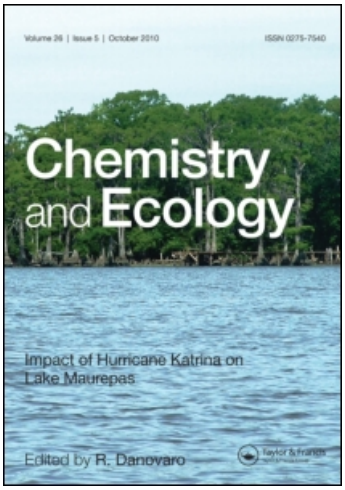

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Determination of Microbial Activity in Marine Sediments by Resazurin Reduction

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A simple method is presented to evaluate microbial activity in aquatic sediments. The method is based on resazurin reduction by microbial electron transport chains and reduced chemical compounds present in the sample. The addition of *m*-cresol, which inhibits enzyme activity, allows one to measure microbial metabolism by difference. Small aliquots of sediment (about 1 g FW) are incubated at 20°C with resazurin solution, with and without *m*-cresol. The sample is then filtered and the unreduced resazurin is measured at 600 nm.

Testing the method with a bacterial suspension gave a resazurin reduction of 89 μg/h/10^9 cells. In a few marine coastal sediment samples, the resazurin reduction in aerobic conditions was in the range of 0.31 to 219.3 μg/h/g DW, which is equivalent to an oxygen consumption of 0.02 to 15.32 μg/h/g DW.

INTRODUCTION

Microbial activity, acting both directly and indirectly, greatly affects the processes which occur in aquatic sediments.

By carrying out the degradation of organic matter, the microorganisms give rise to simple organic molecules, release nutrients, contribute towards the formation of humic substances and, through the aerobic metabolism, consume molecular oxygen. This leads to a modification of the existing conditions in the sediments, markedly influencing its chemical state and the behaviour of the ionic species which can be either fixed or mobilized. The microbial metabolism leads also to the direct incorporation of the bioavailable ions into

the cells and to the formation of new bacterial biomass which in turn serves as food for benthic organisms.

The measurement of microbial activity is therefore an important parameter in the study of aquatic sediments. Numerous methods have been developed for this purpose based on determination of the number of microorganisms (Dutka *et al.*, 1974), relationship to the quantity of specific cellular constituents like ATP (Holm–Hansen and Paerl, 1972; Pamatmat and Skjoldal, 1974), correlation with the metabolism of labelled compounds (Meyer–Reil, 1978), production of CO_2 (Inniss and Young, 1977; Fontvieille and Renaud, 1982), oxygen consumption (Pamatmat, 1971; Dale, 1978), measurement of dehydrogenase (Pamatmat and Bhagwat, 1973; Wieser and Zech, 1976) or electron transport system activity (Christensen and Packard, 1977; Olańczuk–Neyman and Vosjan, 1977), and microcalorimetry (Pamatmat, 1975). None of these methods is without difficulties including poor reliability of the correlation, unsatisfactory extrapolation of the results, methodological troubles, problems when applied in the field, unstandardized methodologies, etc. (see, e.g., Dale, 1978; Pamatmat, 1980; Bowman and Delfino, 1980; Pamatmat *et al.*, 1981; Es and Meyer–Reil, 1982). So the research for new methods still remains open as do the refinement and the improvement of the existing ones.

With this in mind, we took as base the method of Liu and Strachan (1979) for lacustrine sediments and set up a method which allows the microbial activity in marine sediments to be determined in a simple way. It is based on the same principle used in the determination of electron transport activity, that is the measurement of the reduction of a chemical compound by means of hydrogen ions transported along the cellular respiratory chain. The amount of reduced compound allows the assessment of the metabolic activity of the microorganisms present in the sediment sample under examination. Generally, in the determination of the electron transport system activity (e.g., Christensen and Packard, 1977) the compound which undergoes reduction is the 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) which changes into the water-insoluble INT-formazan; therefore, it is necessary to add some Triton X-100 preliminarily in order to take the INT-formazan into solution as it is produced. This method requires, besides enzymes extraction, centrifugation at low temperature and

addition of substrates, and gives a maximum potential measure as result. Trevors (1984) incubates directly the INT with the sediment without any artificial addition or manipulation; however, in this case the insoluble formazan being produced must be extracted by means of an organic solvent at the end of the incubation period so that it can be measured.

In the method described here, we use the dye resazurin which is reduced quantitatively by means of both the microbial respiratory chains and the reduced chemical substances that may be present in the sample. The remaining unreduced resazurin is then measured spectrophotometrically and the biological and chemical reductions are differentiated by means of a metabolic inhibitor, the *m*-cresol (Liu and Strachan, 1977).

The method described does not require any special manipulation and can be used in the field on sediment samples as they are taken. Besides, as it does not require the addition of substrates, or the preliminary extraction of the enzymatic complex, it can give a suitable measure of the microbial activity, determinable with ease and relative rapidity and inexpensiveness. For this reason, the reduction of the resazurin can be an index of the metabolism of the microorganisms present in the samples, useful for a better knowledge of the ecologically relevant processes occurring in aquatic sediments.

MATERIALS AND METHODS

The sediment samples have been taken by means of a gravity corer and examined in the laboratory within 4 hours.

The invertebrates if present were removed, and then a selected portion of the sediment was accurately homogenized by means of a clean spatula. Sediment aliquots (0.5–2 g FW) were introduced into two test tubes containing 1 ml of 0.45 μm filtered natural seawater and mixed by Vortex mixer for 10 seconds. Then 100 μl of *m*-cresol (Carlo Erba RPE) were added to one tube and 100 μl of filtered seawater to the other. After mixing for 10 seconds, the inhibitor was left to take effect for 15 min. After that, exactly 1 ml of 0.01% resazurin solution (2 tablets, BDH Chemicals LTD Poole, dissolved in 50 ml of filtered seawater) was added and the tubes were shaken

again for 10 seconds. The tubes were then incubated at 20°C for 1 hour. The environmental samples were generally analyzed in duplicate. After the incubation, the samples were filtered through a 0.8 μm pore size Millipore membrane and rinsed with filtered seawater. The liquid passed through the filter was brought to 250 ml with filtered seawater and its absorbance was read at 600 nm by using a colorimeter (Technicon Autoanalyzer). In the case of the samples without *m*-cresol, the readings give the total activity of the sediment, that is the sum of biological and chemical reduction of the resazurin. The chemical reduction was determined from the samples treated with *m*-cresol. By subtracting the chemical activity from the total activity one can calculate the microbial activity expressed as μg of reduced resazurin/h/g DW. The dry weight was determined by drying the sediment overnight in an oven at 60°C, and then weighing to ±1 mg.

The spectrophotometric readings were transformed to resazurin amounts by comparison with a reference curve (Figure 1).

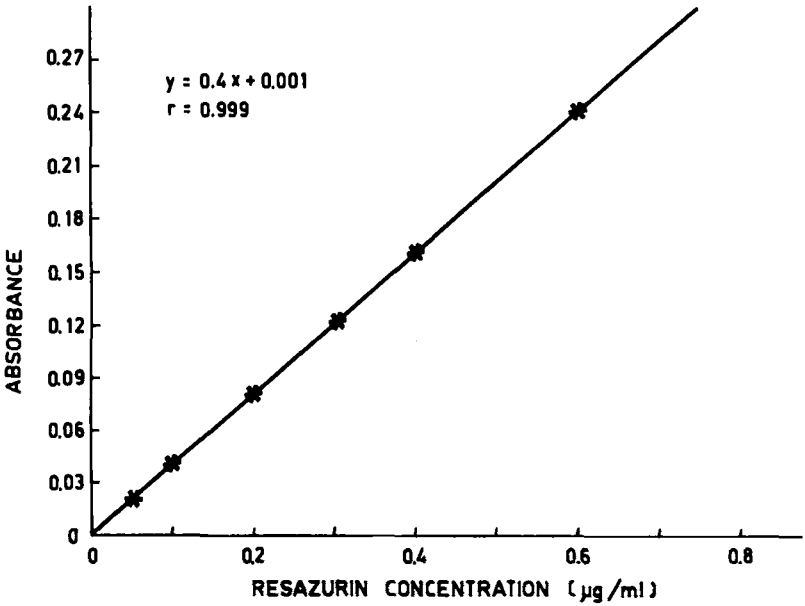

FIGURE 1 Reference curve of resazurin.

The method was tested by means of a bacterial suspension of the strain lambda (*Pseudomonas* genus) of our laboratory collection. The strain was grown in liquid OZ medium (Peroni and Lavarello, 1975) for two days, harvested by centrifugation, washed three times with sterile seawater and resuspended in sterile seawater at a final concentration of 10^9 cells/ml, as determined by plate counts on agar-OZ medium. Various aliquots of bacterial suspension were added to 1 ml of resazurin solution, brought to a final volume of 5 ml with seawater and then treated according to the procedure described above.

Resazurin reduction was also compared with dehydrogenase activity (Peroni and Ruggiero, 1981) and direct oxygen consumption in intact cores using a device as described by Bowman and Delfino (1980), glutaraldehyde being applied to discriminate biological consumption from the chemical one. The samples for the comparison were collected near La Spezia (Ligurian Sea) in late spring at two sampling sites: the "Olivo Bay", inside the Gulf of La Spezia, and the open-sea station "Le Rosse" near the coast at 30 m water depth.

RESULTS

Figure 2 shows that the maximum absorbance of unreduced resazurin is at about 600 nm (a), whereas the maximum absorbance of resorufin, which is the reduced form of resazurin, is at about 570 nm (b). Resorufin contributes to the absorbance at 600 nm insignificantly (c); therefore, the spectrophotometric readings of resazurin are not influenced by the resorufin produced during incubation.

A test of the method with the bacterial culture of strain lambda shows (Figure 3) that there is a good correlation between the reduction of resazurin and the concentration of bacterial cells. On the other hand, no reduction was noted when *m*-cresol was added to bacterial suspension.

By applying the method to sediment samples, a linear relationship can be observed between resazurin reduction and incubation time, at least up to 2 hours (Figure 4). Figure 5 shows the linear relationship between the amount of reduced resazurin and the quantity of sediment as well as the inhibitory effect of *m*-cresol.

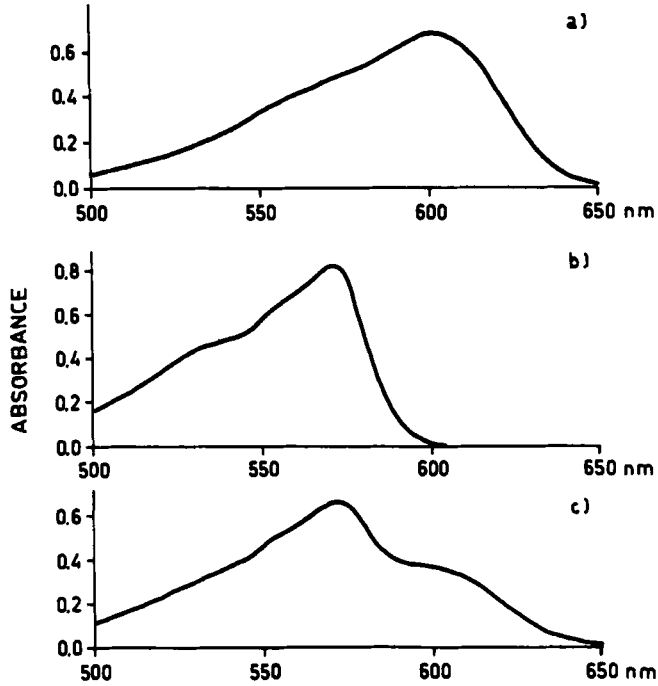

FIGURE 2 Absorption spectra of: (a) resazurin solution (5 μg/ml); (b) resorufin solution (5 μg/ml); (c) mixture of resazurin and resorufin solutions in equal parts, each at a concentration of 2.5 μg/ml.

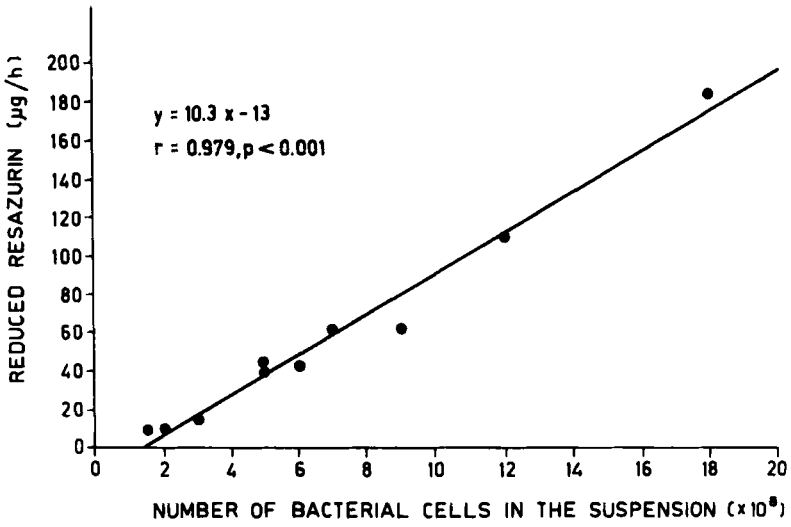

FIGURE 3 Resazurin reduction by bacterial strain lambda suspension.

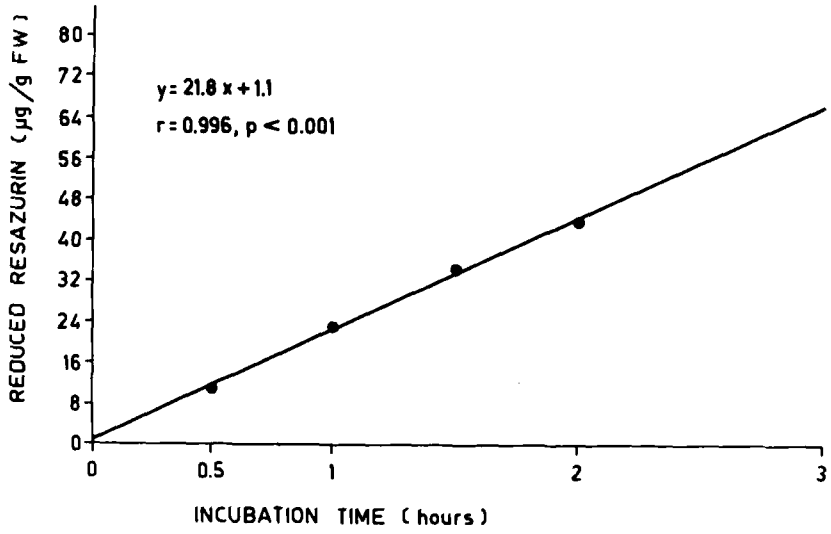

FIGURE 4 Resazurin reduction versus incubation time.

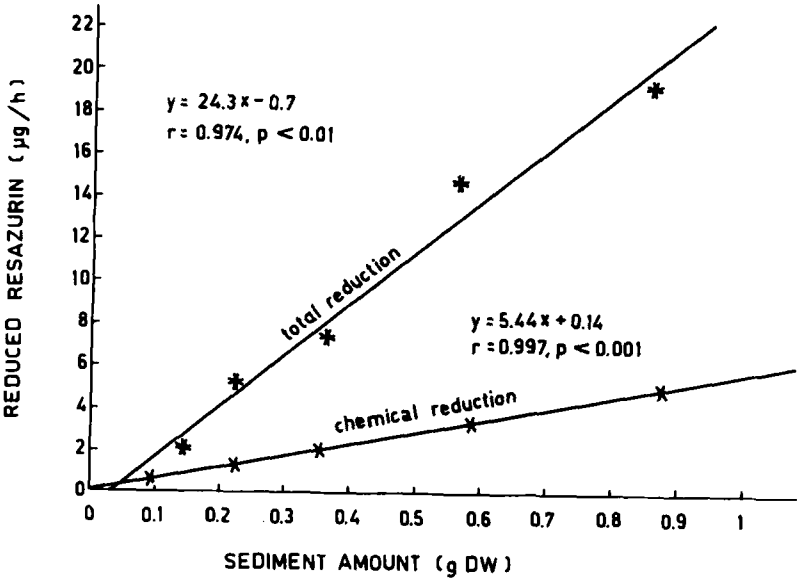

FIGURE 5 Total and chemical reduction of resazurin by a natural sediment sample in relation to sediment amount.

TABLE I
Oxygen consumption by sediment samples as determined by different methods

Sampling site	Resazurin reduction (μg equivalent O_2/h/g DW)	Dehydrogenase activity (μg equivalent O_2/h/g DW)	Direct oxygen consumption ($\mu g/g/cm^2$)
"Olivo Bay"	1.49	5.11	4.34
"Le Rosse"	1.23	3.79	1.12

Table I shows the results obtained by applying the methods of resazurin reduction, dehydrogenase activity, and direct oxygen consumption to samples taken from the same stations.

DISCUSSION

The reduction of resazurin in physiological conditions takes place in two steps, the first of which (the transformation from resazurin to resorufin) is irreversible (Twigg, 1945). This allows the assessment of the biological reduction activity due to dehydrogenasic enzymes of the microorganisms present in the sediment sample. Since the reduction of resazurin to resorufin is irreversible, no caution is necessary to prevent its reoxidation. This, in fact, is an inconvenience of the method described by Jørgensen (1984) who uses methylene blue reduction for the determination of dehydrogenase activity.

The advantages of resazurin reduction procedure over the tests using tetrazolium salts reduction have been stressed by Liu (1983). The method we have adopted modifies that of Liu and Strachan (1979) by eliminating the step of resazurin extraction, which is tedious and furthermore can influence the reaction of resazurin reduction through the sharp lowering of the pH and the consequent alteration of the redox conditions of the sediment. The interference in the spectrophotometric determinations due to the turbidity of the sediment has been avoided by filtering.

Molecular absorbance of resazurin (ε) is high (about 93,000), so it can be measured accurately. The coefficient of variation at a resazurin concentration of 0.4 μg/ml was 1.1% ($n = 6$). The standard deviation of the method is given in Table II.

TABLE II
Microbial activity in the surface layer of sediment samples collected near La Spezia (Ligurian Sea)

Sampling site	Microbial activity in μg/h/g DW as reduced resazurin	Microbial activity in μg/h/g DW as O_2 equivalents	Sediment type
Portovenere	16.3 ± 2.1 ($n = 5$)	1.14	mud
Punta Bianca	25.6 ± 4.1 ($n = 6$)	1.86	fine silt
Le Grazie	2.3 ± 0.1 ($n = 4$)	0.16	sand
Lerici	5.0 ± 0.8 ($n = 5$)	0.35	silt

No apparent toxicity of resazurin against bacteria could be observed. In fact 1 ml of 0.025% resazurin solution added to 5 ml of fresh sediment suspension gave no difference in colonies number by plate counts as compared to a similar suspension without resazurin.

The inhibition by *m*-cresol was simply assessed by plating a fresh sediment suspension after incubation with 100 μl of *m*-cresol for 15 minutes; less than 1% of colonies was counted in comparison with the same suspension incubated without *m*-cresol.

Since the reduction of resazurin to resorufin is, like the reduction of INT, a 2-electron process, the biological reduction of one molecule of resazurin may be equated with the reduction of one half molecule of oxygen, assuming that biological reduction is entirely due to aerobic respiration under our experimental conditions. Hence, from the quantity of the biologically reduced resazurin it is possible to calculate the amount of oxygen consumed by the microorganisms present in the sample under examination.

Gillett *et al.* (1983) found that dissolved oxygen uptake and resazurin reduction assays gave highly comparable results with pure or mixed cultures of microbes in estimating pollutant toxicity. This confirms the assumption that resazurin reduction can be a measure of respiratory activity of microorganisms. Also, the test with the bacterial strain lambda suspension (Figure 3) points out that cellular dehydrogenase activity is in effect responsible for the observed reduction of resazurin. Oxygen consumption by strain lambda suspension, calculated from resazurin reduction measurement, is equal to about 6.4 μg/h/10 cells, a value comparable to that obtained in growing bacterial cultures by Christensen *et al.* (1980) with the INT reduction method.

Also the comparison with dehydrogenase activity and direct oxygen consumption (Table I) shows the reliability of resazurin reduction method. In the case of the "Le Rosse" sample, oxygen consumption calculated from resazurin reduction is similar to direct consumption, whereas it is lower, approaching 35%, in the case of the "Olivo Bay" sample. It is to be considered, however, that the samples collected in the "Olivo Bay", which were tested without any manipulation, contained a few small invertebrates which likely contributed to the high oxygen consumption, whereas they were removed before the resazurin reduction test. Furthermore, the measure units are not strictly the same, being related to the surface for direct oxygen consumption and to the dry weight for resazurin reduction. Also this fact may partly account for the difference observed in the "Olivo Bay" sediment, due to the heterogeneity of its redox conditions in the 0–1 cm layer. The "Le Rosse" sediment is more homogeneous and its aerobic layer extends much more in depth; therefore, resazurin reduction and direct oxygen consumption measurements are more comparable and in fact give similar results. Resazurin reduction and dehydrogenase activity are well correlated in both stations, though the latter is higher since it is a measure of potential activity.

Figure 4 and 5 show the suitability of the resazurin reduction method for determining biological activity in the sediments. In the tests described in these figures the maximum quantity of sediment used was 1 g FW. The linearity, however, can persist even with larger quantities since both the optimal incubation time and the quantity of sediment are dependent on the type of sediment under examination. In fact a higher microbial activity is usually found in fine-grained sediments compared to sandy sediments (e.g., Hargrave, 1972; Dale, 1974; Peroni and Ruggiero, 1981) and in shallow coastal sediments in comparison with deep and offshore sediments (Christensen and Packard, 1977), since it is linked to the specific surface area and to the supply of metabolizable organic matter. Therefore, muddy sediments may require lower amounts of sample and/or shorter incubation times to give an appreciable reaction as compared to those necessary for sandy sediments. However, it is possible to assess visually a change in the colour whether or not there has been any reduction of resazurin, and one can decide to prolong the incubation time accordingly.

Table II summarizes the results obtained with the resazurin reduction method in a few coastal sediment samples from the La Spezia area. The fine-grained sediments show a higher microbial activity. The highest activity was found in the sample taken at Punta Bianca off the mouth of the Magra River. The waters of the river could exert an analogous action to that described by Valdés and Albright (1981) in a similar situation, in which fluvial waters activate the bacterial metabolism in the marine waters into which they flow, through the supply of nutrients and organic matter.

Table III summarizes the data obtained by applying the resazurin reduction method to the determination of microbial activity in the surface layer (0–1 cm) of sediment samples collected during the oceanographic cruises carried on by our laboratory. When expressed as biological oxygen consumption, the values vary from 0.02 μg/h/g DW in samples made of coarse sand to 15.32 μg/h/g DW in a muddy station off the Po River mouth in the North Adriatic Sea. These values, as far as the muddy samples are concerned, are comparable to those obtained from measurements of *in situ* biological consumption of oxygen (Pamatmat, 1971; Smith *et al.*, 1972; Dale, 1978; Es, 1982), while most of them are lower by one order of magnitude than those of Christensen and Packard (1977) for samples taken off the coast of the Spanish Sahara at depths of 30 m and treated according to the INT reduction method which gives a maximum potential activity.

TABLE III

Microbial activity in the surface layer of sediment samples collected during the oceanographic cruises carried on by S. Teresa ENEA Center

Sampling area	Period	Microbial activity in μg/h/g DW as reduced resazurin	Microbial activity in μg/h/g DW as O_2 equivalents	Sediment type
South Adriatic Sea (Apulian Coast)	Oct. 82	0.31– 8.03	0.02– 0.56	sand
(Apulian Coast)	Oct. 82	6.80–173.02	0.47–12.09	mud
North Adriatic Sea	Feb. 83	29.24– 75.29	2.04– 5.38	mud
North Adriatic Sea	Oct. 83	18.52– 48.08	1.29– 3.36	mud
North Adriatic Sea	June 84	111.10–219.32	7.76–15.32	mud
Tyrrhenian Sea	Feb. 84	9.42– 43.21	0.66– 3.02	mud
(Gulf of Gaeta)	Feb. 84	8.86	0.62	silt

The simplicity of this method, its relative speed, its inexpensiveness and its sensitivity are factors which contribute to making it a suitable method of assessment of microbial activity in the sediments, especially in the field, when it is necessary to process many samples quickly. Furthermore, the sensitivity of the method may be increased by determining fluorometrically the amount of produced resorufin instead of that of disappeared resazurin (Guilbault and Kramer, 1965).

It must be pointed out, however, that the present method allows the assessment of the metabolic activity of the aerobic and facultative anaerobic bacteria, but not that of the strict anaerobic bacteria. Since the metabolism of the latter is important in the subsurface layers and in reducing environments, which, according to Jørgensen (1977), are present as microniches even in the oxidized surface layers of the sediments, we are now studying the possibility of treating and incubating the samples also in anoxic conditions. In this way, it would be possible to obtain an estimation of the total microbial activity resulting from the aerobic and anaerobic resazurin reduction by aliquots of the same sediment sample. Furthermore, the chemical activity under anoxic conditions could represent a measure of the reduction depot of the sediment under examination (Dechev and Matveeva, 1981).

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