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A technique to assess the harmful effects of sampling and containment for determination of primary production[1,2]

Abstract—We address here the hypothesis that massive mortality, photosynthetic debility, or both may be associated with sampling and enclosing open ocean phytoplankton. We propose that the hypothesis can be tested by measuring in vivo Chl *a* fluorescence of enclosed phytoplankton assemblages. Experiments on the effects of acute copper toxicity and mechanical disruption were performed to validate our approach. Reports in the literature and results from the experiments show that the difference between in vivo fluorescence before and after treatment with the photosynthetic inhibitor DCMU is a reliable, albeit qualitative, indicator of photosynthetic ability. Fluorescence measurements are thus suitable to assess damage to phytoplankton during measurement of photosynthesis. An example from the tropical ocean demonstrates that the low measured rate of primary production is probably not an artifact of sampling and containment.

Considerable controversy is associated with the determination of primary production in the open ocean. Central to the problem is uncertainty regarding measurements that require sampling and containment. Containment in bottles can be associated with deleterious effects on open ocean phytoplankton (Venrick et al. 1977; Gieskes et al. 1979). Some studies have indicated that trace-metal contamination associated with sampling and handling is responsible for substantial underestimation of primary production determined with the ^{14}C method (Carpenter and Lively 1980; Fitzwater et al. 1982). It is also a concern that confinement of plankton in bottles disrupts natural pathways of nutrient regeneration, perhaps leading to erroneous estimates of planktonic rate processes (Gieskes et al. 1979; Eppley 1982).

The above and other criticisms have been considered and rebutted (e.g. Sharp et al. 1980; Jackson 1983; Marra and Heinemann 1984). An unacceptable level of uncertainty persists, however, as evidenced by a steady flow of papers with conflicting conclusions, backed by arguments that to a large extent are independent of sampling methodology (e.g. Shulenberger and Reid 1981; Jenkins 1982; Platt 1984; Welschmeyer and Lorenzen 1985).

At the root of the problem is the conclusion based on conventional ^{14}C productivity measurements that growth rates of phytoplankton in the open ocean are slow—about 0.2 d^{-1}—a rate about a tenth that inferred on the basis of other measurements (Eppley 1980; Sheldon 1984 and references therein). Progress in understanding planktonic rate processes in the open ocean greatly depends on resolution of the validity of those ^{14}C-based primary production measurements. In response, considerable effort (most notably, the PRPOOS program: Eppley 1982; Marra and Heinemann 1984; Laws et al. 1984) has been expended to compare the ^{14}C method with other means of measuring primary production. Due primarily to logistical constraints, however, little comparison of methods has been performed in environments where conventional measurements suggest low growth rates of phytoplankton. Estimation of primary production in the open ocean thus remains problematic.

We address here a hypothesis that has been considered (Venrick et al. 1977; Gieskes et al. 1979), but has yet to be effectively refuted: that massive mortality and photosynthetic debility may be associated with sampling and enclosing open ocean phytoplankton. Because the discrepancy between estimates of growth rates is about an order of magnitude, we believe that we can test this hypothesis by assessing the photosynthetic capacity of enclosed phytoplankton assemblages with in vivo fluorescence techniques. We will show that if

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sampling and bottle incubations reduce photosynthetic output by a substantial amount, photosynthetic debility will be clearly manifest in easily measured fluorescence parameters.

The fluorescence of Chl *a* in vivo should be an appropriate indicator of photosynthetic competence. It has been postulated that by measuring the increase of fluorescence upon addition of the photosynthetic inhibitor DCMU ($F_d - F$), one can obtain a relative measure of the capacity for photosynthesis or, more specifically, operational photosystem II reaction centers (Vincent et al. 1984). The reasoning is that DCMU blocks noncyclic photosynthetic electron flow and thus eliminates photosynthetic quenching of fluorescence; the increase of fluorescence upon addition of the inhibitor should therefore be related to the photochemical capacity of photosystem II. Several studies relating parameters of in vivo fluorescence to the photosynthesis or physiology of phytoplankton (*see* Leftley et al. 1984) have been based on similar theoretical arguments. Results have been mixed and discussions rife with caveats. Nonetheless, good correlations between fluorescence parameters and photosynthetic performance have been demonstrated experimentally with respect to the circadian rhythm of photosynthetic capacity, photoinhibition of photosynthesis (Vincent et al. 1984), thermal stress (Sellner et al. 1982), nutrient depletion (Roy and Legendre 1979), and short term changes of photosynthesis in natural populations (Oquist et al. 1982). Although various ways to parameterize fluorescence response were used in those studies, in each case a decrease in photosynthetic ability could be related to reduction of ($F_d - F$) relative to a control.

The experiments described here provide new examples of the relationship between fluorescence and photosynthesis by showing a strong correlation between fluorescence parameters and photosynthetic inhibition associated with acute copper toxicity and mechanical disruption. An example from the tropical ocean is used to demonstrate that low measured rates of primary production are probably not artifacts of sampling and containment.

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Experiments were done to determine the effects of acute copper toxicity during 4-h incubations for measurement of primary production. Each sample of seawater collected was immediately passed through a 186-μm mesh into a polyethylene jug, then dispensed into a series of clean polycarbonate bottles. Containers and mesh had been cleaned by rinsing with deionized water, a soak of at least 2 days in 2% Micro detergent, thorough rinsing with Milli-Q water, a soak of at least 2 days in 2% HCl, and thorough rinsing with Milli-Q water. Samples were experimentally treated with $CuSO_4$ in Milli-Q water. Dilution of the sample was always $<1:100$.

Those experiments labeled "Aransas Pass" were done on the Marine Science Institute (University of Texas) pier. Sampling was by clean bucket (washed with Micro detergent, rinsed with deionized water, rinsed with 10% HCl, rinsed well with deionized water, then Milli-Q water) during floodtide. Other experiments were done on the Texas shelf (27°30′N, 96°30′W; depth, 60 m) and over the continental slope (27°10′N, 95°24′W; depth, 1,300 m) on samples taken with a clean but otherwise unmodified 30-liter Niskin bottle. Laboratory experiments were done with exponential-phase unialgal cultures of the diatom *Skeletonema costatum* (UTEX LB 2308) grown in IMR/2 medium (Eppley et al. 1967) on a 12:12 light/dark cycle at 20°C. Photon flux density was 130 μEinst m^{-2} s^{-1}.

Chlorophyll *a,* corrected for pheopigment, was measured fluorometrically in samples collected on Whatman GF/F filters and extracted in 10 ml of 90% acetone in the dark at 0°C for 24 h. As part of one experiment, we determined chlorophyll concentration directly in unfiltered water samples (Phinney and Yentsch 1985): 1 ml of sample was added to 9 ml of 100% ace-

tone, extracted 48 h at 0°C in the dark, and filtered (Whatman GF/F) before fluorescence was measured.

The uptake of [^{14}C]bicarbonate was determined as follows. Each subsample received a high-specific-activity (0.5 mCi ml^{-1}) solution of $NaH^{14}CO_3$ in distilled water and was incubated at 41% surface irradiance in a clear acrylic incubator cooled with surface water and covered with neutral-density screen. Several experiments included dark bottle incubations. After incubation, samples were filtered onto GF/F filters, placed in a scintillation vial containing 1 ml of 0.5 N HCl, agitated in a fume hood for 20 min, and scintillation fluor added. Disintegrations per minute were calculated by the channels-ratio method. Total CO_2 was measured by titration (Strickland and Parsons 1972). Total radioactivity in the samples was determined by subsampling into a scintillation vial containing scintillation fluor plus 0.2 ml of phenethylamine. Results were corrected for time-zero controls.

The fluorescence of Chl *a* in vivo was determined with either a Turner 111 or Turner Designs 10-005R fluorometer after at least 15 min of dark adaptation. Fluorescence was measured in both presence and absence of DCMU, [3-(3,4-dichlorophenyl)-1,1-dimethylurea], 10^5 M added as ethanol solution 60 s before measurement (*see* Vincent 1980).

We used data from a station in the eastern tropical Pacific Ocean (9°45'N, 93°45'W), considered characteristic of broad expanses of the oligotrophic tropical open ocean (Li et al. 1983), to examine the implications of our experimental study. Samples were pumped (Herman et al. 1984) into 200-liter polyethylene tanks and subsamples removed for analysis and incubations. Incubations were in glass bottles suspended in incubators covered with neutral-density screens. Fluorescence was measured as above. Primary production was determined by W. K. W. Li (Li et al. 1983).

Each experiment described here resulted in acute copper toxicity at the higher concentrations. Figure 1A shows a typical result, confirming severe inhibition of photosynthesis by copper (e.g. Steemann Nielsen et al. 1969; Knauer and Martin 1983; Wood 1983). The amount of copper necessary to inhibit photosynthesis and fluorescence was higher than ambient concentrations in coastal waters and probably much more than would be associated with inadvertent contamination during sampling and handling (cf. Fitzwater et al. 1982). Our results are consistent with the findings of others, however, and can be explained by the high level of chelation capacity in coastal waters (Wood 1983).

The response of in vivo fluorescence to copper was similar to that of photosynthesis in that both untreated fluorescence (F: not shown) and DCMU-poisoned fluorescence (F_d: Fig. 1B) declined sharply with increasing concentration of added copper. Because F_d and F both declined proportionally in response to copper, the parameter $(F_d - F)$ (cf. Vincent et al. 1984) also reflected inhibition of photosynthesis. But parameters of relative fluorescence such as $(F_d - F)/F_d$ (cellular photochemical capacity or CPC: Vincent 1980; likewise similar parameters: Harris 1980; Oquist et al. 1982) were not good indicators of photosynthetic stress attributable to acute copper toxicity.

Consistent with a laboratory study (Samuelsson and Oquist 1980), Chl *a* concentration at the end of the 4-h incubation also showed a sensitivity to copper poisoning (Fig. 1C). In a follow-up experiment we examined the fate of the "missing" pigment: after a typical experiment, the chlorophyll content of samples was determined in unfiltered water added directly to acetone (Phinney and Yentsch 1985) and in particulate material captured on GF/F filters. There was no significant difference between the two, both of which indicated copper-dependent disappearance of chlorophyll. One can thus infer that the filter-retained chlorophyll declined as a function of the added copper because it was degraded rather than broken up and passed through the filter.

We did several experiments to assess the generality of the above results. Both in the laboratory and in the field, nearshore and offshore, the parameter $(F_d - F)$ measured after 4 h was a good indicator of the inhibition of photosynthesis by copper (Fig. 2A).

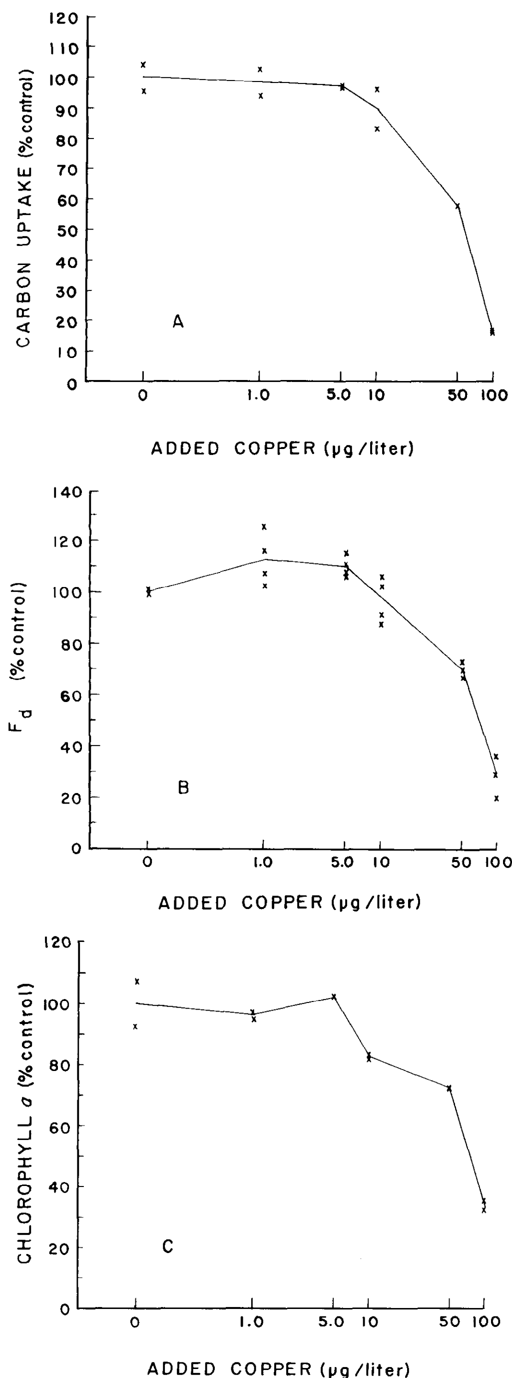

Fig. 1. Effects of added copper on natural phytoplankton during 4-h incubations. Sample from Aransas Pass. A. Uptake of [^{14}C]bicarbonate. B. Fluorescence in vivo of samples treated with DCMU. C. Chlorophyll *a*. All values are percent of control. Lines connect the means of individual measurements.

Chlorophyll *a* concentration, as a percentage of the control after 4 h, was also strongly correlated with photosynthetic inhibition, but the relationship was neither one-to-one nor linear (Fig. 2B), suggesting that in vivo fluorescence is a better indicator of acute copper toxicity than Chl *a*. Experiments with light and dark bottles also show that fluorescence reflects copper toxicity more reliably than chlorophyll concentration: the decline of fluorescence was equivalent in light and dark (in two experiments), whereas the apparent destruction of Chl *a* did not always show light dependence. Although the unscaled parameters F_d, F, and $(F_d - F)$ were strongly correlated with photosynthetic inhibition, the relative measure of increase in fluorescence with the addition of DCMU, $(F_d - F)/F_d$ (cf. Oquist et al. 1982), was not a useful indicator of relative photosynthetic performance in any of our experiments.

A time-course of fluorescence and photosynthesis (Fig. 3; *see also* Steemann Nielsen et al. 1969 and Fig. 4 discussed below) shows that the full effect of copper on fluorescence is not immediately realized, but rather that the decline relative to the control extends over several hours. The slow response of fluorescence to copper poisoning is useful because a measurement made soon after sampling can be considered as a time-zero control representative of an unpoisoned sample.

A time-zero sample is not the only control that can be used to detect physiologically significant contamination by copper. Chelators such as EDTA counteract the toxic effect of dissolved copper (Steemann Nielsen and Wium-Andersen 1970; Jackson and Morgan 1978) and can be added to samples to eliminate the possibility of acute copper poisoning (cf. Sharp et al. 1980). We did an experiment to see to what extent EDTA can alleviate acute copper toxicity, even if the chelator is added some time after the sample is poisoned or inadvertently contaminated (Fig. 4). Surface water from Aransas Pass was dispensed into polycarbonate bottles for determination of [^{14}C]bicarbonate uptake over 4 h. Samples treated with 2×10^{-5} M EDTA were identical to the control with respect to photosynthesis. Adding $CuSO_4$ at

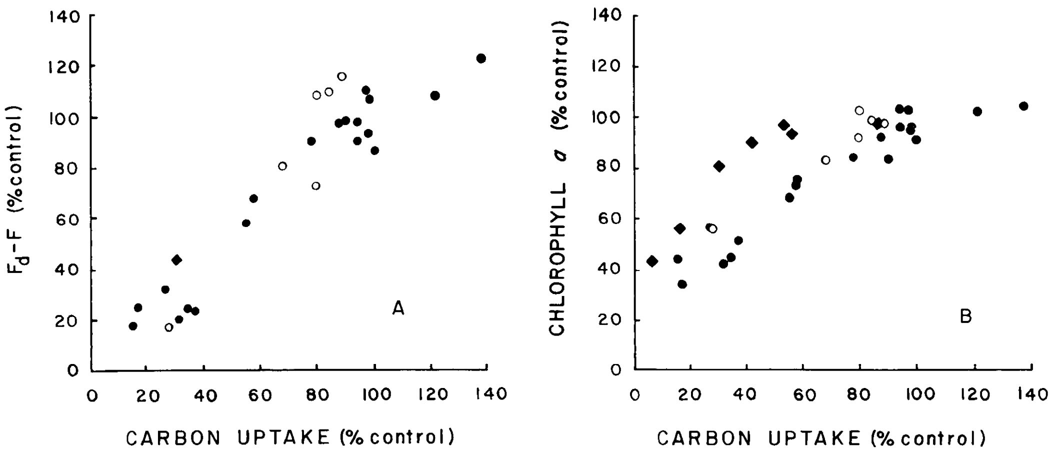

Fig. 2. Relationships between empirical parameters and cumulative [^{14}C]bicarbonate uptake after 4-h incubations. Results of several experiments compiled. A. $(F_d - F)$, where F is in vivo fluorescence and F_d is fluorescence after treatment with DCMU. B. Chlorophyll *a* concentration. Results from Aransas Pass—●; Texas shelf and continental slope—◆; laboratory results—O. Each pair of variables has a significant correlation at $P = 0.01$, but only panel A reflects a 1:1 relationship at $P = 0.01$.

50 μg liter^{-1} inhibited inorganic carbon uptake by 42%. As expected, the toxic effect of copper was almost entirely prevented by adding EDTA before the $CuSO_4$. However, the chelator alleviated the toxic effects of copper even if added after the $CuSO_4$, as if the time-course of inhibition (Fig. 3) were halted with the addition of EDTA. Thus, the fluorescence of an untreated incubated sample can be compared with a sample treated with EDTA to assess the physiological consequences of suspected contamination by copper.

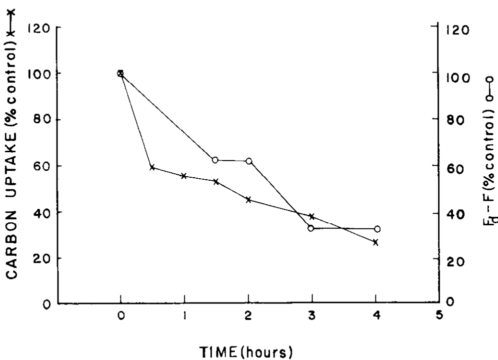

Fig. 3. Time-course of acute copper toxicity. Sample from Aransas Pass. Copper sulfate added at time zero (50 μg liter^{-1}). Uptake of [^{14}C]bicarbonate, cumulative % of control to each time point—×; $(F_d - F)$—O.

A sample from Aransas Pass was divided for experiments to see if mechanical disruption of phytoplankton reduced DCMU-enhancement of in vivo fluorescence to a similar extent as photosynthetic capacity and to determine changes in chlorophyll concentration associated with disintegration of the cells. A 500-ml subsample was sonicated for 5–10 min, long enough to depress in vivo fluorescence. $(F_d - F)$ of the sonicated sample was 18% of control at the beginning of the 4-h incubation and changed only slightly, to 16% of control, at harvest. Consistent with fluorescence measurements, bicarbonate uptake over 4 h for the sonicated sample was 16% of control. The chlorophyll concentration in unfiltered water (*see methods*) was 72% of the control shortly after sonication, declining to 20% of it after 4 h. Of the chlorophyll in the sonicated sample, 89% was retained on a GF/F filter at T_0, 98% at 4 h.

These results show that mechanical disruption of phytoplankton is manifest immediately in the magnitude of $(F_d - F)$ to a degree quite consistent with the destruction of photosynthetic capacity. Chlorophyll concentration is also affected. Disruption of phytoplankton leads to an immediate decrease in total and filterable chlorophyll, followed by a slower decline, presumably

due to photooxidation or enzymatic degradation of photosynthetically inactive pigment.

Coastal phytoplankters are generally much more resistant to physical disruption than many oceanic forms, so we had to use harsh sonication to reduce photosynthetic capacity in our samples. To make useful inferences from our experiments, we assume that the effects of mechanical disruption on photosynthesis and fluorescence are the same regardless of how the structural integrity of the cells is destroyed.

We consider now the open ocean environment in which conventional measurements of primary production suggest slow growth of phytoplankton, i.e. chlorophyll-specific primary productivity achieves a maximum rate of only 1–3 g C (g Chl *a*)$^{-1}$ h^{-1} (e.g. Sharp et al. 1980). Is this measurement a gross underestimate? Specifically, can artifacts of sampling and handling be responsible for severe photosynthetic debility in the confined assemblage of autotrophs? Because the magnitude of discrepancy in primary production and growth rate estimates is easily a factor of 10 (Eppley 1980; Sheldon 1984), we can address a pertinent problem by considering here only gross damage to photoautotrophic processes, i.e. a factor >2.

Time-course measurements of primary production made during incubations of open ocean samples show clearly that the low rates measured were not due to a progressive decrease of photosynthetic rate over several hours—^{14}C uptake was fairly linear during the day and resumed at a similar rate the following morning (figure 3A, Li et al. 1983; Sharp et al. 1980). If one is to ascribe the low measured rates of primary productivity to artifact, then it must be postulated that any damage to photosynthetic systems occurred during or soon after sampling. Observations of changes during incubations (Venrick et al. 1977) might not detect damage incurred during sampling. Such damage would be difficult to detect by measuring rate processes because it is not possible to make appropriate measurements on an unsampled control, save for calculating primary production from noninvasive observations (e.g. Postma and Rommets 1979; Tijssen 1979; Shulenberger and Reid 1981; Platt et al. 1984). The problem could be addressed, however, if a reliable and easily measured indicator of photosynthetic ability were available.

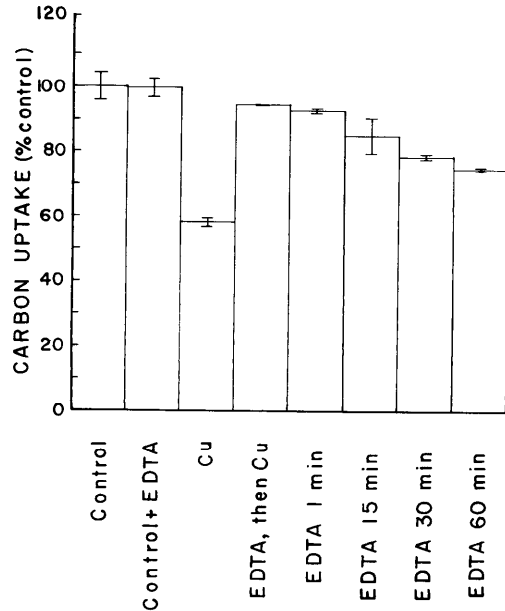

Fig. 4. Influence of EDTA on acute copper toxicity. Sample from Aransas Pass incubated for 4 h to determine uptake of [^{14}C]bicarbonate. Control is an untreated sample. Copper was added to all other samples (except control + EDTA) at 50 μg liter^{-1}. EDTA (2×10^{-5} M) was added at various times during the experiment: before the addition of copper, just after, and 15, 30, and 60 min into the incubation. Error bars represent the range of duplicate samples.

Our experimental results show clearly that photosynthetic debility from acute copper toxicity is reliably reflected by a decrease in the parameter ($F_d - F$) during the first few hours after sampling. Significantly, the parameter ($F_d - F)/F_d$ is largely insensitive to copper over short incubations. The patterns of fluorescence that we have observed can be reconciled with the proposition that a major site of toxic action for copper is between the water-splitting enzyme and the reaction center of photosystem II and that the maximal rate of photosystem II activity is affected much more than efficiency of functional photosystem II units (Shioi et al. 1978).

Because we are concerned only with phys-

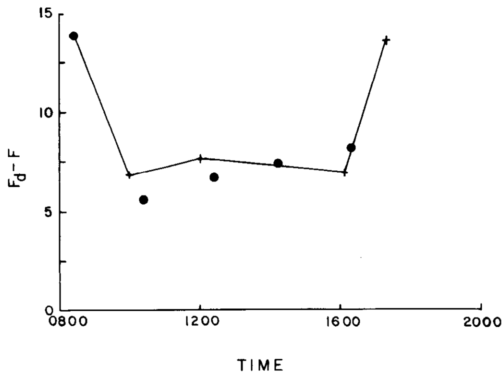

Fig. 5. In vivo fluorescence ($F_d - F$) of samples from the eastern tropical Pacific (*see text*). A sample was obtained by pump at 0830 hours and dispensed into bottles for on-deck incubation. The line connects subsequent measurements on the incubated water. Direct measurements on samples obtained by pump from the same stratum—●. There is no qualitative difference between fluorescence of a sample confined through the day and samples obtained from the same stratum periodically through the day.

iological responses of phytoplankton and because cupric ion activity rather than copper concentration per se affects phytoplankton physiology (e.g. Anderson and Morel 1978; Jackson and Morgan 1978; Huntsman and Sunda 1980), our technique of measuring fluorescence to assess acute copper toxicity is more appropriate than approaches dependent on chemical analyses. If the activity of copper in a sample is increased sufficiently to inhibit photosynthesis, fluorescence will be similarly influenced. Our technique is thus not limited by analytical sensitivity except to the extent that in vivo fluorescence can be measured precisely.

Our simple experimental approach adequately describes some empirical manifestations of acute copper toxicity. However, problems may be encountered in interpreting time-course measurements, because copper added as $CuSO_4$ takes time to equilibrate with chelators in the medium, resulting in a decline of copper activity with time after addition (Anderson and Morel 1978). We used time-course data from experiments on a sample from Aransas Pass to conclude that the toxic effect of copper on phytoplankton was expressed relatively slowly, over hours. This conclusion is consistent with the findings of Steemann Nielsen et al. (1969) but does not necessarily apply to copper poisoning of open ocean samples, in which chelation capacity may be much less than in coastal waters (cf. Wood 1983). Time-course measurements of copper toxicity in chelated and unchelated water would resolve the uncertainty. We expect, however, that signs of copper toxicity would develop even more slowly in poorly chelated water because the activity of copper would remain relatively steady at a toxic level rather than declining sharply after addition, thereby reducing the rate of change of toxicity with time.

We did not examine the effects on fluorescence of other toxic metals. Nonetheless, there is reason to believe that the parameter ($F_d - F$) should reflect inhibition of photosynthesis by other metals, as any inhibition of electron flow through photosystem II should be accompanied by changes in F, F_d, or both. For example, Chl *a* fluorescence and fluorescence after DCMU treatment were inhibited concurrently with photosynthesis after poisoning with zinc (Tripathy and Mohanty 1980).

We have shown that easily measured empirical manifestations of photosynthetic debility are associated with harmful artifacts that may accompany sampling and containment of phytoplankton. Reports in the literature and our experimental results support the suggestion that if low measured rates of open ocean primary productivity are an artifact of sampling and containment, the parameter ($F_d - F$) should be much lower for a contained sample than for the unaffected natural seawater. The problematic measurement of an unaffected control is discussed below.

Measurements made in the eastern tropical Pacific Ocean illustrate our approach. We collected a sample at 0830 hours from 22 m, corresponding to a relative light level of about 25% surface irradiance, at a temperature of 27.3°C, and incubated subsamples in glass bottles on deck at 25% surface irradiance for time-course measurements of [^{14}C]bicarbonate uptake, Chl *a* concentration, and in vivo fluorescence. Periodically throughout the day the depth of the 27.3°

isotherm was again sampled with the pumping system for fluorescence and Chl *a* determinations. The samples ranged from 27.1° to 27.4°C, indicating that the same stratum was sampled each time.

The primary productivity measurements indicated a photosynthetic rate of 1.0 g C (g Chl $a)^{-1}$ h^{-1} (W. K. Li pers. comm.), consistent with the "slow growth" view of planktonic processes in the oligotrophic ocean. Fluorescence parameters from the incubated sample were quite similar to those of fresh samples pumped during the day (Fig. 5). Notably, the incubated sample showed a morning decrease and an evening recovery of $(F_d - F)$, consistent with photoinhibition (Vincent et al. 1984) and indicative of dynamic physiological response by the phytoplankton. There was no indication of a large, irreversible decline of fluorescence or chlorophyll over the first few hours of incubation. Thus, severe copper toxicity, unnatural nutrient stress, mechanical disruption in the bottles, and thermal stress can probably be excluded as causes for spuriously low measured rates of primary production. In fact, the fluorescence parameters and chlorophyll concentrations suggested no substantial differences between the phytoplankton confined for 10 h and the natural population from the same stratum.

If the samples pumped through the day can be regarded as legitimate controls, our results can be interpreted to support the idea that the low measured rates of primary production at the open ocean site are reasonably representative of natural phytoplankton. That is the case with respect to copper toxicity, as the empirical response to copper poisoning (Figs. 3, 4) demonstrates that pumped samples analyzed for in vivo fluorescence minutes after sampling can be regarded as unaffected controls.

A remaining question is whether catastrophic mortality and fragmentation of phytoplankton might accompany the physical disruption associated with sampling. This concern might be significant when sampling is by pump, but a thorough comparison of primary production measurements showed no difference between samples taken by pump vs. bottle (samples from Scotian Shelf waters: Herman et al. 1984). Further, analyses from bottle samples taken in the same region as our example produce similar values for chlorophyll and primary production (W. G. Harrison pers. comm.), and our rates are consistent with the well documented pattern of typical tropical structure (Herbland and Voituriez 1979). Thus the artifacts of sampling must be quantitatively similar for conventional sampling methods and for pumps.

Changes of in vivo fluorescence have been related to many harmful effects of sampling and containment, but initial measurements are made on-deck and may not be truly representative of unaffected controls. Fortunately, measurements of chlorophyll fluorescence in situ with a submersible fluorometer can serve as the appropriate control to assess damage associated with bringing a sample onboard. Problems arising from differences in dark adaptation can be obviated by sampling at night. With respect to our example from the tropical Pacific Ocean, appropriate data are available to determine the influence of sampling on in vivo fluorescence for water pumped from the upper euphotic zone (Herman et al. 1984; A. W. Herman pers. comm.). The fluorescence of Chl *a* measured in situ with the Batfish vehicle (Herman and Denman 1977) was well correlated with that measured at the pump outflow; i.e. the output of the in situ fluorometer corresponded with that of the on-deck fluorometer and with extracted Chl *a* and was consistent with quantitative relationships determined in the laboratory and at other, more eutrophic sites. If significant cell disruption had occurred during transit through the pumping system, one would expect anomalously high readings from the in situ fluorometer and a decline of chlorophyll during incubation over several hours after sampling.

We concluded that the fluorescence of Chl *a* reflects reliably those artifacts that might lead to serious underestimation of primary production during short term incubations. By measuring in vivo fluorescence in situ before sampling, soon after the sample is captured, and periodically during incubations, one can detect severely harmful influences on the determination of primary production. Acute copper toxicity can be

assessed by comparing the fluorescence of an untreated incubated sample and of a parallel sample with added EDTA. A low rate of chlorophyll-specific primary production measured in the oligotrophic eastern tropical Pacific Ocean does not seem to be an artifact of sampling or containment.

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A simple method to detect bacterial associations in bivalve gills

Abstract—Recent discoveries have shown that endosymbiotic bacteria may be widespread among bivalve species in different habitats. A simple method is described which allows the detection of bacterial-gill association with an ordinary light microscope.

The discovery of a rich fauna in the vicinity of hydrothermal vents raised several important questions about food chain relationships at great depths. A symbiotic association was first shown between chemolithotrophic sulfur-oxidizing bacteria and the gutless worm *Riftia pachyptila* Jones, one of the most abundant species of this vent fauna (Cavanaugh et al. 1981; Felbeck 1981; Southward et al. 1981). Later, two large species of bivalve Mytilidae were found to carry similar bacteria in their gill tissue (Cavanaugh 1983; Fiala-Medioni 1984; Le Pennec and Hily 1984; Le Pennec and Prieur 1984). These chemolithotrophic bacteria are thought to constitute a major source of the energy and nutrition of their hosts (cf. Jannasch 1985). Symbiotic bacteria and possible chemosynthetic capabilities have also been described for animals living in shallow sulfide-rich environments. These include groups such as oligochaetes (*Phallodrilus leukodermatus* and *Phallodrilus planus*:

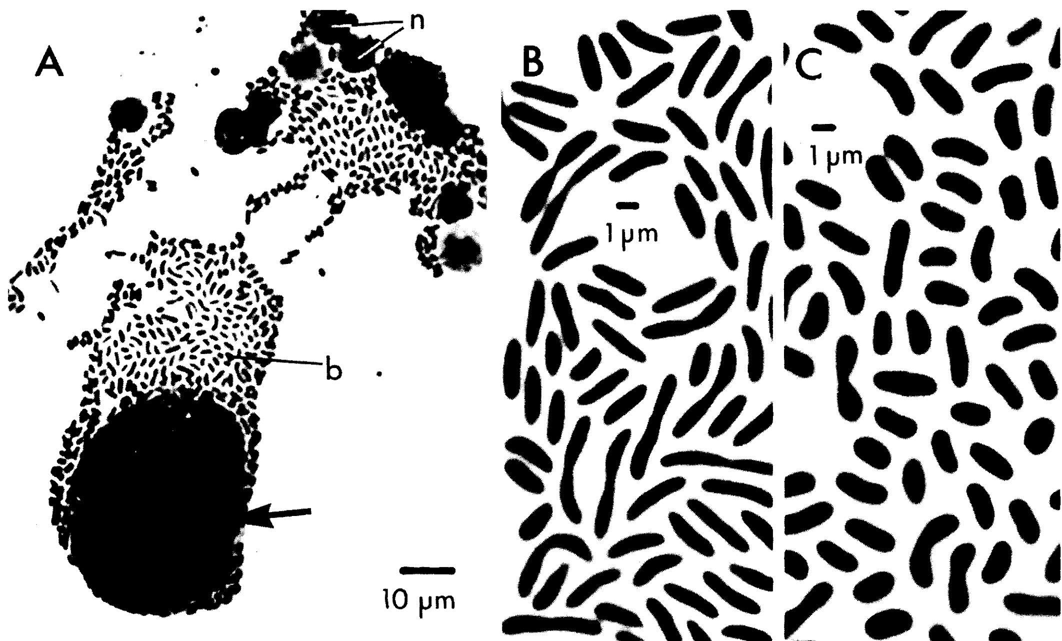

Fig. 1. Results of the method using a gill tissue from *Spisula subtruncata*: photomicrographs of cell suspension, Giemsa staining. A. Dispersed bacteria from packed assemblages (arrow); b—bacteria; n—nuclei of gill tissue. B and C. Two different types of bacteria.

Giere 1981; Felbeck et al. 1983), nematodes (*Astomonema jenneri*) and turbellarian *Parecatenula* sp. (Ott et al. 1982), and bivalves of the family Solemyidae (Cavanaugh 1983; Felbeck 1983; Powell and Somero 1985) and Lucinidae (Cavanaugh 1983; Berg and Alatolo 1984; Fisher and Hand 1984; Vetter 1985). Finally, Dando et al. (1985) have described endosymbiotic bacteria in the gill tissue of the bivalve lucinid *Myrtea spinifera,* a species which inhabits shallow sea sediments with a low concentration of free sulfides. They also suggested that the bacterial-gill association might be more widespread than hitherto suspected and might contribute in general to the energy needs of the host animals.

The intracellular symbionts in bivalve gill tissue have until now been demonstrated by TEM observations that involve elaborate fixations and special techniques not always available in the field. We describe a simple method to detect bacterial associations in bivalve gill tissue with a transmitted light microscope. Using this method, we have found two different types of bacteria in gill tissue of the surfclam *Spisula subtruncata* (Da Costa) (Mactridae) and verified the results by TEM observations that showed their intracellular and endosymbiotic character (Bouvy et al. 1986).

The steps of our method follow.

1. Dissect gills from live specimens.
2. Wash in filtered seawater (0.22 μm).
3. Transfer to distilled water or 25% seawater for 30 min (the latter gives better results on photomicrographs).
4. Remove supernatant.
5. Fix in freshly prepared solution: three parts absolute ethanol to one part acetic acid, 4×10 min (can be stored at 4°C before further steps).
6. Transfer gill tissue to a depression slide containing 50% acetic acid and macerate with forceps to obtain a cell suspension.
7. Release one drop of cell suspension with a Pasteur pipette from a height of about 10 cm onto a cleaned microscope slide that has been warmed to 44°C ($\pm$2°C) (the temperature necessary to split cell membranes).
8. Quickly aspirate excess liquid.
9. Air-dry.

10. Stain for 10 min in freshly prepared Giemsa solution: 4 ml of Giemsa (Merck, Art. 9204); 4 ml of phosphate buffer 0.2 M; pH 6.8; 92 ml of distilled water.

11. Wash in water for 5 min and air-dry.

The preparation can be viewed immediately with the light microscope. For high magnification (×100 optics), immersion oil is placed directly on the dried droplet without need of a cover glass. If wanted, the slide can be made permanent: keep 10 min in xylol and mount in Euparal (or another resin).

This procedure yields preparations of disrupted cells at the periphery of the drop sites. Giemsa stains DNA of nuclei and bacteria. Results with *S. subtruncata* show in each droplet of gill cell suspension many assemblages of bacteria-shaped organelles. We have used a minimum of 10 animals (each gill gave one droplet of cell suspension). We also tested mantle and foot tissue from *S. subtruncata,* and gill tissues from other bivalves (*Mytilus edulis, Mytilus galloprovincialis, Ostrea edulis, Crassostrea gigas, Ruditapes philippinarum, Venus gallina*); bacteria-shaped organelles were not observed. The results with *S. subtruncata* are shown in Fig. 1. Large numbers of bacteria-shaped organelles were confirmed by TEM to be Gram-negative bacteria (Bouvy et al. 1986) which had been released from their packed assemblages (Fig. 1A). Two different types of bacteria are present, one rod-shaped (about 3 × 0.6 μm) (Fig. 1B) and the other shorter and wider (about 2 × 1 μm) (Fig. 1C).

It is also possible to use the Gram method directly with fresh tissue. The procedure used is as above except that step 5 is omitted and after air-drying, the preparation is Gram-stained. Gram-negative bacteria were found in *S. subtruncata* (Bouvy et al. 1986).

The method can be useful in a systematic search for bacterial associations among bivalves and could also be used to detect surface bacteria as well as bacteria in other tissues of other species. However, it should be considered as a first step to detect bacteria-shaped organelles; further TEM techniques should be used to confirm the characteristics of procaryotic cells. After that, enzymatic assays, chemical analyses, carbon dioxide fixing, nitrogen fixing, etc. are necessary to determine metabolic pathways and actual symbiotic relationships.

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A simple fiber-optic microprobe for high resolution light measurements: Application in marine sediment[1]

Abstract—A fiber-optic microprobe is described which is inexpensive and simple to build and use. It consists of an 80-μm optical fiber which at the end is tapered down to a rounded sensing tip of 20–30-μm diameter. The detector is a hybrid photodiode/amplifier. The probe has a sensitivity of 0.01 μEinst m^{-2} s^{-1} and a spectral range of 300–1,100 nm. Spectral light gradients were measured in fine-grained San Francisco Bay sediment that had an undisturbed diatom coating on the surface. The photic zone of the mud was only 0.4 mm deep. Measured in situ spectra showed extinction maxima at 430–520, 620–630, 670, and 825–850 nm due to absorption by chlorophyll *a*, carotenoids, phycocyanin, and bacterio-chlorophyll *a*. Maximum light penetration in the visible range was found in both the violet and the red at ≤400 and ≥700 nm.

Most of our knowledge about light distribution and microalgal photosynthesis in aquatic ecosystems is based on studies in the water column. In many lakes and coastal marine environments, however, benthic microalgae contribute significantly to primary production (e.g. Hunding 1971; Gargas 1971; Cadée and Hegeman 1974; Hartwig 1978). The benthic systems differ from the plankton in certain respects which make the study of microbenthic photosynthesis and its regulation by light experimentally difficult: the photic zone is very narrow in sediments, light scattering is strong, and many benthic microalgae migrate vertically in response to changing environmental conditions such as light.

Earlier techniques for measuring light in sediments have not been able to resolve the spectral light gradients with a high spatial resolution. Light penetration has been studied by covering a large light sensor with millimeter-thin layers of sediment or benthic microalgae (e.g. Hoffmann 1949; Taylor 1964; Taylor and Gebelein 1966; Jørgensen et al. 1979; Haardt and Nielsen 1980) or by inserting a small light probe into the sediment (Fenchel and Straarup 1971). Only in the leaves of higher plants have light measurements recently been made on a very small scale by the use of fiber-optics (Vogelmann and Björn 1984).

Since the development of microelectrode techniques for the measurement of chemical gradients and of photosynthesis at ≤100-

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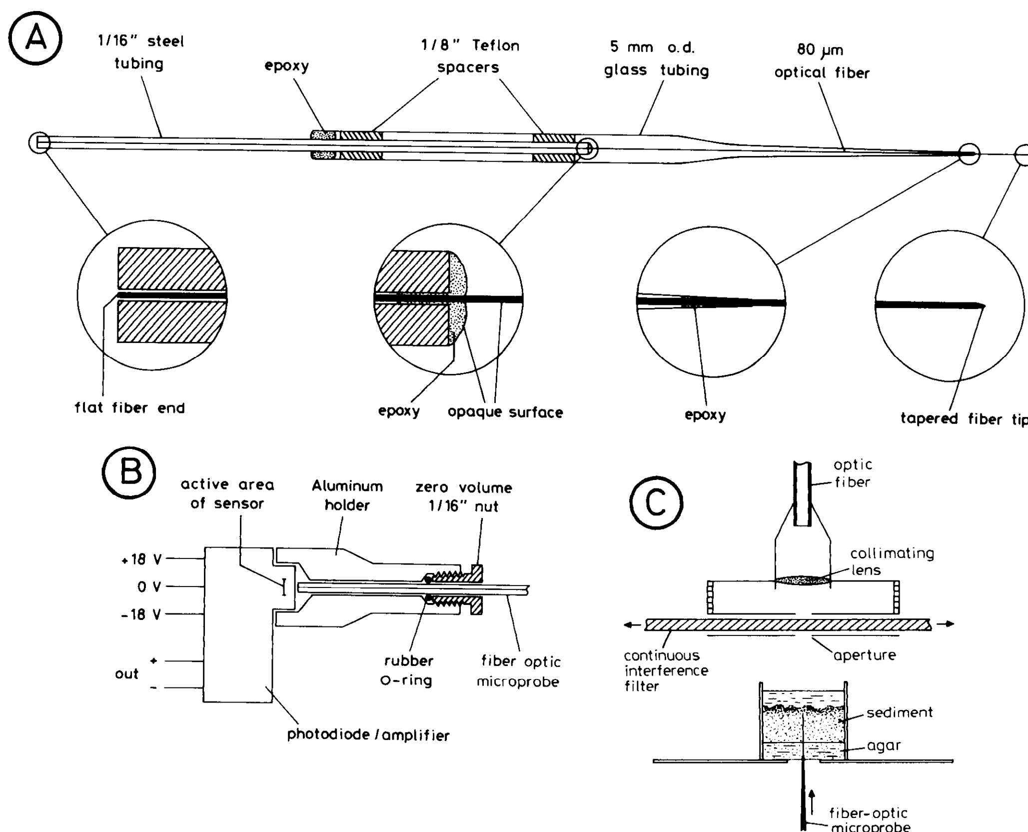

Fig. 1. Fiber-optic microprobe. A. Probe with tapered optical fiber and steel and glass shaft. B. Hybrid photodetector with holder for fiber-optic microprobe. C. Experimental setup for measurement of spectral light gradients in sediments.

μm resolution in sediments, in epiphytic communities etc. (Revsbech and Jørgensen 1986), a need has emerged for a similar resolution in light measurements. We have therefore constructed a fiber-optic microprobe that is simple and yet very sensitive and have tested its applicability in a marine sediment.

We thank C. Arneson, R. Booth, Y. Cohen, K. H. Nealson, N. P. Revsbech, and M. Seyfried for helpful discussions on the techniques and O. Holm-Hansen for donating optical fibers.

Single-stranded optical fibers of 80–250-μm diameter can be used to construct the probes. The graded index fibers used consist of a light-guiding core, in which the refractive index increases toward the axis, surrounded by a cladding of lower refractive index. Only fibers with silica core and cladding (e.g. Corning or American Optical) were used. These fibers generally have a loss of light energy of only a few dB per km (depending on the wavelength). Quartz fibers (e.g. Schott) can be used for UV detection. The uncoated 80-μm fibers we used were obtained from the multistranded light guide of a halogen lamp. Other fibers used were coated with a polymer jacket; a few centimeters of the coating on these was first removed from the fiber end, which was submerged in methylene chloride for 1 min and the coating then wiped off with clean tissue.

To reduce mechanical disturbance of the sediment and algae by the advancing fiber tip, the end of the fiber was tapered down to a tip diameter of 20–30 μm and rounded. The fiber was suspended vertically and stretched with a 1–2-g weight in the lower end. A heating loop made of 0.1-mm plat-

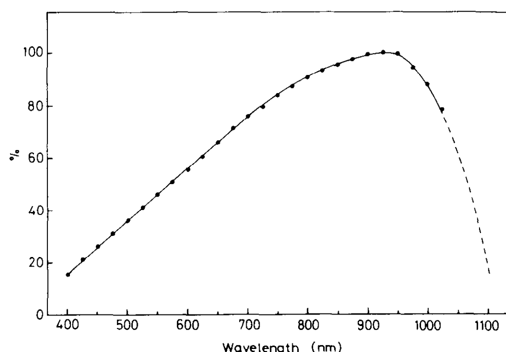

Fig. 2. Spectral sensitivity of fiber-optic microprobe with hybrid semiconductor detector.

inum wire (Revsbech and Jørgensen 1986) was attached to a micromanipulator and the fiber observed under a dissecting microscope at 50× magnification. The red-hot loop was carefully advanced toward the fiber until partial melting and drawing created a fine constriction. (Only a few fibers could easily be drawn in this manner without cracking of the outer glass layer.) The fiber was then broken at the constriction and the fine tip rounded by approaching it with the hot loop again. Due to the surface tension of the molten glass, the small tip automatically rounded into a hemisphere of good optical axial symmetry.

The tapered fiber was broken 18–20 cm from the rounded tip. A fine score was first made on the naked fiber with the edge of carborundum paper and the fiber was pulled apart. With some practice, this procedure creates a perfectly flat surface perpendicular to the fiber axis, which is necessary to obtain a good optical coupling to the detector.

The fiber was then mounted in a shaft (Fig. 1A). It was first inserted into a 10-cm piece of $\frac{1}{16}$-inch (0.32 cm) stainless steel tubing with 0.010-inch (250 μm) i.d. We used precut lengths of cleaned tubing with burr-free ends, made for HPLC (e.g. Alltech). The fiber was aligned with the flat end flush with one end of the steel tubing and sealed with a small drop of fast-hardening epoxy resin at the other end. The epoxy and most of the fiber was painted black to prevent stray light from channeling through the steel tubing up to the detector; the whole fiber surface, however, does not need an opaque coating as light entering it from the side will not be propagated internally along its axis. Two pieces of $\frac{1}{8}$-inch (0.64 cm) Teflon tubing were positioned on the steel tubing as spacers for the following mounting.

An outer glass casing was made from 5-mm-o.d. sodium glass. The glass tubing was pulled in several steps over a small flame into a fine, tapering capillary. A fiber was inserted until it got stuck inside the capillary, which was broken at that point under a dissecting microscope so that the capillary tip fit exactly around the fiber. The tapered fiber was mounted in the capillary with 5–10 mm protruding from the capillary end and sealed at both ends with epoxy resin.

A hybrid semiconductor detector was used for the light measurement (EG&G Electro Optics, TCN-1000-93). Such combined low-noise photodiode/amplifier units of only a few cubic centimeters are available at low cost and require a minimum of power supply and output measuring equipment. A simple holder was constructed for mounting the fiber probe precisely over the detector window (Fig. 1B). The aluminum body of the holder was painted black inside and sealed over the detector window. The optical geometry ensured that all light fell on the 5-mm^2 active area of the detector. The probe was fastened to the holder by tightening a rubber O-ring with a $\frac{1}{16}$-inch (0.32 cm) zero volume nut (standard HPLC fitting). Four 9-V batteries were used for a ±18-V-d.c. power supply. The supply current was <1 mA and the output voltage 0–500 mV, read on a mV-meter to an accuracy of <0.1 mV. We used an HP model 3421A datalogger as a high-precision, integrating meter to increase the sensitivity of detection.

The sensitivity depended on the tip diameter of the optical fiber, which determined the cross-sectional area of light acceptance. Calibration was with a LiCor light meter with either a pyranometer detector or a 400–700-nm quantum detector. Using a 150-W halogen lamp as a source of collimated white light, we measured output currents at different light intensities with the fiber probe pointing directly toward the light source (angle 0°). With 20–30-μm tip diameters the response was 40–80 mV at 1 W

m^{-2} or 5–10 mV at 1 μEinst m^{-2} s^{-1}. With a typical noise level of 0.05–0.1 mV, the detectability of white light was about 0.001 W m^{-2} or about 0.01 μEinst m^{-2} s^{-1}. The response was linear up to 500 mV (i.e. 10 W m^{-2} or 100 μEinst m^{-2} s^{-1} of white light) and the dynamic range was thus 10,000. At higher light intensities a neutral-density filter can be used to step down the quantum flux reaching the detector. The photodiode detector potentially covers a spectral range of 300–1,100 nm. The spectral sensitivity of the fiber-optic microprobe is shown in Fig. 2.

The acceptance angle of the tapered fibers is important for quantitative discrimination between collimated light and scattered light from different directions. Optical fibers with a flat end generally have a numerical aperture, NA, in air of 0.4–0.6 (NA is the sine of the angle between the axis of the fiber and the point at which the intensity has fallen to 5% of its own axis value). The directional sensitivity of the fiber-optic microprobes in air was analyzed by fixing the fiber tip in a collimated light beam and varying the light-to-fiber angle over +90 to −90 degrees. Figure 3 shows how the acceptance angle increased (the NA increased from 0.53 to 0.65) and became less sharply defined by tapering and rounding the fiber tip. Measurements in monochromatic light showed that the acceptance angle increased only slightly with wavelength (about 2 degrees between 450 and 1,000 nm: Fig. 3B). For aquatic applications the NA should be corrected for the refractive index of water.

We used the fiber-optic microprobes to measure light gradients and spectra in sediments and cyanobacterial mats. It is essential that these measurements be done from different angles, especially with the fiber ascending from below, without disturbing the sediment–water interface. Sediment samples were carefully collected by hand in 5-cm-i.d. coring tubes, brought to the laboratory, and subcores of 1–2-cm depth taken from the sediment surface in 3-cm-long transparent tubes of 2.5-cm i.d. To avoid leakage of porewater, especially from sandy sediments, we sealed the subcores in the bottom with an agar plug through which the optical fiber could penetrate. A 1.5% agar solution in water from the sampling locality was poured hot into Petri dishes and cooled. The subcores were pressed down over the 5-mm agar layer. The lower edge of the coring tubes had been tapered on the inside to ensure a tight seal of the agar plug. The subcore with about 5 mm of overlying water was then positioned on a plastic plate over a 10-mm hole through which the fiber could penetrate up into the core (Fig. 1C).

The sediment was illuminated on the surface with monochromatic light. White light from a halogen lamp was guided via a fiber-optic cable and a condensing lens through a continuous interference filter (Schott) sliding between two apertures of 3-mm diameter (Fig. 1C). A 400–700- and a 400–1,025-nm interference filter were used, the former in combination with a heat protection filter (Schott No. 158300) to remove IR radiation interfering at wavelengths <700 nm. The half-band widths were 12–14 and 25–32 nm for the two filters and spectral measurements were taken at 10- and 25-nm intervals. The filters were calibrated with a scanning spectrophotometer (Cary model 14).

The fiber-optic microprobe was attached to a micromanipulator with which it was advanced in 50- or 100-μm increments at $\pm$5-μm accuracy. The fiber penetrated vertically up through the sediment core and was observed through a dissection scope. The exact position of the measurements relative to the sediment surface was determined by reading the micromanipulator scale when the tip was just visible at the sediment–water interface.

Measurements of spectral light gradients were made in different types of sediments, of which one example will be presented here. Cores of organic-rich silt-clay sediment were collected along the south shore of San Francisco Bay. The sediment had a dense coating of pennate diatoms together with many filamentous cyanobacteria. At 1–2 mm depth there was a sparse population of purple sulfur bacteria. Below these, the sediment was black due to high iron sulfide concentration.

Light gradients at four wavelengths are shown in a semilog plot in Fig. 4. The blue light of 450 nm was extinguished more rapidly than red (670 nm) and orange (600 nm), while infrared (1,000 nm) penetrated deep-

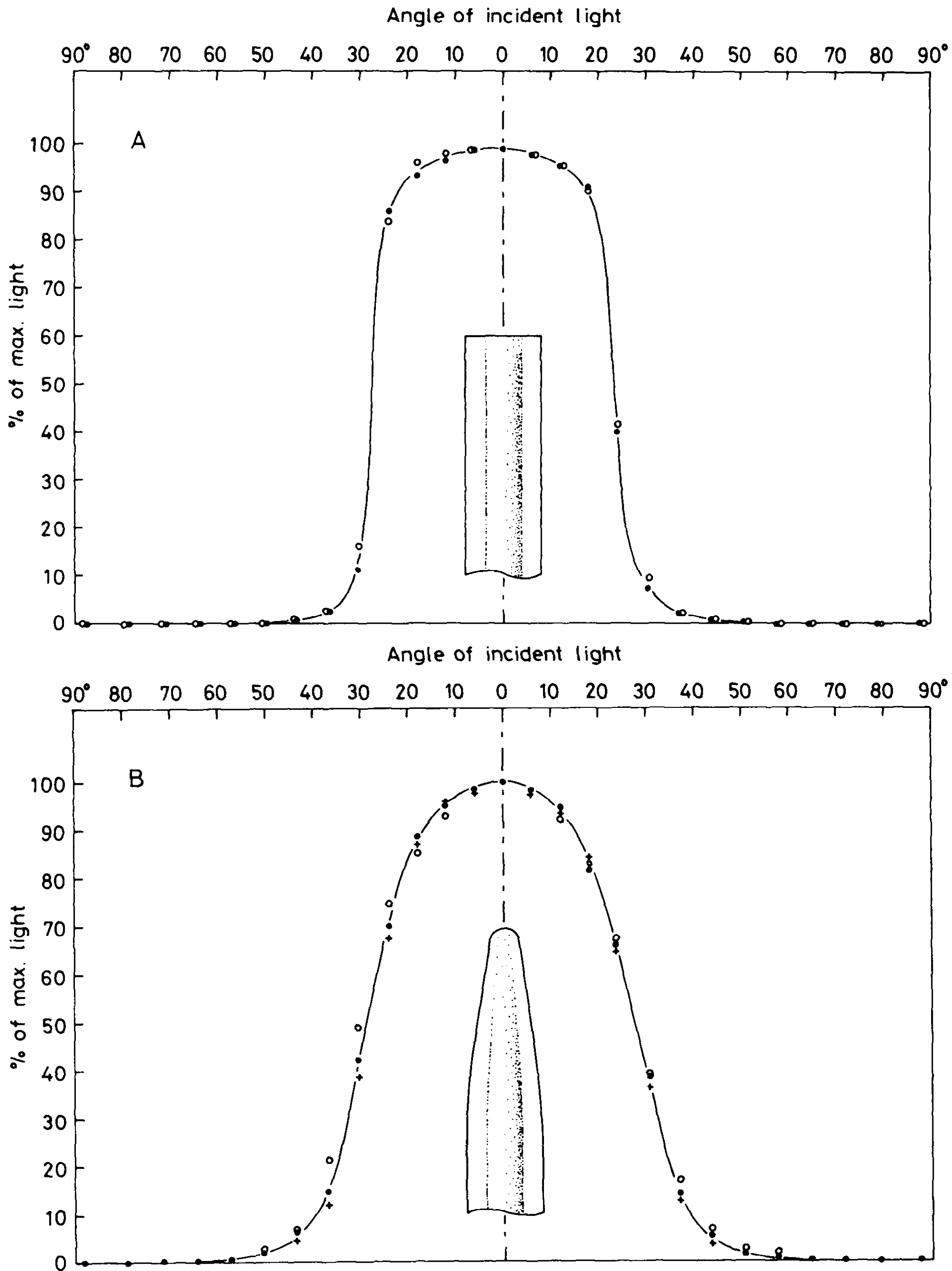

Fig. 3. Acceptance angles in air of fiber-optic microprobes with flat fiber end (A) or tapered and rounded fiber end (B). A. The 80-μm-wide fiber was rotated 90° between the first (○) and the second (●) set of measurements of white light. B. Measurements were made in monochromatic light at 400 (○), 700 (●), and 1,000 nm (+).

est into the sediment. The blue light was reduced to 1% of the surface illumination at 0.3-mm depth and the red at 0.4, while the infrared reached 1% only at 1.2-mm depth. The attenuation coefficients, k, for the four wavelengths were calculated from $E_z = E_0 \times \exp(-kz)$, where E_z and E_0 are the measured light intensities at depth z and

at the surface. Near the sediment surface the attenuation coefficients were 20.3, 8.0, 16.8, and 2.3 mm^{-1} for 450, 600, 670, and 1,000 nm, about an order of magnitude higher than reported for other marine sediments (Fenchel and Straarup 1971; Haardt and Nielsen 1980).

The spectral composition of light at different depths in the sediment is shown in Fig. 5. The spectra of visible light (Fig. 5, left) showed a broad minimum in the blue-green range of 420–530 nm, a distinct minimum in the red at 670 nm, and a slight minimum around 630 nm. The spectral extinction corresponded well to the main light-absorbing pigments of the dominant phototrophic organisms. Chlorophyll *a* absorbs mostly in the blue around 430 nm and in the red around 670 nm. Carotenoid pigments in the diatoms absorb in the green between 450 and 530 nm. Phycocyanin in the cyanobacteria absorbs in the orange around 620 nm. The near-infrared light penetrated much deeper than visible light (Fig. 5, right). There was a broad minimum between 800 and 900 nm corresponding to the absorption region of bacteriochlorophyll *a* in the purple sulfur bacteria. The infrared absorption was most pronounced below 0.5-mm depth where most of the purple sulfur bacteria were positioned. The absorption spectra in Fig. 5, right, are less distinct than those in Fig. 5, left, due to the lower spectral resolution of the broad-range interference filter.

These results, obtained from fine-grained coastal sediment, show how rapidly the light spectrum may change with depth in sediments. By 0.4 mm below the sediment surface the light of wavelengths absorbed by Chl *a* or carotenoids had been reduced to 1% of the surface intensity (Fig. 5, left). As 1% seems roughly to be the level of compensation light intensity in coastal ecosystems, the photic zone in this sediment was only 0.4 mm thick. It is remarkable that both the shortest and longest wavelengths in the visible spectrum penetrated deepest into the sediment. Light scattering by mineral grains, which might be expected to cause the shorter wavelengths to be extinguished faster (Fenchel and Straarup 1971), thus

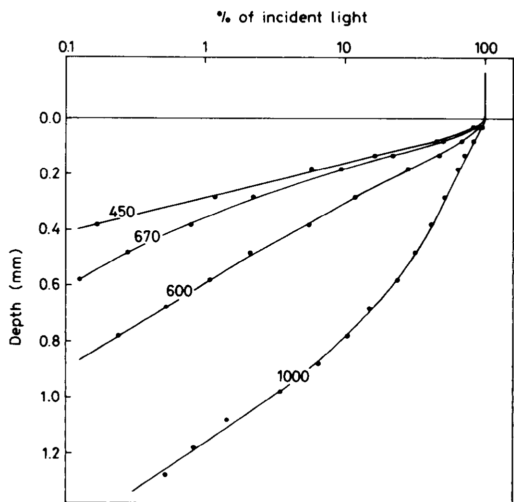

Fig. 4. Distribution of monochromatic light in diatom-rich silt-clay sediment from San Francisco Bay. The light flux is expressed as percent of surface illumination for each wavelength. Vertical illumination and 0° light-to-fiber angle (fiber tip facing light source).

seemed to have less effect than absorption by pigments. A quantitative discrimination between the effects of scattering and absorption is, however, not simple.

A spatial resolution of ≤ 0.1 mm has not previously been approached in in situ studies of light distribution in microalgal communities. The data presented here show that such a high resolution was necessary to demonstrate the steep light gradients within the photic zone. A spectral analysis with a resolution of at least 10–15 nm was also found to be necessary to show the selective extinction of certain wavelengths. It is evident from Fig. 5 that measurements only of the "white" light distribution would not be very useful, or would even be misleading, in a sediment like this with dense phototrophic communities.

One important advantage of the microprobe technique is that it is nondestructive, in contrast to most previous techniques. It is thus possible to perform continued light measurements and microscopic observations of the same sample. In spite of its small dimensions, the optical fiber may cause some mechanical disturbance, which can become a problem when measurements are taken at 50–100-μm depth increments. It is there-

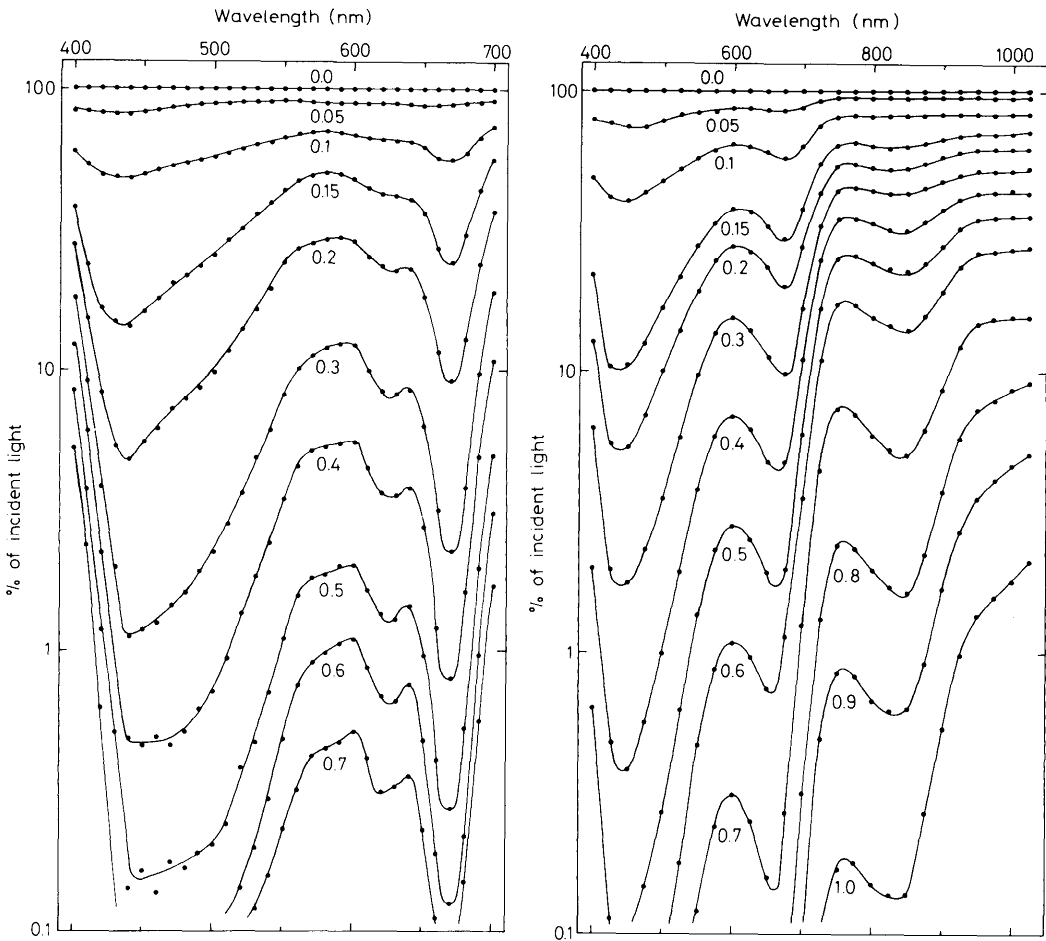

Fig. 5. Spectral light distribution in sediment from San Francisco Bay at 400–700-nm (left) and 400–1,025-nm (right) range. Numbers on curves indicate depths (mm) in sediment. The light flux is expressed as percent of surface illumination for each wavelength on a log scale. Same data as in Fig. 4.

fore important to check carefully for such disturbances under the dissecting scope. Migration of the benthic microalgae may also be a serious problem when longer series of measurements are taken. A more advanced detecting system, which would rapidly scan the complete spectrum, in combination with a motor-driven micromanipulator, would help to solve this problem. However, the present technical solution has the advantage of being simple to build, inexpensive, and straightforward to use (provided that a stable mechanical support can be provided with a vibration level $\ll 100$ μm).

The fiber-optic microprobe has other obvious applications to the study of light in epiphytic communities and other aggregates of microalgae, in macroalgae with relatively soft tissues, in microbial mats, in animals with photosynthetic symbionts, etc. We have now combined the high-resolution light measurements with microelectrodes that can be used to measure chemical gradients and photosynthetic activity at similar spatial resolution (Revsbech and Jørgensen 1986; Jørgensen and Des Marais 1986). With these microtechniques we have been able to carry out more precise in situ studies of light saturation, action spectra, and light-in-

duced zonation and migration in cyanobacterial mats (Jørgensen et al. in prep.).

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Announcement

Call for Nominations: G. Evelyn Hutchinson Medal

Nominations are now solicited for the sixth award, to be presented at the June 1987 meeting of ASLO in Madison, Wisconsin. The medal is awarded annually for continued excellence in research in any aspect of limnology or oceanography, with emphasis on work done during the preceding 5 years. The award is intended to symbolize the quality and innovations toward which ASLO strives and to remind its members of these goals.

Anyone may nominate a candidate, but the recipient must be a member of ASLO. Nominations and supporting documents (curriculum vitae, statement of merit, list of publications) should be submitted before 1 March 1987 to John Hobbie, ASLO Awards Committee Chair, Ecosystems Center, Marine Biological Laboratory, Woods Hole, Massachusetts 02543.