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Measurement of plankton O_2 respiration in gas-tight plastic bags

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ABSTRACT: Two opaque and two transparent types of plastic material, developed for the food industry, were examined for applicability in plankton experiments. The plastic bags were tested for oxygen penetration, physical strength, and bacterial growth in the water phase and on the walls. The oxygen concentration in the bags was determined by a mini-Winkler method, where titrations were carried out in 5 ml pycnometer bottles. The standard error of replicate determinations was 0.2 % achieving significant respiration measurements down to 0.6 µM O_2 d^{-1}. The oxygen penetration into a 3 l opaque aluminium foil bag was 0.08 µmol O_2 d^{-1} or < 1.3 % of a typical plankton respiration rate of 2 µM O_2 d^{-1}. When the oxygen concentration in the bags was lowered to about 10 % of air saturation, no oxygen penetration could be recorded after 16 d. The penetration rate into 3 l transparent ethylvinyl alcohol bags under the same oxygen gradient was 0.7 µM O_2 d^{-1} when dry on the outside. An incubation effect on bacterial growth and respiration was observed after 3 d in the bags, and after 14 d the walls were heavily colonized by bacteria. In a coastal field study where plankton respiration was determined using alufoil bags, oxygen uptake rates >4 µM O_2 d^{-1} were measured during a winter resuspension event at 5 °C, and values >10 µM O_2 d^{-1} were recorded in the bottom water at 14 °C following a subsurface *Ceratium* bloom. The use of aluminium foil bag material is recommended for short-term pelagic experiments, with a maximum incubation period for respiration measurements of 48 h.

INTRODUCTION

Oxygen deficiencies due to eutrophication in many coastal waters (Rosenberg 1985) have increased the interest in the role and dynamics of oxygen in the marine environment. Measurements of size-fractionated plankton respiration rates are an important tool for studying microbial heterotrophic activity and in evaluating the marine carbon cycle (Hopkinson et al. 1989). The transport of oxygen into sediments is important for reoxidation of the reduced products of anaerobic decomposition processes (Jørgensen 1983). Much effort has been expended to improve the techniques for measuring natural oxygen uptake rates, which are a few µM O_2 d^{-1} or around 1 % d^{-1} in coastal waters.

Parallel dark incubation and Winkler analysis of 2 sets of 3 to 6 calibrated bottles has been the method of choice in most plankton respiration studies (Williams 1984, Jensen et al. 1990). Respiration studies utilizing sensitive pulsed oxygen electrodes (Langdon 1984) have been reported just a few times. The bottle incubation method can only be carried out under the assumption that the rather time-consuming filling of parallel bottles is a true parallel incubation and that there are no concentration differences between the 2 sets of bottles either at the beginning or at the end of the incubation period.

The aim of this study was to develop an incubation technique for plankton respiration studies which avoids parallel bottles in which gases other than oxygen (e.g. carbon dioxide) and biological parameters (e.g. heterotrophic activity and grazing) could be measured in the same water at the same time. Plastic bags have previously not been used in experiments which include rate measurements of dissolved gases, presumably because of expected problems with gas penetration through the plastic and concern for toxic effects of plasticizers leaching from the inner side. In this

study, different laminated plastic materials developed for the food industry were examined for oxygen penetration, physical strength, and bacterial growth. If transparent material could be used, this would open possibilities for simultaneous measurements of photosynthesis and respiration.

Since the introduction of a precise Winkler procedure (Bryan et al. 1976) and the first shipboard use of microprocessor-controlled titration systems (Williams & Jenkinson 1982) the rapid technical development of auto-titration systems has made it possible for many authors to carry out pelagic oxygen respiration experiments (Culberson & Huang 1987, Oudot et al. 1988). The applicability of seawater incubation in plastic bags under *in situ* conditions for respiration investigations and the use of computer-controlled O_2 mini-Winkler titrations are discussed.

MATERIALS AND METHODS

Bag incubation setup. The characteristics of the 4 materials tested are given in Table 1. The inner side of 3 of the bags tested consisted of a low-density polythene (polyethylene) laminate. This material is free of plasticizers and approved for use in direct contact with foodstuff. Polyethylene is generally accepted as non-toxic for marine organisms, and widely used in water samplers (Grasshoff 1983) and as the material for the Chopstic sterile bag water sampler developed by General Oceanics. The toxicity of the fourth bag material (polyvinyl fluoride) for marine organisms was not indicated by the supplier.

The laminated plastic sheaths were cut into 2 identical pieces, and the sides with the inner sealing-laminate were determined. A 0.5 cm diameter hole was cut in the center of one piece and a pipe stub of stainless steel, with an inner teflon washer and an outer nylon washer, was attached. The pipe stub was connected to a 1 m long (1 mm i.d.) gas-tight Tygon tube, which was protected from light by an outer 5 mm rubber tube. A steel clamp kept the inner tube closed at the end. The inner sides of the plastic were sealed together by use of an impulse-heat-sealing machine with 30 cm sealing jaws at 190 °C on 3 sides. The sealing time took approximately 1 s.

Immediately before filling and incubating, the inner side of the bags and tubes was washed with water from the water sampler. The bags were then filled, allowing 1 l of overflow, through a 0.5 cm diameter silicone tube which was immediately placed at the bottom of the bag to minimize contact with the air. No air bubbles were allowed to attach to the inner surface of the bag during the filling procedure. The filled bags were then sealed approximately 10 cm from the opening so that no air was trapped inside. The bags were placed in dark water-baths (± 0.1 °C), and subsamples were taken after the temperature had equilibrated to *in situ* conditions (Fig. 1A).

Mini-Winkler technique. Subsamples for oxygen concentration analysis were taken from the bags using the gas-tight Tygon tubing, which was carefully placed at the bottom of acid-washed 5 ml pycnometer bottles, after 1 volume washing and 4 volumes overflow. The degree of overflow was chosen after evaluation of results from a small test series, in which coloured sea-

Table 1. Characteristics of the bag materials examined. The O_2 penetration from outer to inner surface is calculated from data given by the manufacturer in µmol O_2 m^{-2} d^{-1} at atmospheric pressure, 75 % relative humidity, and 25 °C

Type, name, and description	Laminated structure (outer to inner)	Thickness (µm)	O_2 penetration
Metal film: 'Conotainer, 4-ply, metallic'; opaque	Polyamide	15	<12.2
	Aluminium	0.03	
	Polyester	12	
	Low-density polythene	70	
	Total:	ca 100	
Alufoil: 'Lamofoil M'; opaque	Polyester	12	<0.16
	Aluminium	9	
	Low-density polythene	70	
	Total:	ca 90	
EVA: 'Riloten X'; transparent	Polyamide	36	6.2
	Ethylvinyl alcohol	8	
	Polyamide	36	
	Low-density polythene	100	
	Total:	ca 180	
PVF: 'Tedlar'; transparent	Polyvinyl fluoride (solid)	50	200

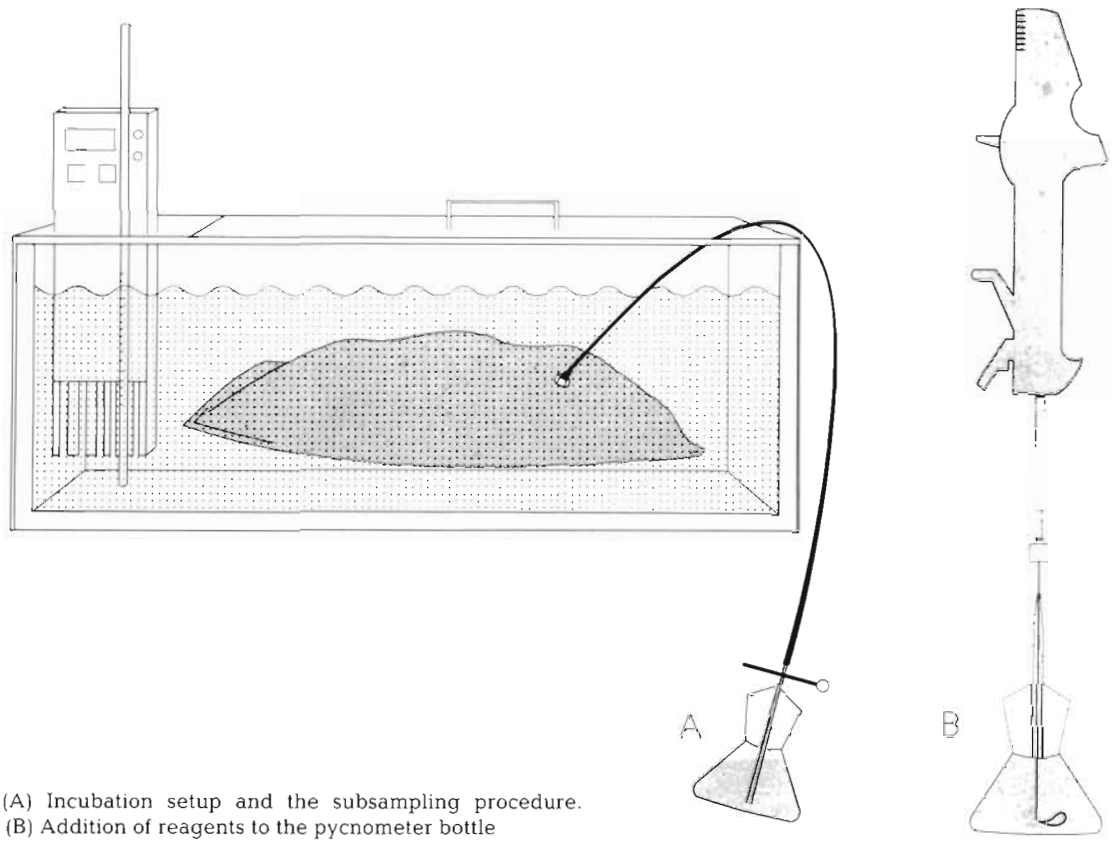

Fig. 1. (A) Incubation setup and the subsampling procedure. (B) Addition of reagents to the pycnometer bottle

water was washed out of the pycnometer. Also, the Tygon tubing was washed to remove its toxic effect on bacterial growth, reported by Price et al. (1986). Winkler reagents were added immediately and the pycnometer was stored under water within 10 min until analysis. The general principles and reagents of Carrit & Carpenter (1966) were followed, except that we added only 50 µl of reagents I and II and 100 µl of reagent III in the 5 ml samples. The addition was performed through the narrow pycnometer stopper channel by a GC hypodermic needle mounted on an automatic pipette (Fig. 1B). The stoppers were carefully removed before titration so that none of the iodine complex formed in the stopper-channel fell down into the pycnometer itself. The titration with thiosulfate was performed directly in the pycnometer with an autotitrator for potentiometric titration, where the platinum and kalomel electrodes and the delivery tip were modified for entering the narrow pycnometer neck. The apparatus was programmed to carry out 3 overtitration steps to calculate the inflection point. Parallel subsampling and titrations of water from the same plastic bag were continued until the oxygen concentration was determined with a coefficient of variation (CV) of around 0.3 %. This was normally achieved after 3 to 5 subsamples.

Oxygen penetration tests. The oxygen concentration in 0.22 µm filtered seawater (28‰ salinity) was lowered to about 10 % of air saturation by bubbling with N_2. The seawater was transferred to 3 l bags made of the different laminated materials. Apart from one set of alufoil bags, formalin was added to a 2 % final concentration to ensure sterility. The bags were sealed and incubated in the setup described above. To measure the penetration of oxygen into the bags, a minimum of 3 samples for mini-Winkler analyses were taken at intervals for up to 16 d. One set of transparent EVA laminate bags was also tested by incubating them dry in a cold room.

Physical strength of bag materials. Bag material strength was monitored because small cracks were always seen in the metal film after the incubation period. These cracks represented a possible route for enhanced oxygen penetration into the bags and therefore a potential artifact for the respiration measurements. The area involved was determined by carefully cutting up 4 alufoil bags after the incubation and counting the very small cracks in the metal film, while holding it up against the light. The diameter of the cracks was categorized as greater or smaller than 0.25 mm, and the areas around 10 cracks were inspected under a microscope.

Linearity of plankton respiration. An experiment was set up for testing the influence of incubation period on oxygen respiration, in order to identify an optimal incubation time. Two 4 l bags, prepared with the alufoil laminate, were filled with coastal water from the Baltic Sea and incubated for 11 d during which subsamples were collected for O_2 concentration analysis. This experiment was carried out in December at a water temperature of 5°C and in late April at 12°C.

Bacterial growth test. The growth of free-living bacteria during the incubation was measured by adding well-mixed seawater to bags of 3, 1 and 0.1 l incubated at 10°C. For the determination of bacterial abundance in the seawater, 10 ml subsamples were collected at time 0 and after 8, 20, and 48 h. Samples were placed in acid-washed vials, fixed with borate-buffered formalin to 2% final concentration and treated as in Hobbie et al. (1977) for counting of bacteria by acridine orange. To measure bacterial colonization of the inner bag walls, five 1 l alufoil bags without tubing were filled with seawater, sealed and incubated. A bag was then cut open after 8 h, 4 d, 8 d and 15 d. Bacterial densities were examined by staining directly 2 cm^2 pieces of bag material with acridine orange for 5 min. After washing with 5 ml of sterile filtered seawater >400 bacteria were counted on the laminated material under epifluorescence microscope.

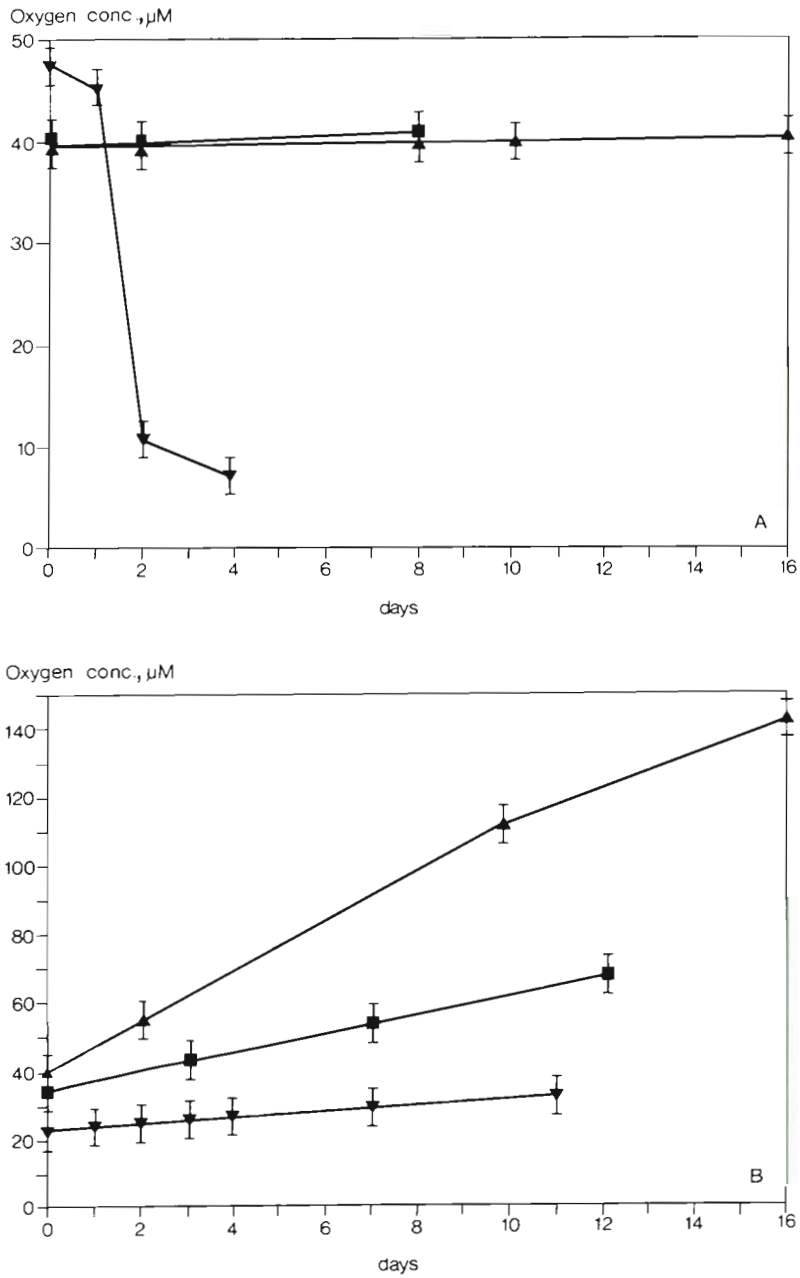

Fig. 2. Change of oxygen concentration in filtered, formalin-treated, and oxygen-depleted seawater, in 3 bags at 10°C. (A) Alufoil (▲), alufoil where no formalin was added to the sample (▼), metal film (■). (B) EVA laminate (▲), PVF (■), EVA laminate, incubated dry (▼)

Plankton respiration in Aarhus Bay. From February 1990 to June 1991 an investigation of the primary production and heterotrophic processes in both seawater and sediment was conducted in central Aarhus Bay, Denmark, at 15.5 m depth. The study area is situated in the shallow transition zone between the North Sea and the Baltic Sea where brackish water (14 to 17‰ S) overlies and mixes with more saline water masses (25 to 31‰ S). As a part of the pelagic study, seawater samples were taken from 10 depths by a rosette sampler with 5 l Niskin bottles every week during the spring bloom, every second week in spring and autumn and every month in the winter. Besides hydrographic and biological parameters (Kruse unpubl.), daily phytoplankton primary production was measured *in situ* by means of algal uptake of $NaH^{14}CO_3$ and later acidification and bubbling (Riemann & Jensen 1991), bacterial gross production by use of the tritiated thymidine incorporation (TTI) method (Fuhrman & Azam 1980b), and plankton oxygen uptake by analysis of seawater incubated in plastic bags.

To follow the plankton respiration, 2 l laminated alufoil bags were filled with seawater from each sampling depth at the station and incubated in the dark at the *in situ* temperature. Subsamples were taken for mini-Winkler analysis upon collection and after incubation for 24 to 48 h. The distribution of plankton respiration in the water column and a depth-integrated respiration value at the station were calculated. Results from winter after a storm period with a totally mixed water column, from the spring bloom period, and from late summer have been selected here for presentation.

RESULTS

Oxygen penetration test

The change in oxygen concentration in the undersaturated seawater of the bags with aluminium laminates is shown in Fig. 2A. In the first test, in which no formalin was added, a rapid decrease in the oxygen content was seen after 2 d of incubation in the alufoil bags. We could detect no increase in oxygen concentration in the formalin-treated alufoil or metal film bags over a period of 7 to 16 d. The change in oxygen concentration in the transparent bags is shown in Fig. 2B. In the EVA bags incubated in water the oxygen concentration increased by 7.4 µM O_2 d^{-1}. When the EVA bags were incubated dry in a dark, cold room, the oxygen concentration increased only by 0.7 µM O_2 d^{-1}. The PVF bag in water showed a linear increase in oxygen concentration of 2.8 µM d^{-1}.

Physical strength of bags

The mean area of cracks in the alufoil after the incubation of four 3 l bags for 48 h was 4 mm^2. By microscopic inspection it was confirmed that only the aluminium layer was cracked and the polyester and polythene laminates were undamaged.

Linearity of plankton respiration

At a surface water temperature of 5 °C in the Baltic Sea in December, the decrease in oxygen concentration was linear during the first 7 d of alufoil bag incubation (Fig. 3). The plankton respiration was initially 2.0 µM O_2 d^{-1} and increased to 5.1 µM O_2 d^{-1} after 7 d. The respiration in the surface water at the end of April at 12 °C increased after 2 d from 3.4 µM O_2 d^{-1} to 3.6 µM O_2 d^{-1} (Fig. 3).

Bacterial growth

Fig. 4A shows the change in bacterial numbers per ml seawater over 48 h with different sizes of alufoil bags. Only in the smallest bags (0.1 l) was an increase in bacterial numbers recorded. Bacterial colonization on the inner walls of 1 l and 3 l alufoil bags is shown in Fig. 4B. Here a minor increase could be seen by microscopic observation after 24 h, and after 1 wk the bacterial density was doubled. After 2 wk of incubation, the inner walls were heavily colonized by bacteria.

Plankton respiration measurements

In February, the whole water column consisted of mixed North Sea and Baltic Sea water (26 ‰ S) and the primary production was very low and restricted to only the upper 4 m (Fig. 5A). In contrast, high oxygen uptake values (4 to 7 µM O_2 d^{-1}) and bacterial gross production rates (over 5 µM C d^{-1} at 15 m, 1 m above the sediment) were found at the same time at most depths. However, lower bacterial gross production was found at 13 to 14 m. POC measurements gave high val-

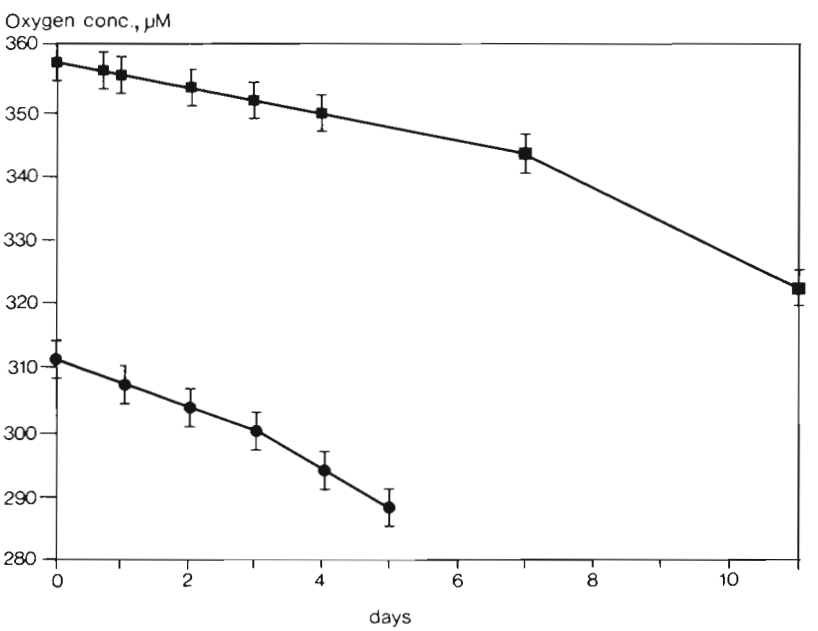

Fig. 3. Decline in oxygen concentration over time in two 4 l alufoil bags containing natural surface seawater incubated at *in situ* temperature. (■) Water sampled in December 1990 at 5 °C. (●) Water from April 1990 at 12 °C. Linear regression lines are shown

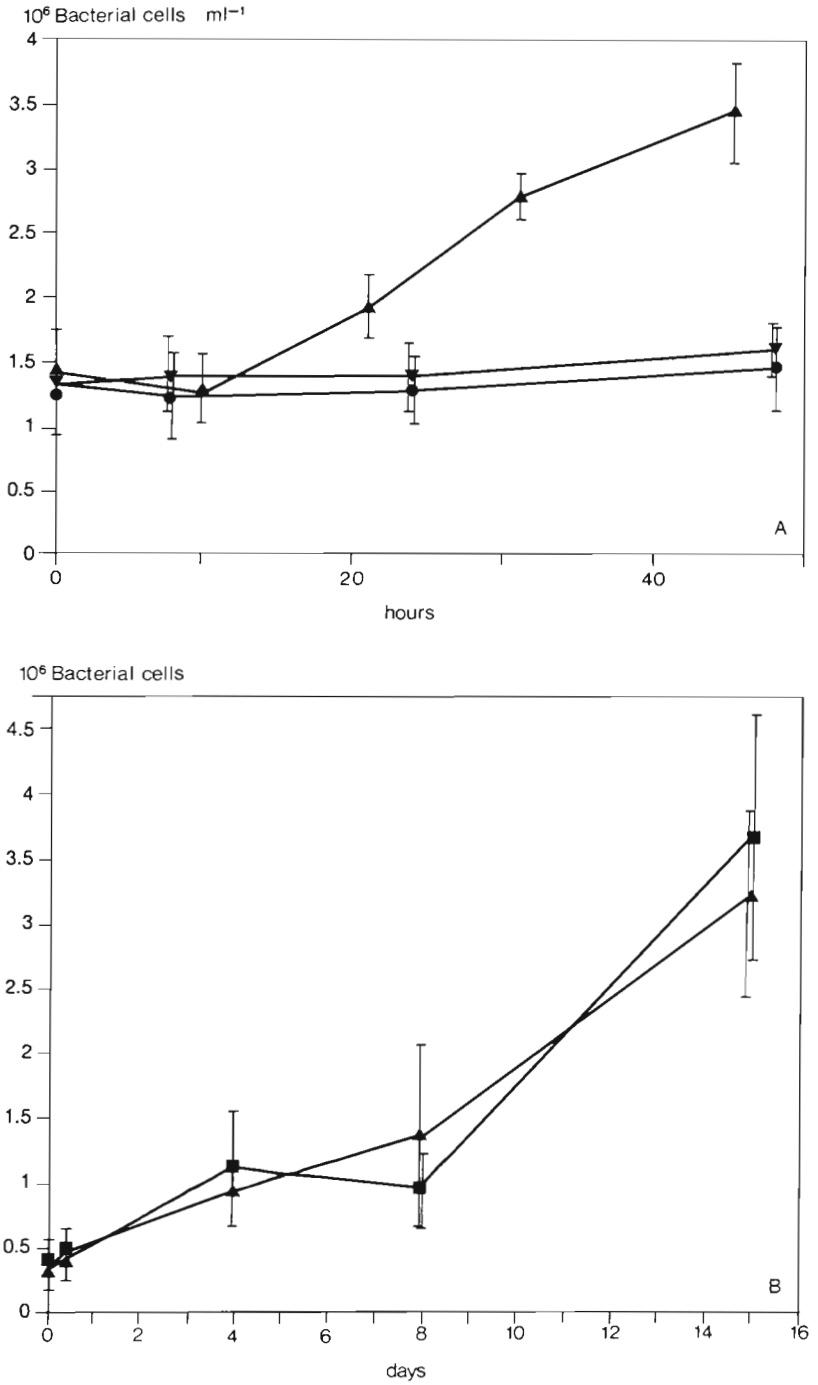

Fig. 4. Development of bacterial abundance in seawater and on the inner surface of bags of different sizes. (A) Bacteria in the seawater in 0.1 l (▲), 1 l (▼) and 3 l (●) bags over 48 h. (B) Increase in bacterial density on the plastic wall per cm^2 in 1 l (■) and 3 l (▲) bags over 15 d

ues of approximately 800 µg C l^{-1} in the upper layers, decreasing to around 400 µg C l^{-1} a few meters above the bottom, increasing however to 2700 µg C l^{-1} at 1 m above the sediment surface. Oxygen saturation was 100 % in the surface water. From 2 m to the bottom the O_2 saturation was at 94 to 96 %.

The water column was stratified during March (Fig. 5B). Primary production, bacterial production, and plankton respiration were high in the brackish surface water, but low in the high-saline North Sea water near the bottom. The upper part of the water column was supersaturated with oxygen. High plankton respiration was also measured in the lower part of the pychnocline, at ca 10 m depth.

In August (Fig. 5C) the highest rates of biological activity were found in the lower part of the water column. Primary production was low at the surface, but high down to 13 m. Bacterial gross production was 4 µM C d^{-1} and respiration was up to 10 µM O_2 d^{-1} at 10 to 12 m, the same depth at which a weak salinity stratification began. The seawater was undersaturated with oxygen at all depths.

The integrated values for the 3 process rates per m^2 are given in Table 2. Respiration exceeded 100 mmol O_2 m^{-2} d^{-1} in August in connection with a subsurface *Ceratium* bloom, but was also high during a winter storm with sediment resuspension. If one assumes an RQ of 0.75, between 39 and 67 % of the oxygen consumption was due to the bacteria.

DISCUSSION

Bag incubation setup

In coastal and stratified waters the inhomogeneity of the water column can be a serious problem for achieving a starting value for measurements. The amount of seawater needed for replicates at each sampling depth for plankton respiration measurements can be considerable. During our field measurements oxygen gradients were recorded several times in the 5 l water samplers. Kuparinen (1987) solved the problem by mixing the seawater in an open container before incubation. He later used a water splitter. The best way to solve the inhomogeneity problem is, however, by

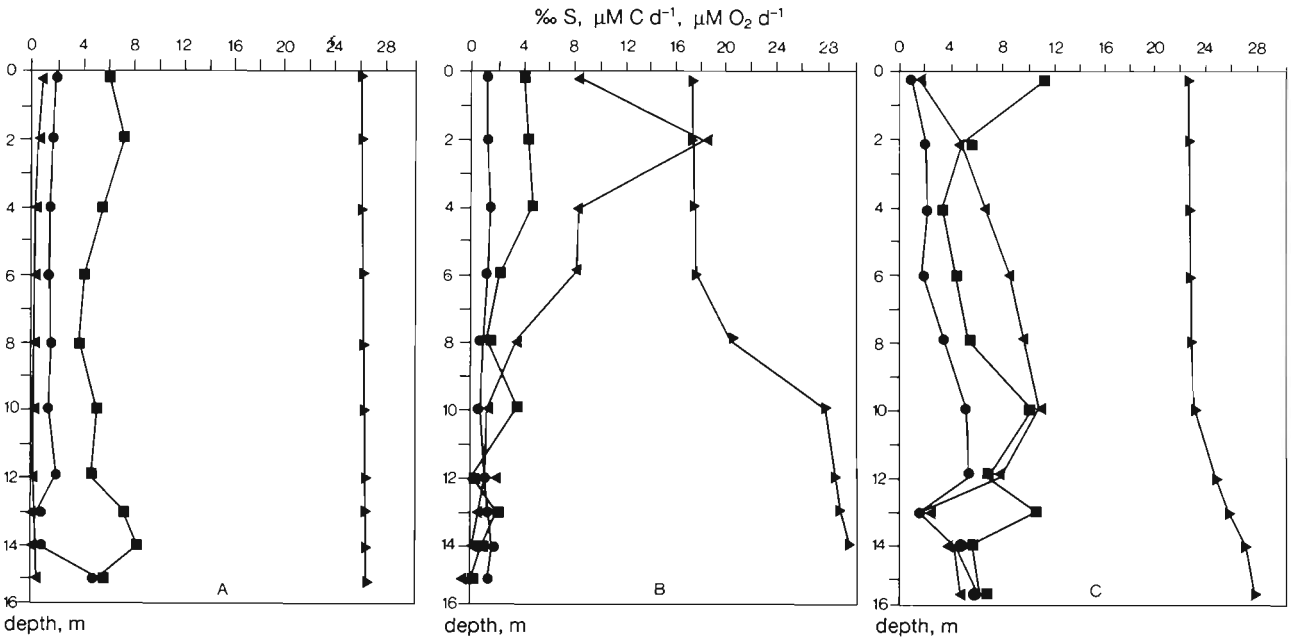

Fig. 5. Depth distribution of salinity (▸), primary production (◂), bacterial gross production (●) and plankton respiration (■), at a station in central Aarhus Bay. (A) Profiles on 19 February, with a mixed water column and resuspension after a storm. (B) Profiles on 18 March, with stratified water masses and a diatom bloom in the surface layer. (C) Profiles from 28 August, with stratification in the bottom layer after a subsurface algal bloom at 10 m depth

measuring the oxygen concentration repeatedly in the same water volume, a principle followed in the bag incubation method described in this study. The mixing of the seawater samples was ensured through the circulation of the cooling water, which moved the bags constantly during the incubation period.

Oxygen penetration test

Only bag materials having an aluminium barrier completely prevented measurable oxygen penetration (Fig. 2A). The rapid oxygen disappearance in the alufoil bag without formalin was probably due to an unsuccessful filtering process followed by a rapid growth in the bags of bacteria remaining in the seawater. In the aluminium bags, the only oxygen leakage is by diffusion through the side-sealing and through cracks in the aluminium layer caused by mechanical wear. Diffusion through 75 µm of the compressed low-density polythene layer and a double 8 mm sealing around a 3 l bag (120 cm in circumference) account for < 0.05 µmol O_2 d^{-1}, when incubated in air (Otto Nielsen Emballage AS 1990). Similarly the diffusion through the 80 µm thick polyester and the polythene layer, where the aluminium layer was broken (4 mm^2), could only be responsible for a penetration < 0.03 µmol O_2 d^{-1}. In the water baths these values will be lower because of the low temperature. The background penetration of oxygen into the 3 l bag is therefore lower than 0.08 µmol O_2 d^{-1}. Compared with a typical plankton respiration rate of 2 µM O_2 d^{-1} measured in the same size of alufoil bag, the background oxygen penetration represents only 1.3 % of this value.

In accordance with the technical specifications, the PVF bags provided an insufficient barrier to oxygen for plankton respiration measurements (Fig. 2B). The EVA bags also provided an insufficient barrier when incubated in water, but permitted less oxygen transmission when incubated in air. The outer polyamide layer is not able to withstand the water which enters, which makes the bag laminates more open to diffusion (P. Togeskov pers. comm.). Further studies will be required to deter-

Table 2. Depth-integrated primary production (PP, mmol C m^{-2} d^{-1}), gross bacterial production (GBP, mmol C m^{-2} d^{-1}) and plankton respiration (PR, mmol O_2 m^{-2} d^{-1}) throughout the water column. Measurements from a station in central Aarhus Bay made on 19 February, 18 March and 28 August 1990 respectively

	Winter resuspension	Spring bloom	Autumn subsurface max.
PP	3	90	104
GBP	25	19	51
PR	85	40	102

mine whether this transparent bag type could be used dry under weaker oxygen gradients in e.g. photosynthesis studies.

Mini-Winkler technique

The small pycnometers were found to be very useful, because they were much easier to fill than e.g. small syringes. Their volumes were also well calibrated, no air bubbles were trapped during sampling and the small amounts of reagents could easily and carefully be added. In one experiment, 2 ml calibrated glass syringes were tested instead of pycnometers, and no significant differences in mean standard error of replicate determinations were found. Since the handling of the syringes, however, was much more time-consuming, this procedure was discontinued.

Most of our oxygen concentration determinations with the mini-Winkler technique have standard errors of replicate determinations of ±0.2%. In a respiration study in a highly productive Danish inlet, standard errors of oxygen determination of ±1% could be accepted (Jensen et al. 1990). On the other hand, as early as 1981 Tijssen & Wetsteyn obtained mean standard errors of replicates of 0.06% in shipboard samples from the open sea (Tijssen & Wetsteyn 1984). Such a low standard error of replicate determinations was not possible in the present study due to the small subsample volume. A minimum decrease in oxygen concentration in the incubated seawater (water temperature at 12°C) of 0.6 µM O_2 d^{-1} over 48 h incubation is necessary to obtain a significant respiration determination. This value is satisfactory for oxygen uptake measurements in coastal waters where the respiration scale is generally 1 to 10 µM O_2 d^{-1}.

Bacterial growth test

Aspects of so-called 'bottle effects' have been discussed in a number of papers. For example, Fuhrmann & Azam (1980a) found no difference in bacterial growth when incubating seawater in 150 and 600 ml bottles. In our study, however, bacterial abundance did increase in the smallest bags, probably because of the high surface-to-volume ratio. The larger bags showed constant bacterial numbers in the seawater. Bacterial wall growth on the inner surface caused a steady but slow rise in numbers in both 1 l and 3 l bags. The numbers of attached bacteria after 2 and 3 d was too small to significantly affect the oxygen respiration. On the other hand, the number of bacteria on the inner walls after 15 d represents an abundance of the same magnitude as occurred in the water phase at the beginning of the experiment, 1.5×10^6 cells ml^{-1}. A bacterial wall growth of this magnitude should be taken into account in pelagic experiments, as it will influence process studies employing large *in situ* plastic enclosures, which have been utilized in many coastal areas within the last decade.

Oxygen uptake showed weak 'bottle effects' after 7 d in cold water and after 3 d in warmer water (Fig. 3). Taking the bacterial growth parameters and the linearity of oxygen consumption into account, it is recommended that seawater incubation not exceed 48 h, in agreement with Hopkinson et al. (1989).

Field plankton respiration measurements

The distribution of biological activity was very different during the 3 seasons, and depended on the hydrographic conditions.

In February, when the numbers of algae and heterotrophs in the water column are normally very restricted, the high oxygen uptake rates at all depths indicated a unique event. The water column was mixed after a storm and the surface water was turbid, suggesting resuspension of sedimentary material locally or from a nearby area. This interpretation is supported by the high POC values and high bacterial gross production rates in most depths. But from results in Table 2 one can calculate that only 39% of the oxygen uptake is due to bacterial activity (assuming an RQ of 0.75). This suggests a rapid and purely chemical oxidation of resuspended substances in the water column, apparently with especial intensity 2 to 3 m above the sediment surface. Chemical oxidation was calculated to be responsible for nearly half of the oxygen uptake rate. This hypothesis is also supported by the fact that the water column was undersaturated with oxygen at most depths, which is rather unusual for this time of the year.

In March the primary production in the brackish Baltic Sea surface water was higher than the pelagic respiration in the water column. Around 45% of the primary production was decomposed here by grazing or heterotrophic activity. However more than half of the production must have been transported either to the bottom or horizontally out of the area. Small peaks in respiration in transition zones between water layers have also been observed elsewhere (Kruse & Rasmussen unpubl.) as a result of accumulation of decomposing plankton.

In August, the field measurements reveal intense phytoplankton primary production, bacterial activity and plankton respiration at 8 to 13 m depth. The depth-integrated plankton respiration on 28 August (102 ± 15 mmol O_2 m^{-2} d^{-1}; Table 2) in Aarhus Bay rep-

resents a high value relative to more open marine areas. In a Danish inlet, very high values, > 127 µM O_2 d^{-1}, have recently been reported (Jensen et al. 1990), whereas in Laholm Bay, 100 nautical miles NE of Aarhus Bay, values around 12 to 60 mmol O_2 m^{-2} d^{-1} were indirectly calculated (these include benthic oxygen uptake; Rydberg et al. 1990). In Laholm Bay the few direct measurements in seawater showed results around 10 mmol O_2 m^{-2} d^{-1}. Although temporal and spatial displacements of production and degradation are larger in coastal pelagic systems than in more open systems, this difference in results stresses the need for more direct oxygen uptake measurements in coastal areas, with high resolution in space (vertically) and time.

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