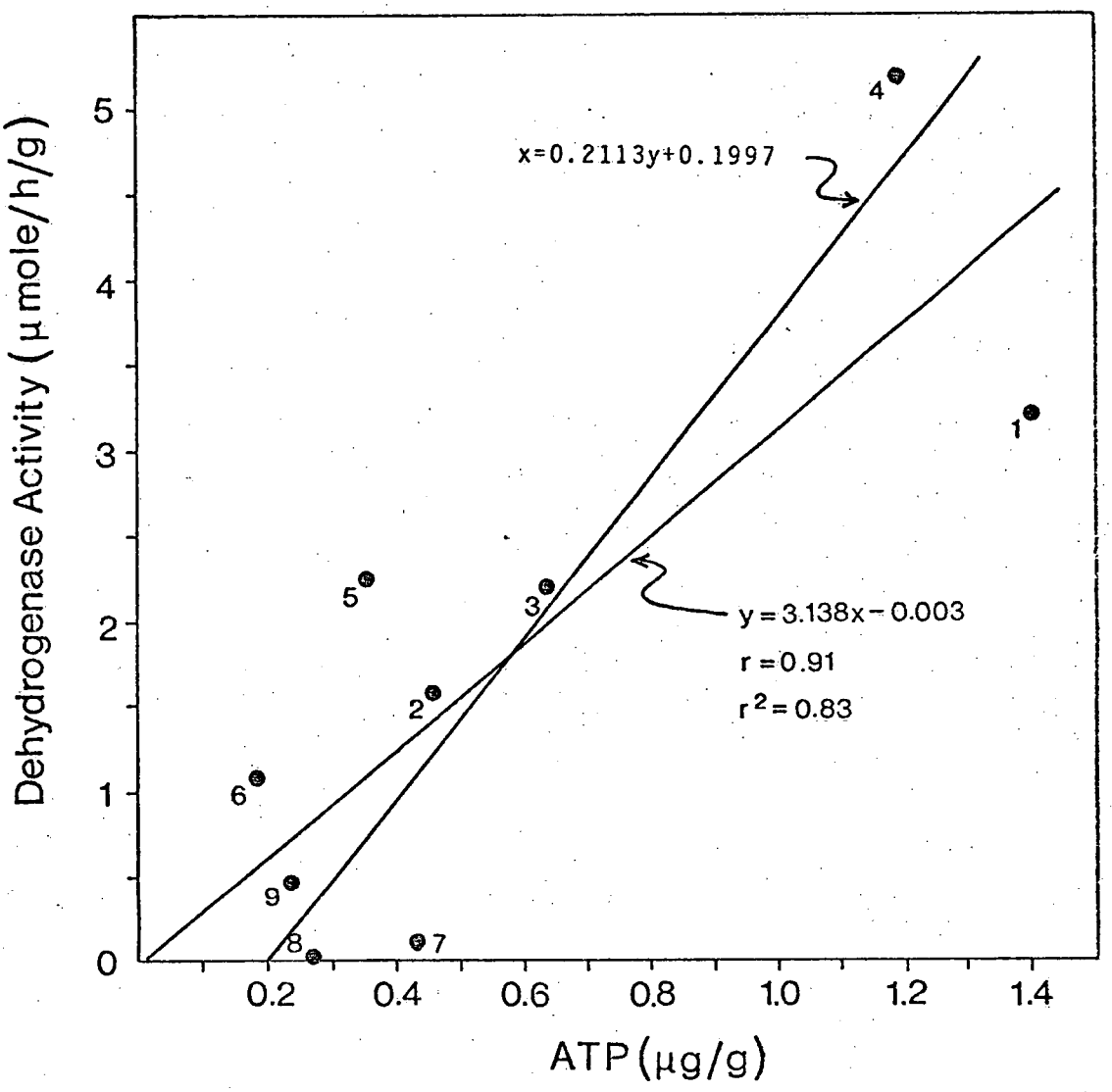

Figure 3. Correlation of dehydrogenase activity with ATP content.

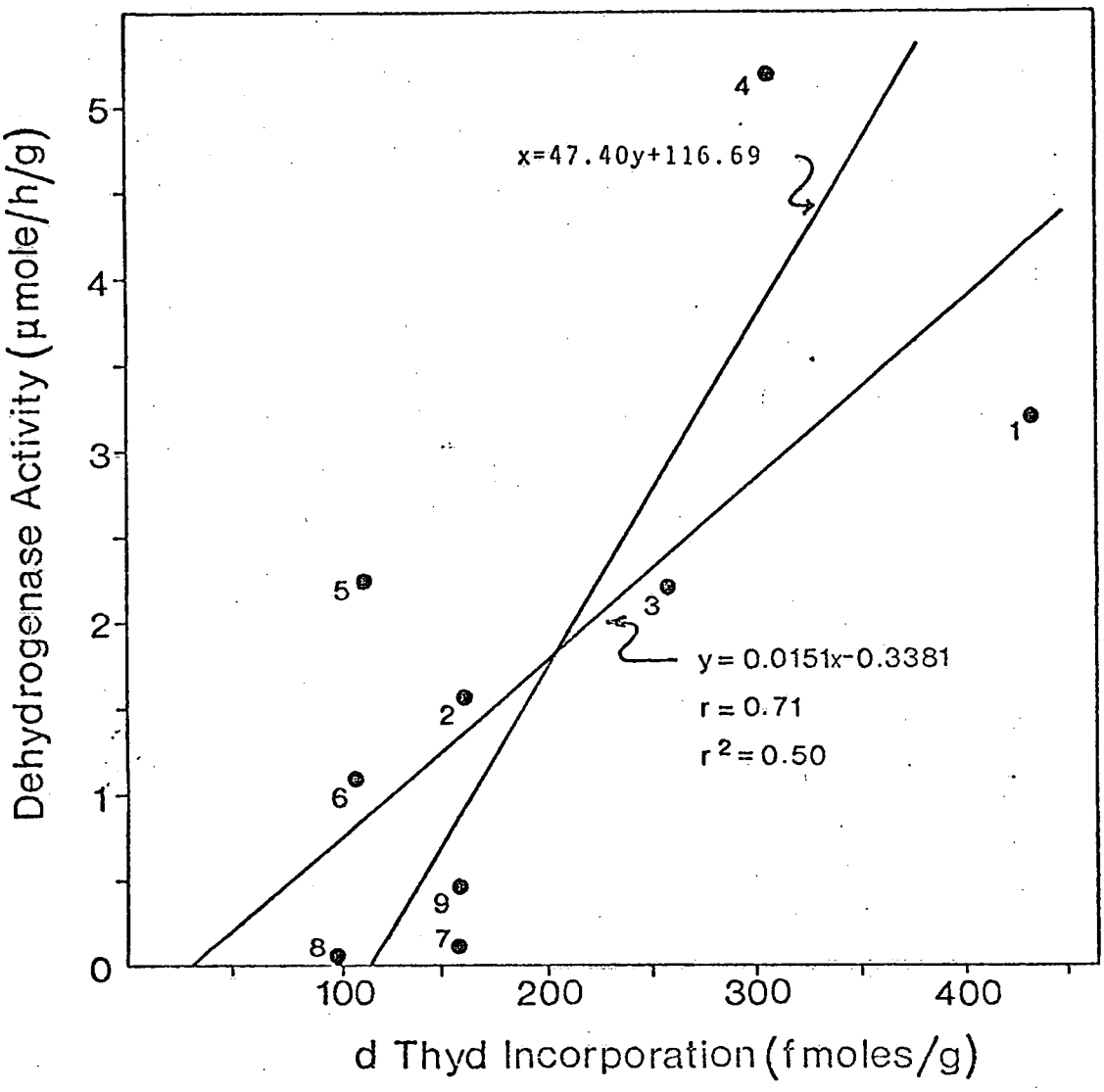

Figure 4. Correlation of dehydrogenase activity with DNA synthesis.

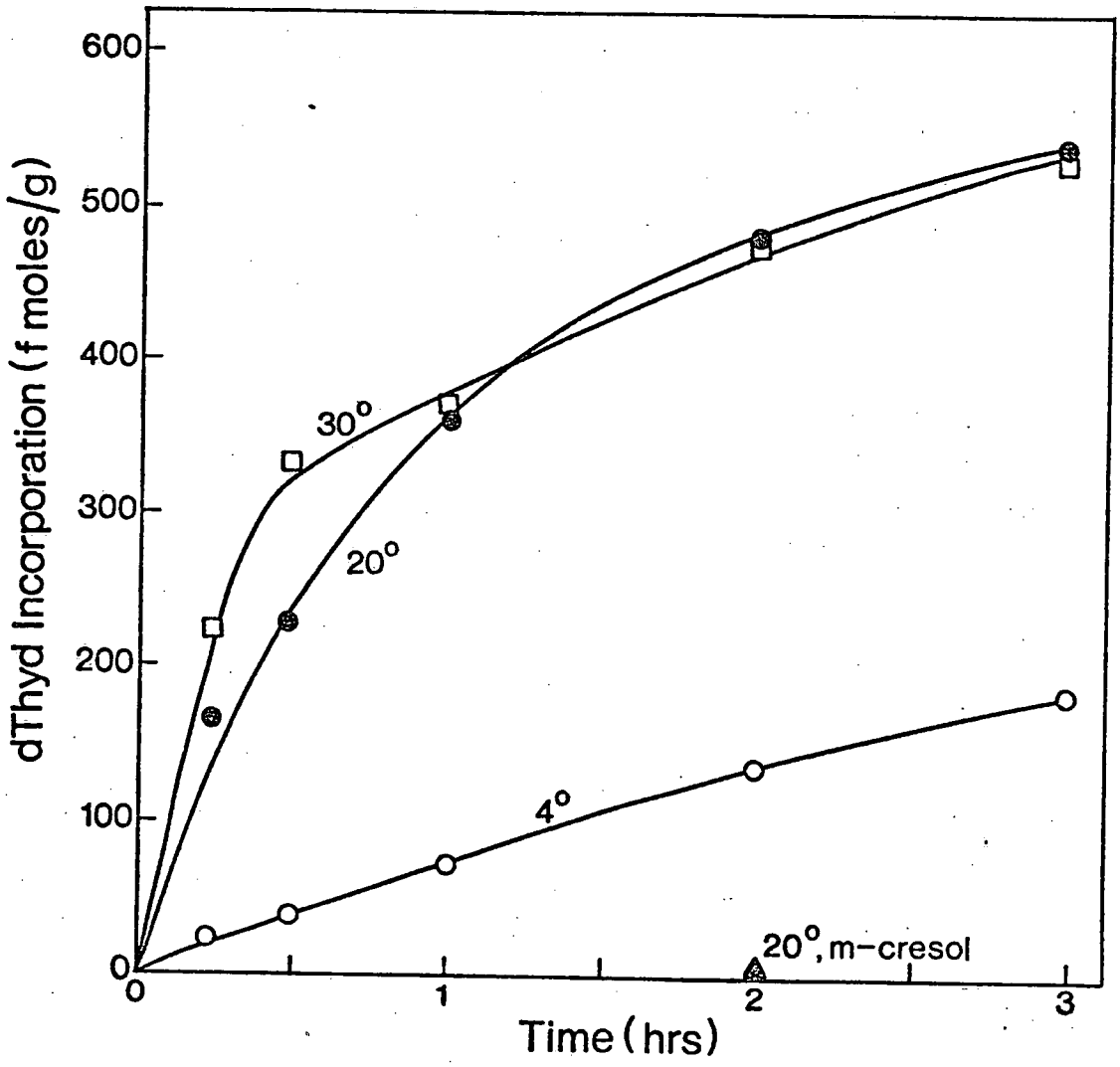

Figure 5. DNA Synthesis at different temperatures. Sediment from station 4 was incubation for various times up to 3 hours at 4°C (○), 20°C (◉) or 30°C (□). The control was incubated at 20°C for 2 hours in the presence of m-cresol (▲).

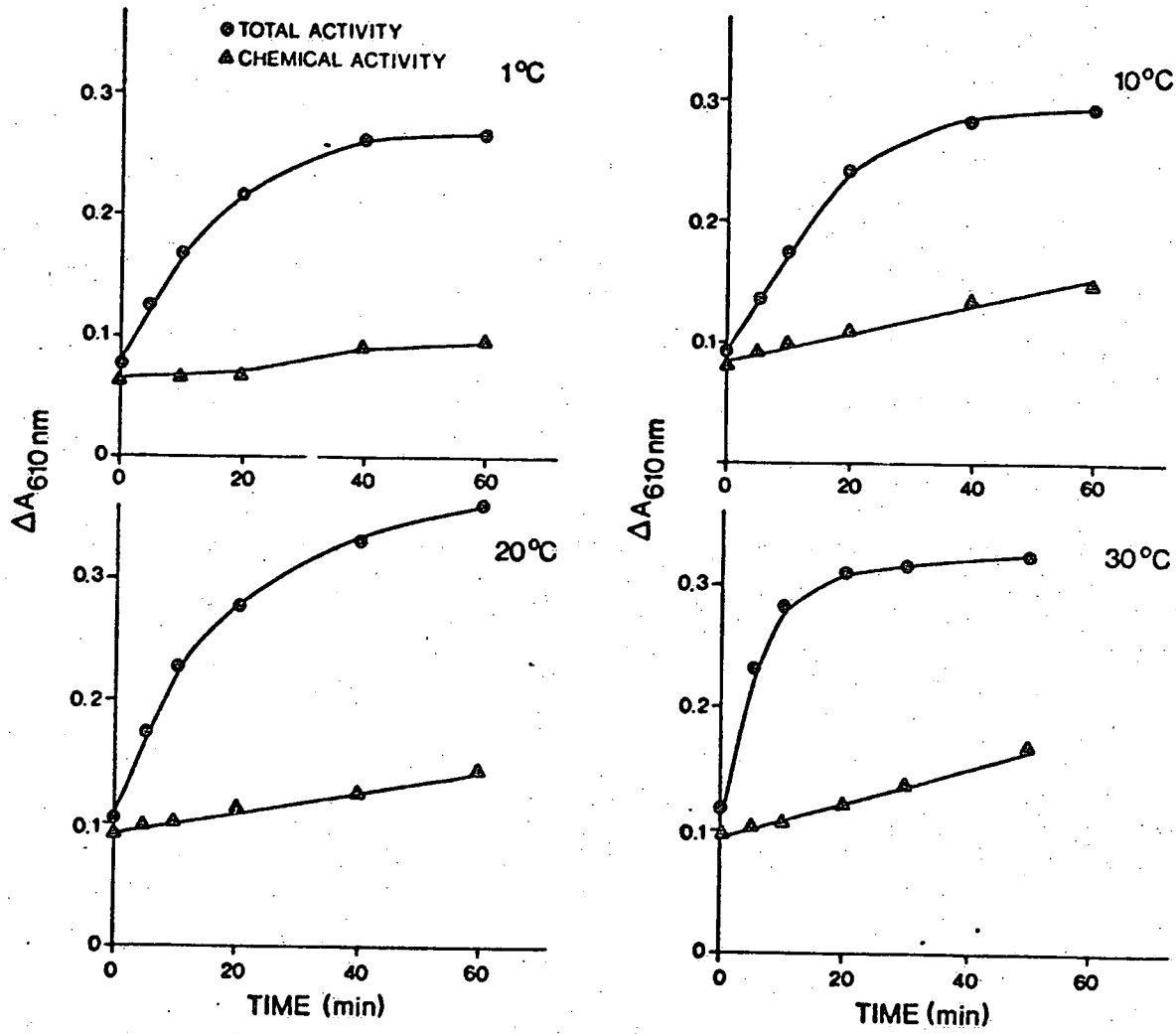

Figure 6. Dehydrogenase activity of sediment at several temperatures. Sediment from station 4 was incubated for various times up to 60 min in the presence (▲, chemical activity) or absence (●, total activity) of m-cresol. Biological activity is the difference between the total activity and chemical activity at a given time.

Appendix "A". Detailed procedure for the determination of DNA synthesis (dT incorporation).

1. Weigh 5.0g sediment directly into 50 ml capacity polycarbonate centrifuge tubes. Bring to room temperature.
2. Add 2.0 ml [^{3}H]dT to each tube. (Make [^{3}H]dThyd solution by diluting 1 mCi/ml, 5 Ci/mmole [methyl-^{3}H] deoxythymidine (Amersham-Searle TRA 120) 1000x with sterile deionized, distilled water and filter-sterilize with a 0.2 μm membrane in a disposable Nalgene filtration unit).
3. Mix sediment and [^{3}H]dThyd solution well with a glass rod.
4. Incubate 2h at 20°C. Mix by swirling racks twice during the incubation period.
5. Terminate the reaction with 8 ml 0.38N NaOH. Mix well and incubate 16h at 37°C to hydrolyze RNA and solubilize DNA.
6. Cool tubes to 4°C and maintain this temperature except where indicated. Centrifuge at 45,000 xg 15 min. Decant the supernatant into a 50 ml "Oak-Ridge" type screw top centrifuge tube, add 50 μl 2 mg/ml DNA (made up in 0.01N NaOH), mix, add 1.2 ml 4N HCl (to give final HCl concentration of 0.2N. After 10 min. in the cold, centrifuge at 45,000 xg, 15 min.
7. Aspirate the supernatant and wash the pellet 3x with 20 ml, 20 ml and 10 ml of 5% trichloroacetic acid, dispersing the pellet the third time with a glass rod. Centrifuge 45,000 xg for 15 min.

8. Aspirate supernatant. Add 1 ml 5% trichloroacetic acid and put in a boiling water bath for 25 min. to hydrolyze the DNA.
9. Cool to 4°C. Centrifuge at 45,000 xg for 15 min.
10. Transfer the supernatant to scintillation vials, add 15 ml PCS coctail (Amersham-Searle) and count with a scintillation counter. Counting efficiency must be determined for each sample because of variable quenching.
11. Calculations:

$$DPM_s = (CPM_s - CPM_{BG}) \times \frac{1}{E_s}$$

$$DPM_c = (CPM_c - CPM_{BG}) \times \frac{1}{E_c}$$

Incorporation (fmoles dThyd/g sediment, dry wt)

$$= \frac{(DPM_s - DPM_c)}{} \times \text{ratio } \frac{1}{\frac{\text{dry wt.}}{\text{wet wt.}}} \times \frac{1 \text{ fmole}}{11 \text{ DPM}}$$

Where CPM_{BG}, the counting rate of the machine with a clean vial, 15 ml PCS, but no sample, in counts per minute

CPM_s, the counting rate of the sample in counts per minute

CPM_c, the counting rate of the control in counts per minute

DPM_s, disintegrations per minute in the sample

DPM_c, disintegrations per minute in the control

E_s, sample counting efficiency

E_c, control counting efficiency

Appendix "B". Procedure for measuring dehydrogenase activity in sediment.

Reagent

(1) Dissolve 1 resazurin tablet (5 mg/tablet from B.D.H.) in 50 ml of glass distilled water (use 50 ml size volumetric flask) and keep the solution in brown bottle. The dye solution is stable for two days at room temperature and five days at 4°C.

(2) n-Amyl alcohol (Analytical reagent grade)

(3) m-cresol (Analytical reagent grade)

Procedure

1. Total activity (biological + chemical)

   (a) Weigh 0.10, 0.20 and 0.50 g sediment (wet wt) directly into 2x15 cm clean test tubes.

   (b) Add 3 ml of filtered lake water (use 0.45 µm filter) to each tube.

   (c) Add 1 ml of dye to each tube and the tubes are mixed with a vortex mixer for 5 sec.

   (d) The tubes are incubated at 20°C for 30 min. Followed by the addition of 10 ml of n-Amyl alcohol to extract the dye from the aqueous phase.

   (e) Use a pasteur pipette transfer approximately 5-7 ml of the top layer (organic solvent) into a clean test tube containing approximately 1 g $NaHCo_3$.

(f) Read absorbance at 610 nm on a spectrophotometer and subtract it from A_{610nm} of an unreacted sample = ΔA_{610nm} (sample).

2. Chemical activity

(a) Same as 1 (a).

(b) Same as 1 (b).

(c) Add two drops of m-cresol to each tube and mixed content vigorously with a vortex mixer for 30 secs.

(d) Same as 1 (c).

(e) Same as 1 (d)

(f) Same as 1 (e).

(g) Same as 1 (f).

Biological activity = total activity (A_{610nm}, sample) - chemical activity (A_{610nm}, sample + m-cresol).

3. Construction of standard curve

(a) Add 0.100, 0.200, 0.300, 0.500, 0.700 and 1.00 ml of resazurin solution to the marked test tubes. (1.00 ml dye = 100 µg).

(b) Add 3.90, 3.80, 3.70, 3.50, 3.30 and 3.00 ml of distilled water to the tubes. (final fluid volume in each tube = 4 ml)

(c) Same as 1 (d).

(d) Same as 1 (e).

(e) Same as 1 (f).

(f) Plot ΔA_{610nm} vs resazurin concentration.

Resazurin formula wt = 229.18

Dehydrogenase activity = μmole resazurin reduced /hr/g sediment dry wt.