MARINE BACTERIA AS A SOURCE OF DISSOLVED FLUORESCENCE IN THE OCEAN

by

Paula G. Coble

B.A., Biology and Geology
Mount Holyoke College, South Hadley, MA
(1973)

M.S., Marine Studies
University of Delaware, Newark, DE
(1977)

SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE
DEGREE OF

DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

and the

WOODS HOLE OCEANOGRAPHIC INSTITUTION
Woods Hole, Massachusetts 02543

October 1989

Signature of Author _____

MIT-WHOI Joint Program in Oceanography
Woods Hole, MA
November 1989

Certified by _____
Robert B. Gagosian, Thesis Supervisor

Accepted by _____
Philip M. Gschwend, Chairman, Joint Committee for Chemical Oceanography, Massachusetts Institute of Technology-Woods Hole Oceanographic Institution

MARINE BACTERIA AS A SOURCE OF DISSOLVED FLUORESCENCE IN THE OCEAN

by

Paula G. Coble

Submitted to the Joint Committee for Chemical Oceanography, Massachusetts Institute of Technology and the Woods Hole Oceanographic Institution on October 6, 1989 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

ABSTRACT

The hypothesis that marine bacteria produce sufficient quantities of highly fluorescent compounds to permit mapping of their distribution in low oxygen oceanic waters using fluorescence profiling has been investigated. Individual fluorescent compounds produced by natural seawater isolates in the laboratory include flavins, which are produced in sufficient quantities to explain in situ concentration in the ocean. Maxima in riboflavin concentrations were found at the depth of the oxic/anoxic interface in both the Black Sea and the Cariaco Trench, coincident with maxima in other indicators of elevated microbial biomass, including bacteriochlorophyll e concentration and electron transport system (ETS) activities.

In the Black Sea, simultaneous, continuous profiles of fluorescence due to dissolved organic matter (DOM) (Ex/Em = 355/500), flavins (Ex/Em = 445/525) and chlorophyll (Ex/Em = 445/ >660) from 0 to 300 m show DOM and flavin fluorescence increase with depth. Sharp increases were observed at density boundaries. Local maxima in flavin fluorescence were found in the zone of denitrification. A secondary maximum in chlorophyll fluorescence was due to bacteriochlorophyll e from Chlorobium and was associated with maxima in beam attenuation coefficient and electron transport system (ETS) activity.

High performance liquid chromatography (HPLC) analysis of individual dissolved fluorescent compounds (DFCs) in samples from both the Black Sea and the Cariaco Trench showed 15-20 peaks superimposed on a high background of unresolved material. The compositional patterns displayed remarkable similarity at the two sites. Individual components identified included several flavin compounds. There was good agreement between estimates of dissolved fluorescence as measured by both HPLC analysis and in situ fluorescence profiling.

Overall, results indicate that important information regarding the source and nature of marine DOM can be obtained using the combined approach of fluorescence spectroscopy and individual compound analysis.

Thesis Supervisor: Dr. Robert B. Gagosian
Associate Director of Research
Woods Hole Oceanographic Institution
Woods Hole, Massachusetts 02543

ACKNOWLEDGEMENTS

I have so many people to thank for help, equipment, and encouragement that it is hard to know where to start or end. Ed Peltzer, Gale Nigrelli, Nelson Frew, Carl Johnson, Stuart Wakeham, and Liz Canuel each taught me some of what I know about laboratory procedures for analysis of trace levels of organic compounds. Clarice Yentsch, John Waterbury, Neil Blough, and Rob Olsen all provided vital pieces of equipment at various stages of the project. Collection of the pump profile data in the Black Sea would have been impossible without the assistance of John Christensen, Lou Codispoti, and Gernot Friederich. Valuable discussions of analytical techniques with Ken Mopper and Sue Vastano hastened my progress with flavin analysis and provided moral support. Discussions with Hilary Glover in the early stages of planning this thesis project regarding fluorescent bacteria provided much of the initial excitement and enthusiasm, which helped to carry me through the many years to completion. Special thanks to Hovey Clifford for helping me move my laboratory to Istanbul via Pan American Airlines.

I would like to thank each one of my committee members Craig Taylor, Charlie Yentsch, Penny Chisholm, Dan Repeta, and Ollie Zafiriou, for contributing their time and highly individual expertise to this project. Most of all, I must thank my advisor, Bob Gagosian, for his endless support and patience through some very trying times.

Financial support was provided by the Ida Green Fellowship, WHOI Education Office, and NSF Grants OCE-84-15724 to RBG and OCE-87-01386 to RBG and PGC.

Thanks also to the Captain, crew and scientists on R/V Columbus Iselin cruise #CI8601 and the Captain, crew and scientists on R/V Knorr cruise #134-12. Each person on the staff of the Graphics Department at WHOI made some contribution to the figures in this thesis, and I thank them for their cheerful professionalism.

Above all, I must thank my parents for instilling in me the belief that I could accomplish whatever I set out to do, and my son, Nathan, for reminding my everday of the most important things in life.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

INTRODUCTION

The total fluorescence of seawater can be divided into two fractions: "dissolved" fluorescence due to the presence of dissolved organic matter (DOM) in seawater (Kalle 1949) and "particulate" fluorescence due to the presence of photosynthetic pigments of phytoplankton (Yentsch and Menzel 1963; Lorenzen 1966). Previous measurements of *in situ* fluorescence have relied upon differences in the positions of excitation and emission maxima to discriminate between the two fractions. The blue fluorescence of dissolved organic matter (DOM) in seawater has an excitation maximum centered at 350 nm and an emission maximum between 400 and 450 nm. Chlorophyll fluoresces at 685 nm when excited at 435 nm. Further discrimination between the signals arises from the fact that particulate fluorescence is highest in the upper 100 m of the water column (euphotic zone) because phytoplankton require light, whereas dissolved fluorescence is highest below 100 m due to photodegradation of DOM in surface waters (Gjessing and Gjerdahl 1970; Kramer 1979; Hayase *et al.* 1987).

Several recent reports of deep fluorescence maxima have led to speculation that in low oxygen environments, interpretation of *in situ* fluorescence may not be straightforward. Although measured using fluorometers optimized for chlorophyll fluorescence, the deep fluorescence maxima in the eastern tropical Pacific Ocean occur below the euphotic zone at the depth of the minimum in oxygen concentration (Anderson 1982; Broenkow *et al.* 1983; Lewitus and Broenkow 1985).

In addition to fluorescence maxima, other features associated with the oxygen minimum zone in the eastern tropical Pacific are maxima in: nitrite concentrations (for example, Brandhorst 1959; Wooster et al. 1965), suspended particles (Pak et al. 1980; Kullenberg 1981, 1984; Garfield et al. 1983; Broenkow et al. 1983), particulate protein (Garfield et al. 1979), phaeopigment concentration (Blasco et al. 1979), rates of microbial activity (Garfield and Packard 1979; Garfield et al. 1983), bacterial biomass (Spinrad et al. 1989), and particulate sterenes (Wakeham et al. 1984). These observations suggest that fluorescence in low oxygen waters may be due to a unique combination of dissolved and particulate components and that the indigenous microbial community may be at least partially responsible for the fluorescence maxima.

Bacteria are known to produce a wide variety of photosynthetic and non-photosynthetic pigments including bacteriochlorophylls, carotenoids, phycobillins, pteridines, flavins, cytochromes, phenazines, pyoverdine and pyocyanin. Several species of marine bacteria which produce yellow fluorescent pigments and are capable of nitrate reduction have been reported by ZoBell and co-workers (ZoBell and Feltham 1934; ZoBell and Upham 1944). Denitrifying bacteria are known to be important in oceanic oxygen minimum zones, and, if present in the water column in sufficient quantities, these bacteria could potentially contribute to the observed fluorescence maximum.

This thesis project was designed to test the hypothesis that an indigenous population of marine bacteria could be a source of fluorescent compounds of sufficient concentration to be detected using in situ fluorometry.

1. Fluorescence and particle maxima in oxygen-deficient oceanic regions

In stratified water masses, a phytoplankton-derived primary fluorescence maximum occurs in the euphotic layer at the pycnocline/nitracline (e.g. Pingree et al. 1978; Cullen and Eppley 1981; Holligan et al. 1984). However, the existence of much deeper fluorescence maxima has only recently been recognized by using fluorometers attached to pumping systems (Anderson 1982) or fluorometers lowered over the side of the ship (Broenkow et al. 1983; Lewitus and Broenkow 1985; Broenkow et al. 1985; Spinrad et al. 1989). Off the west coast of Mexico, secondary (>125m) and tertiary (200-400m) fluorescence maxima have been observed to have a weaker fluorescence signal than that of the primary fluorescence peak in the photic zone. These deep fluorescence maxima were associated with high nitrite and low oxygen concentrations (Anderson 1982; Broenkow et al. 1983; Lewitus and Broenkow 1985). Secondary and tertiary fluorescence maxima have also been observed to be associated with high nitrite and low oxygen waters off the Peruvian coast (Spinrad et al. 1989), but in the central North Pacific Ocean, Lewitus and Broenkow (1985) observed only one deep (950m) fluorescence maximum in the oxygen minimum zone. Previous studies of in situ fluorescence at sea have been conducted using fluorometers with broad band excitation (336-572nm) and emission (645-750nm) filters, which allow compounds other than chlorophyll a to be detected, provided they are present in sufficient concentrations. Other compounds which may contribute to the fluorescence signal include chlorophyll degradation products, phycobiliproteins, bacteriochlorophylls and cytochromes in the particulate fraction, and

dissolved organic compounds (Anderson 1982; Broenkow et al. 1983; Lewitus and Broenkow 1985). Broenkow et al. (1985) used a variety of optical filter combinations with an in situ fluorometer, which indicated that there may indeed be a number of different fluorescent sources.

2. Sources of dissolved fluorescence

Significant correlation between the total beam attenuation coefficient and in situ fluorescence suggests that particles are a major source of the observed fluorescence in the secondary and tertiary maxima (Broenkow et al. 1983; Lewitus and Broenkow 1985; Spinrad et al. ' 39), however dissolved fluorescent substances may also be important. Fluorescence measurements have been used for many years to measure dissolved organic matter (DOM) concentrations in seawater (Kalle 1949; Duursma 1974). Dissolved fluorescence shows an inverse correlation with salinity in estuaries and has been attributed to runoff of terrestrial humic material (Duursma 1974, Laane 1981; Berger et al. 1984; Hayase et al. 1987). Vertical distributions of dissolved fluorescence in the open ocean generally show a monotonic increase with depth (Ivanov 1962; Duursma 1974; Hayase et al. 1987, 1988; Chen and Bada 1989), however increased DOM fluorescence at the surface has also been found in association with areas of high productivity (Karabashev 1977).

The chemical nature of fluorescent DOM is not well understood. It represents a fraction of the "gelbstoff" or yellow substance which is found in all natural waters and has three known sources in the ocean. Terrestrial humic material derived from land plant degradation

is delivered to estuaries and coastal waters via river runoff (Kalle 1966). Phytoplankton and macroalgae release gelbstoff into seawater as a metabolic by-product (Yentsch and Reichert 1961; Traganza 1969; Sieburth 1969; Sieburth and Jensen 1969; Carlson and Mayer 1983). Small molecules such as amino acids and carbohydrates have been shown in laboratory studies to condense to form larger molecules which have been called "melanoidins" (Kalle 1966). This process presumably also occurs in the ocean and may be accelerated on the surface of clay particles (Hedges 1978).

Gelbstoff has also been equated with humic and fulvic acids, two operationally-defined classes of organic matter in the ocean which can be of allochthonous (terrestrial) or autochthonous (marine) origin. The similarity between the excitation maximum (350 nm) and emission maximum (460 nm) for dissolved fluorescence in the ocean and those for humic and fulvic acids has led to the assumption that the latter are the principal components of the fluorescence signal (Laane and Koole 1982 and references therein). Marine humic material is thought to be formed in a manner analogous to melanoidin formation and a wide variety of structures has been proposed for this material (Kalle 1963; Duursma 1965; Stuermer and Harvey 1974; Hedges 1978; Harvey et al. 1983; Harvey 1984; Laane 1984).

3. Pterins and flavins in seawater

Identification and measurement of individual fluorescent compounds in the ocean has been limited to a few pterins and flavins (Momzikoff 1969a; Dunlap and Susic 1985; Mopper and Zika 1987; Vastano 1988), however the concentrations of these are too low to explain the

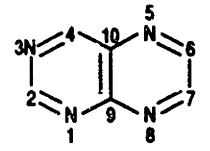
magnitude of the total dissolved fluorescence signal. Flavins and pterins are constituents of coenzymes which are required by all living organisms, e.g. riboflavin and folic acid. Figure 1.01 shows the structures of a few of these compounds, most of which have been reported to be present in seawater.

Momzikoff (1969a), was the first to measure isoxanthopterin, riboflavin, and lumichrome in seawater and subsequently reported these same compounds to be present in (Momzikoff 1969b; Momzikoff and Legrand 1973) and excreted by marine copepods (Momzikoff 1973). Pterins and flavins have also been found to be in the bodies of and excreted by marine ascidians (Gaill and Momzikoff 1975). Demonstration of the effects of light and cation degradation of pteridines in seawater was used to challenge Momzikoff's original results (Antia and Landymore 1975; Landymore and Antia 1978), however later studies have confirmed the presence of these compounds in the ocean.

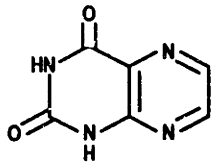
A routine method for analysis of pterins and flavins from seawater which requires only 0.1% of the volume used in Momzikoff's (1969a) method has been described recently (Dunlap and Susic 1985). Results from this study show measurable quantities of six pterins and four flavins in surface waters surrounding the Great Barrier Reef in Australia. Some of these compounds were also found to be excreted by one species of coral and one sponge. Vastano (1988) has proposed that the distributions of flavins and their degradation products may provide useful tracers of water masses and biological productivity in certain environments.

Figure 1.1. Structures of the flavin and pteridine compounds referred to in this thesis. Lumazines are 2,4-dihydroxypteridines and pterins are 2-amino-4-hydroxypteridines.

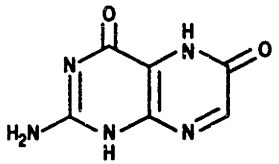
PTERIDINES



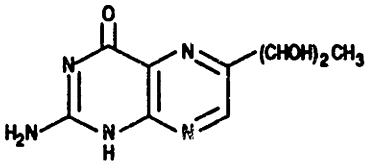
Pteridine



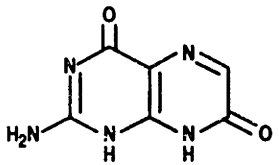
Lumazine



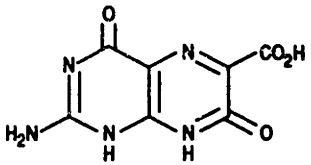
Xanthopterin



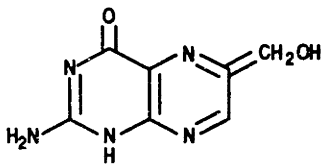
6-biopterin



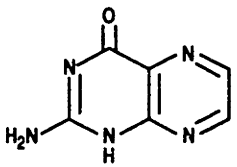
Isoxanthopterin



Pterin-6-carboxylic acid

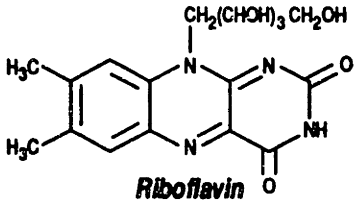


6-hydroxymethylpterin



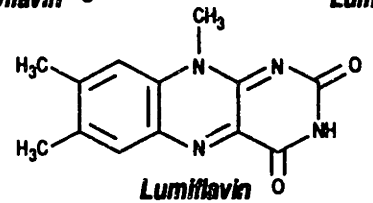
Pterin

FLAVINS

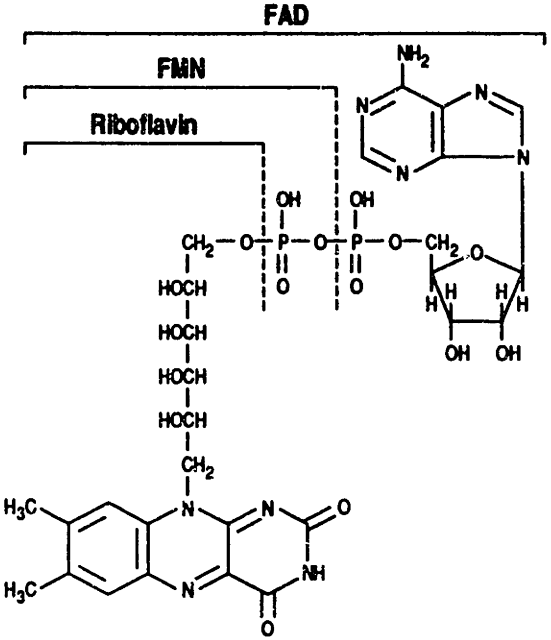


Riboflavin

Lumichrome



Lumiflavin



In summary, methods are now available to isolate pterins and flavins from a few liters of seawater and quantify them in the water column. These compounds originate from biological activity, but may be photo-degraded to fluorescent and non-fluorescent products in the euphotic zone.

4. Bacteria which may contribute to subsurface fluorescence maxima

Many marine bacteria belong to the family Pseudomonaceae, some of which are denitrifiers and some of which are notorious for the production of non-photosynthetic fluorescent pigments. The production of a yellow, water-soluble pigment which fluoresces green has long been used for taxonomic classification of one group of species called the fluorescent pseudomonads (Jessen 1965). Pseudomonas fluorescens is the best-known member of the group producing this pigment, which is said to be "freely diffusible". It is released into the surrounding growth medium, however whether this is due to active excretion or to passive diffusion across the cell membrane has never been examined. The structure of the fluorescent pigment has never been adequately elucidated. It has been called "fluorescein" or "pyoverdine" in the literature and various structures such as pteridines, (Giral 1936; Chakrabarty and Roy 1964) flavins, (Birkhoffer and Birkhoffer 1948), pyrroles (Lenhoff 1963; Greppin and Gouda 1965), purines, and others have been proposed. Since the deep fluorescence maxima are located in the oxygen minimum zones where denitrification occurs, marine pseudomonads may contribute to the total fluorescence signal observed in the eastern tropical Pacific Ocean. The pigments from these

bacteria, if present in seawater, would be part of the dissolved fluorescence fraction.

Other types of bacterial pigments might also contribute to the fluorescence signal in anoxic basins such as the Cariaco Trench and the Black Sea where the bottom waters contain hydrogen sulfide. These are the bacteriochlorophylls produced by the photosynthetic bacteria, and flavins and nickel porphyrins produced by methanogens. These pigments are intracellular and would therefore be expected to be part of the particulate fluorescence fraction.

One of the major obstacles which has prevented analysis of individual fluorescent compounds in seawater has been the lack of adequate analytical methods. Several significant advances in separation technology were made during the course of this project which greatly improved both the sensitivity and resolution of analysis of two classes of dissolved compounds, pterins and flavins (Dunlap and Susic 1985; Vastano et al. 1987). As applied in this study and in those cited aboved, this new technology provides more detailed information regarding the nature and concentration of the individual components of dissolved fluorescence. Continued development of new column packing materials for high performance liquid chromatography (HPLC) and improvements in existing materials will undoubtedly result in very rapid expansion of our knowledge in this area of Chemical Oceanography.

This thesis project was designed to answer the following questions:

1. What are the fluorescent compounds produced by marine denitrifying bacteria and how do they compare to

pyoverdine, the pigment produced by the fluorescent pseudomonad Pseudomonas fluorescens?

2. Are these fluorescent bacterial pigments present in seawater in regions of low oxygen concentrations?
3. Do these compounds make a significant contribution to the total dissolved in situ fluorescence signal?
4. How useful are these compounds as markers of specific environments or bacterial communities in the water column?

These questions are the framework of a research problem which spans a broad range of scientific disciplines, including natural products chemistry, marine microbiology, and marine optics. Rather than concentrate on the details of any one area, the approach taken in this thesis was to gather information relating to the larger issue, that is, the potential importance of fluorescent DOM produced by bacteria to the in situ concentrations in the ocean. The potential significance of the problem relates to our ability to map three-dimensional distributions of marine bacteria in real time using in situ fluorometry, to increase our understanding of the nature and distribution of dissolved fluorescence in the ocean, and to gain a better understanding of the role of bacteria in cycling of organic carbon in the ocean.

The thesis is organized around the answers to the questions listed above, namely: identification of individual fluorescent compounds produced by laboratory cultures of marine bacteria isolated from the low-oxygen waters of the Cariaco Trench (Chapter 2); a comparison of fluorescent pigments produced by marine isolates used in

Chapter 2 with pyoverdine, the pigment produced by the well-known fluorescent bacterium (*Pseudomonas fluorescens*) (Chapter 3); measurement of concentrations of dissolved fluorescent compounds in the water column of two marine anoxic basins using *in situ* fluorometry (Chapter 4) and chemical analysis (Chapter 5); and evaluation of the potential contribution of marine bacteria to dissolved fluorescence in the ocean (Chapter 6).

REFERENCES

Anderson, J.J. 1982. The nitrite-oxygen interface at the top of the oxygen minimum zone in the eastern tropical North Pacific. Deep Sea Res. 29: 1193-1201.

Antia, N.J. and A.F. Landymore. 1975. The non-biological oxidative degradation of dissolved xanthopterin and 2,4,6-trihydroxy-pterine by the pH or salt content of seawater. Mar. Chem. 3: 347-363.

Berger, P., R.W.P.M. Laane, A.G. Ilahude, M. Ewald, and P. Courtot. 1984. Comparative study of dissolved fluorescent matter in four West-European estuaries. Oceanologica Acta 7: 309-314.

Birkhoffer, L. and A. Birkhoffer. 1948. Riboflavin, a component of "bacterial fluorescein." Zeits. Naturforschung 3b: 136.

Blasco, D., J. Paul, and N. Ochoa-Lopez. 1979. Chlorophyll and phaeopigment from the Peru Current, 1977. In: Biological data from JOINT-II, R/V Melville, Leg IV, May 1977. P.C. Garfield and T.T. Packard, eds. CUEA Tech. Rept. 53.

Brandhorst, W. 1959. Nitrification and denitrification in the eastern tropical North Pacific. J. Cons. Int. Explor. Mer 25: 3-20.

Broenkow, W.W., A.J. Lewitus, M.A. Yarbrough, and R.T. Krenz. 1983. Particle fluorescence and bioluminescence distributions in the eastern tropical Pacific. Nature 302: 329-331.

Broenkow, W.W., A.J. Lewitus, and M.A. Yarbrough. 1985. Spectral observations of pigment fluorescence in intermediate depth waters of the North Pacific. J. Mar. Res. 43: 875-891.

Carlson, D.J., and L.M. Mayer. 1983. Relative influences of riverine and macroalgal phenolic material on UV absorbance in temperate coastal waters. Can. J. Fish. Aq. Sci. 40: 1258-63.

Chakrabarty, A.M. and S.C. Roy. 1964. Characterization of a pigment from a Pseudomonad. Biochem. J. 93: 144-148.

Chen, R.F., and J.L. Bada. 1989. Seawater and porewater fluorescence in the Santa Barbara Basin. (submitted to Geophys. Res. Lett.)

Cullen, J. J. and R. W. Eppley. 1981. Chlorophyll maximum layers of the Southern California Bight and possible mechanisms for their formation and maintenance. Oceanol. Acta 4: 23-32.

Dunlap, W.C., and M. Susic. 1985. Determination of pteridines and flavins in seawater by reverse-phase, high-performance liquid chromatography with fluorometric detection. Mar. Chem. 17: 185-198.

Duursma, E. K. 1965. The dissolved organic constituents of sea water, pp. 433-475. In: Chemical Oceanography. J. P. Riley and G. Skirrow, eds. Academic Press. London.

Duursma, E. K. 1974. The fluorescence of dissolved organic matter in the sea, pp. 237-256. In: Optical Aspects of Oceanography. N. G. Jerlov and E. Steemann Nielsen, eds. Academic Press, N. Y.

Gaill, F. and A. Momzikoff. 1975. The presence of riboflavin and two pterins in ascidians (Tunicata) and their excretion into seawater. Mar. Biol. 29: 315-319.

Garfield, P.C. and T.T. Packard. 1979. Biological data from JOINT II R/V Melville, Leg IV. May 1977. CUEA Tech. Rept. 52. 186 p.

Garfield, P.C., T.T. Packard, and L.A. Codispoti. 1979. Particulate protein in the Peru upwelling system. Deep Sea Res. 26: 623-639.

Garfield, P.C., T.T. Packard, G.E. Friederich, and L.A. Codispoti. 1983. A subsurface particle maximum layer and enhanced microbial activity in the secondary nitrite maximum of the northeastern tropical Pacific Ocean. J. Mar. Res. 41: 747-768.

Giral, F. 1936. Sobre los liocromos characteristicos del grupo de bacterias fluorescentes. Anales de la sociedad espanola de fisica y quimica 34: 667-693.

Gjessing, E.T., and T. Gjerdahl. 1970. Influence of ultra-violet radiation on aquatic humus. Vatten 2: 144-145.

Greppin, H. and S. Gouda. 1965. Action de la lumiere sur le pigment de Pseudomonas fluorescens Migula. Archives des sciences, Geneve 18: 721-25.

Harvey, G.R. 1984. Comment on the structure of marine fulvic and humic acids. Mar. Chem. 15: 89-90.

Harvey, G. R., D. A. Boran, L. A. Chesal, and J. M. Tokar. 1983. The structure of marine fulvic and humic acids. Mar. Chem. 12: 119-132.

Hayase, K., M. Yamamoto and H. Tsubota. 1987. Behavior of natural fluorescence in Sagami Bay and Tokyo Bay, Japan - Vertical and lateral distributions. Mar. Chem. 20: 265-276.

Hayase, K., H. Tsubota, and I. Sunada. 1988. Vertical distribution of fluorescent organic matter in the North Pacific. Mar. Chem. 25: 373-381.

Hedges, J. I. 1978. The formation and clay mineral reactions of melanoidins. Geochim. Cosmochim. Acta 42: 69-76.

Holligan, P. M., W. M. Balch and C. M. Yentsch. 1984. The significance of subsurface chlorophyll, nitrite and ammonia maxima in

relation to nitrogen for phytoplankton growth in stratified waters in the Gulf of Maine. J. Mar. Res. 42: 1051-1073.

Ivanoff, M.A. 1962. Au subjet de la fluorescence des eaux de mer. Comp. Rend. Acad. Sci. (Paris) 254: 4190-4192.

Jessen, O. 1965. Pseudomonas aeruginosa and other green fluorescing pseudomonads. A taxonomic study. Copenhagen: Munksgaard.

Kalle, K. 1949. Fluoreszenz und Gelbstoff im Bottnischen und Finnischen Meerbusen. Dt. hydrogr. Z. 2: 117-124.

Kalle, K. 1963. Uber das Verhalten und die Herkunft der in den Gewassern und in der Atmosphare vorhandenen himmelblauen Fluoreszenz. Dt. hydorgr. Z. 16: 154-166.

Kalle, K. 1966. The problem of the gelbstoff in the sea. Oceanogr. Mar. Biol. Ann. Rev. 4: 91-104.

Karabashev, G.S. 1977. Characterization of the distribution of fluorescence and light scattering in the ocean during strong vertical mixing and upwelling. Oceanology 17: 200-204.

Kramer, C.J.M. 1979. Degradation by sunlight of dissolved fluorescing substances in the upper layers of the eastern Atlantic Ocean. Neth. J. Sea Res. 13: 325-329.

Kullenberg, G. 1981. A comparison of distributions of suspended matter in the Peru and northwast African upwelling areas, pp. 282-209. In: Coastal Upwelling. F.A. Richards, ed. Am. Geophys. Union, Washington, D.C.

Kullenberg, G. 1984. Observations of light scattering functions in two oceanic areas. Deep Sea Res. 31: 295-316.

Laane, R.W.P.M. 1981. Composition and distribution of dissolved fluorescent substances in the Ems-Dollart Estuary. Neth. J. Sea Res. 15: 88-99.

Laane, R.W.P.M. 1984. Comment on the structure of marine fulvic and humic acids. Mar. Chem. 15: 85-87.

Laane, R.W.P.M., and L. Koole. 1982. The relation between fluorescence and dissolved organic carbon in the Ems-Dollart Estuary and the western Wadden Sea. Neth. J. Sea Res. 15: 217-227.

Landymore, A.F. and N.J. Antia. 1978. White-light promoted degradation of leucopterin and related pteridines dissolved in seawater, with evidence for involvement of complexation from major divalent cations of seawater. Mar. Chem. 6: 309-325.

Lenhoff, H. 1963. An inverse relationship of the effects of oxygen and iron on the production of fluorescein and cytochrome c by Pseudomonas fluorescens. Nature 199: 601-2.

Lewitus, A.J., and W.W. Broenkow. 1985. Intermediate depth pigment maxima in oxygen minimum zones. Deep Sea Res. 32: 1101-1115.

Lorenzen, C.J. 1966. A method for the continuous measurement of in vivo chlorophyll concentration. Deep-Sea Res. 13: 223-227.

Momzikoff, A. 1969a. Recherches sur les composes fluorescents de l'eau de mer. Identification de l'isoxanthopterine, de la riboflavine et du lumichrome. Cahiers Biol. Mar. 10: 221-30.

Momzikoff, A. 1969b. Etude de quelques substances fluorescentes presentes dans deux echantillons de plancton marin. Cahiers Biol. Mar. 10: 429-37.

Momzikoff, A. 1973. Mise en evidence d'une excretion de pterines par une population naturelle de copepodes planctoniques marins. Cahiers Biol. Mar. 14: 323-328.

Momzikoff, A., and J.-M. Legrand. 1973. Etude de quelques substances fluorescentes presentes dans un lot de plancton marin naturel compose de copepodes. Identification de l'erythropterine, de la drosopterine, de l'isodrosopterine et de la neodrosopterine. Cahiers Biol. Mar. 14: 249-259.

Mopper, K., and R.G. Zika. 1987. Natural photosensitizers in sea water: riboflavin and its breakdown products, pp. 174 - 190 In: Photochemistry of Environmental Aquatic Systems. R.G. Zika and W.J. Cooper, eds. American Chemical Society. Washington, D.C.

Pak, H., L.A. Codispoti, and R.V. Zaneveld. 1980. On the intermediate particle maxima associated with oxygen-poor water off western South America. Deep Sea Res. 27: 783-97.

Pingree, R.D., P.M. Holligan and G.T. Mardell. 1978. The effects of vertical stability on phytoplankton distribution in the summer on the northwest European shelf. Deep Sea Res. 25: 1011-1028.

Sieburth, J.M. 1969. Studies on algal substances in the sea. III. The production of extracellular organic matter by littoral marine algae. J. exp. mar. Biol. Ecol. 3: 290-309.

Sieburth, J.M., and A. Jensen. 1969. Studies on algal substances in the sea. II. The formation of gelbstoff (humic material) by exudates of Phaeophyta. J. exp. mar. Biol. Ecol. 3: 275-289.

Spinrad, R.W., H. Glover, B.B. Ward, L.A. Codispoti, and G. Kullenberg. 1989. Suspended particle and bacterial maxima in Peruvian coastal waters during a cold water anomaly. Deep-Sea Res. 36: 715-734.

Stuermer, D.H., and G.R. Harvey. 1974. Humic substances from seawater. Nature (London) 250: 480-481.

Traganza, E.D. 1969. Fluorescence excitation and emission spectra of dissolved organic matter in sea water. Bull. Mar. Sci. 19: 897-904.

Vastano, S.E., P.J. Milne, W. Stahovec, and K. Mopper. 1987. Determination of picomolar levels of flavins in natural waters by solid phase, ion pair extraction and liquid chromatography. Anal. Chim. Acta. 201: 127-133.

Vastano, S.E. 1988. Processes affecting the distribution of flavins in the ocean. MS Thesis. Univ. Miami. 67p.

Wakeham, S.G., R.B. Gagosian, J.W. Farrington, and E.A. Canuel. 1984. Sterenes in suspended particulate matter in the eastern tropical North Pacific. Nature 308: 840-43.

Wooster, W.S., T.J. Chow, and J. Barrett. 1965. Nitrate distribution in Peru Current waters. J. Mar. Res. 23: 210-21.

Yentsch, C.S., and D.W. Menzel. 1963. A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. Deep-Sea Res. 10: 221-231.

Yentsch, C.S., and C.A. Reichert. 1961. The interrelationship between water soluble yellow substances and chloroplastic pigments in marine algae. Botan. Marina

ZoBell, C.E. and C.B. Feltham. 1934. Preliminary studies on the distribution and characteristics of marine bacteria. Bull. Scripps Inst. Oceanogr., Tech. Rept. 3; 279-296.

ZoBell, C.E. and H.C. Upham. 1944. A list of marine bacteria including descriptions of sixty new species. Bull. Scripps Inst. Oceanogr. 5: 239-292.

CHAPTER 2

STRUCTURAL EVIDENCE FOR IDENTIFICATION OF INDIVIDUAL COMPONENTS OF BACTERIAL FLUORESCENT PIGMENTS

INTRODUCTION

Fluorescent pigments produced by non-marine bacteria have been previously categorized based on color, color of fluorescence and whether or not they freely diffuse out of cells into surrounding growth media. The actual chemical structure of these pigments is still unknown, however flavins and pterins are among the compound classes which have been proposed by some investigators for the pigment from Pseudomonas fluorescens, the classic species of fluorescent pseudomonad (Giral 1936; Birkhoffer and Birkhoffer 1948; and Chakrabarty and Roy 1964).

Flavins and pterins are constituents of coenzymes which are required by all living organisms. Folic acid, which contains the pterin fluorophore, serves as a one-carbon carrier in the synthesis of thymidylate. It is also the essential one-electron carrying cofactor in the oxidation of unactivated aromatic ring compounds such as phenylalanine, tyrosine and histidine. These oxidations are essential steps on the pathway to formation of neurotransmitter compounds such as norepinephrine and dopamine. In addition to these functions, pterins function as accessory vision pigments in insect eyes (Zeigler and Harmsen 1969), as a one-carbon carrier in methanogenesis by a thermophilic bacterium (Keltjens et al. 1983a, b), as a component of carbon monoxide oxidase in carboxydotrophic bacteria (Meyer and Rajagopalan, 1984), and as a chemosensory signal for slime molds (Van Haastert et al. 1982). Glycopteridines have been found to be produced by cyanobacteria under cold or dark shock, but their function has not been established (Forrest et al. 1957, 1958, 1959; MacLean et al. 1966).

Flavin coenzymes are also involved in electron transfer reactions, but are set apart from pterin coenzymes by their unique ability to undergo either one or two electron transfers. This accounts for their importance in respiratory electron transport chains, where they function as one electron/two electron switches. Massey and Hemmerich (1980) have classified flavin coenzyme reactions into 5 types (Walsh 1982): transhydrogenases, dehydrogenases/oxidases, dehydrogenases/oxygenases, dehydrogenases/electron transferases, and pure electron transferases.

Several species of marine bacteria have been reported to produce a water-soluble fluorescent pigment similar to that of P. fluorescens (ZoBell and Feltham 1934; ZoBell and Upham 1944). Many of these species were either isolated from low oxygen waters or shown to be capable of reducing nitrate to nitrite. Deep fluorescence maxima have been found in the oxygen minimum zone where denitrification occurs (Anderson 1982; Broenkow et al. 1983; Lewitus and Breonkow 1985; Spinrad et al. 1989). Production of pigments and flavins is influenced by oxygen tension (Lenhoff 1963; Peel 1958) and by availability of iron (Totter and Moseley 1958; Lenhoff 1963; Demain 1972; Meyer and Hornsperger 1978; Kloepper et al. 1980. Both oxygen concentration and iron solubility undergo drastic changes near the depth where the deep fluorescence maxima have been previously observed. Therefore, if marine bacteria produce fluorescent pigments they may contribute to the total fluorescence signal observed in the eastern tropical Pacific Ocean.

In this chapter, we take the first step towards addressing the overall hypothesis by examining the nature of the water-soluble fluorescent pigments produced by several clones of marine bacteria isolated from the low-oxygen waters of the Cariaco Trench are described. These compounds are present outside the cells, however whether this is due to excretion or simply to diffusion across the cell membrane is not known. Individual "pigment" components are characterized by a variety of analytical methods, including high performance liquid chromatography (HPLC), fluorescence spectroscopy, and mass spectrometry.

METHODS

Culture conditions. Pure and mixed cultures of marine bacteria were obtained from seawater samples collected in the oxygen-deficient waters of the Cariaco Trench between 200 and 260 m (Table 2.01). Mixed cultures have names beginning with an "M". Cultures without an "M" are pure clones. All isolates were obtained from most probable number (MPN) enrichment tubes which gave positive results for the production of nitrogen gas (see Methods Chapter 3). Aliquots for mixed cultures were obtained by direct inoculation of defined media, whereas aliquots from which pure clones were obtained were streaked onto agar plates (1/2 strength Difco Maine Broth with 15 gm/l Difco Bactoagar) and grown in a nitrogen atmosphere. Individual colonies were picked and restreaked until all colonies on the plate were visually identical. After three additional transfers, isolates were assumed to be pure clones. For short-term storage, cultures on agar slants were covered with sterile mineral oil and stored in the dark in the refrigerator. All cultures used for experiments were also preserved in liquid nitrogen in media composed of 1/2 strength Difco Marine Broth with 10% dimethyl sulfoxide (DMSO).

A defined liquid growth medium (Asn-2S) was used for all experiments on marine isolates. This media contained 2.5 g asparagine, 8.3 g potassium monophosphate (dibasic), 2.6 g potassium monophosphate (monobasic), 0.1 g magnesium sulfate, 20 g sodium chloride, and 1 liter distilled water (pH = 7.2). The growth medium was not fluorescent. All cultures were grown aerobically in the dark and sampled in stationary growth phase.

Table 2.01. Data pertinent to isolation of the marine bacteria used in this study, including date and depth from which samples were taken. Dil. and tube refer to the dilution level and replicate tube number of MPN tube from which isolation was made. Gas, nitrite, and growth are rated from 1 (lowest) to 5 (highest) + for presence of gas in Dalham tubes, nitrite concentration in tube after growth, and amount of growth as determined by turbidity. All were measured by visual observation. Oxygen is the oxygen content of the original water sample from which isolates were made, in µM.

Name	Date	Depth	Dil.	Tube	Gas	Nitrite	Oxygen	Growth
Pure clones								
C21g	3/14/87	200	1	3	++	+++	116	+++
C3A	"	210	1	"	++	+++++	21.6	++
D91g	3/16/87	220	2	1	+++	+++++	N/A	+++
D9sm	"	"	"	"	"	"	"	"
D10	"	220	3	3	+++	+++++	"	+++
Mixed cultures								
MB34	3/8/87	250	1	2	++	+++++	0.1	+++
MC4	3/14/87	220	1	2	+++	+++++	9.3	+++
MD1	3/16/87	240	1	1	++++	+	N/A	++++
MD2	"	"	1	3	++++	+++	"	++++
MD10	"	220	3	3	+++	+++++	"	+++

Materials and supplies. All chemicals used were obtained from Sigma, except for the biopterin standard which came from CalBiochem, and all were reagent grade. Potassium permanganate distilled water was used for all reagents, and all steps in pigment isolation and analysis procedures were carried out under red lights.

High performance liquid chromatography (HPLC). Three different types of HPLC were used during the course of this project. Reverse phase (RP) HPLC was performed using a Versapack C18 column (4.6 x 250 mm; 10 µm particle size) with a mobile phase of ammonium acetate (50 mM) : methanol, either with isocratic elution at 85:15 or with gradient elution varying from 85:15 to 50:50 aqueous:solvent and a flow rate of 2.0 ml/min. Strong cation exchange (SCX) HPLC (Stea et al. 1980) was used for analysis of both Florisil and C18 pigment fractions. A Partisil SCX column (4.6 x 250 mm; 10 µm particle size) was eluted isocratically with ammonium phosphate (pH = 2.8), 7 % methanol, and 5 % acetonitrile at 1.5 ml/min. Ion-pairing (IP) HPLC was performed using Alltech Adsorbosphere-HS (ODS) columns, either the 25 cm length with 7µm particle size or 15 cm length with 5 µm particle size. The mobile phase consisted of 70:30 Waters PIC A reagent in water:methanol and isocratic elution at 1.0 - 1.5 ml/min. Detection was either by fluorescence with excitation at 250 nm and emission at > 418 nm using a Kratos Model 980 detector using a deuterium lamp or by absorbance at 254 nm using a Waters Model 440 absorbance detector. Retention times for samples and standards run on the same day using SCX-HPLC varied due to deterioration of the column, so preliminary compound identifications were based on retention times relative to the retention time of bpt standard.

Fluorometry. Fluorescence spectra were run using either a Perkin-Elmer MPF-3 (no baseline correction), or using a computer-controlled SLM-Aminco SPF-500C (baseline correction).

Mass spectrometry. Standards and unknown compounds (100-300 ng) were purified by HPLC and derivatized to their peracetates with 100 µl pyridine and 100 µl acetic anhydride (overnight at 30°C). The reaction was stopped by evaporation of reagents under vacuum. Reaction products were dissolved in a few drops of methylene chloride and purified by column chromatography on silica-gel (Peltzer et al. 1984) eluted with three column volumes of: hexane (f_1), 20% ethyl acetate in hexane (f_2), ethyl acetate (f_3), and 20% ethyl acetate in methanol (f_4). The first two fractions were discarded. The second two fractions contained flavin and pterin acetates and were collected separately.

Mass spectrometric analyses were made on a Finnigan GC-MS system (4510) with an Incos expanded data system in both electron impact (EI) and chemical ionization (CI, methane) mode. Samples were injected via thermal desorption from a glass cup. The probe was heated ballistically to a maximum temperature of 350°C and maintained there briefly to allow the sample to reach the probe temperature.

Hydrolysis of FAD to FMN. Flavin adenosine dinucleotide (FAD) was converted to flavin mononucleotide (FMN) according to the procedure of Chappelle and Picciolo (1971). One ml of solution containing 102 nM FAD/ml distilled water was aliquoted into a brown glass vial and evaporated to dryness under vacuum at <35°C. One ml of 1.0 N perchloric acid was added and the vial was shaken vigorously to dissolve the FAD. The vial was then placed in a boiling water bath

for 30 min. The reaction was stopped by adding 1.3 ml 1.0 N sodium hydroxide to neutralize the mixture. The amount of FMN produced was analyzed by IP-HPLC and was calculated to be 90 nM, for a yield of 89%.

The procedure was repeated using FAD-unknown from D10 (MC18-2) and FAD standard collected by IP-HPLC in 70:30 PIC A (Waters): methanol and concentrated by rotary evaporation to a small volume which was transferred to a 1.5 ml Reactivial (Wheaton), evaporated under vacuum again until dry and treated as described above. Ion-pairing buffer was concentrated and served as a blank for the procedure.

Photochemical degradation of riboflavin to lumiflavin and lumichrome. Riboflavin standard and unknown (MC18-3) from D10 were purified and quantified by HPLC prior to photolysis. Samples were placed in clear glass screw-capped vials and exposed to irradiation from two 30 watt Sylvania Cool White lights at a distance of 4 ft for a period of 66 hours. Photodegradation products were analyzed for riboflavin, lumichrome, and lumiflavin using IP-HPLC.

Sample collection and preparation. Dissolved compounds were collected from seawater and from cultures by the method of Dunlap and Susic (1985). Filtered (0.2 μm) samples were acidified with 0.1 M acetic acid to pH = 3.2 and pumped through Sep-Pak (Waters Assoc.) cartridges which had been precleaned by rinsing with 10 ml each methanol then distilled water. The cartridges were connected in series (C18 nearest the pump outlet followed by Florisil) and a Masterflex peristaltic pump was used at flow rates of about 20 ml/min. Only 50 ml of culture filtrate was required. The cartridges were

rinsed with acetic acid (0.1 M; pH = 3.2), flushed with nitrogen and stored frozen in the dark.

Elution and analysis of samples was not according to any previously published method. Cartridges containing culture samples were thawed and separately eluted with 15 ml each of: methanol, 50 mM ammonium acetate:methanol (85:15), and distilled water in sequence. These fractions were combined for each cartridge and concentrated by rotary evaporation at 30°C. After concentration by rotary evaporation, most of the samples contained a precipitate which was removed by filtration through Milipore GV filter cartridges which had been pre-rinsed with 2.5 ml methanol and 2.5 ml water followed by an air purge to remove any remaining solvent. HPLC analysis of samples before and after filtration showed no significant loss of fluorescent components. All operations were carried out under red illumination. Samples were stored in amber vials or foil-wrapped containers. Distilled water used for reagents was redistilled over potassium permanganate.

RESULTS

Initial experiments to characterize the pigments produce by cultures of marine bacteria were performed using both pure and mixed cultures. Pigment components were classified as "pterin-like" or "flavin-like" based on whether they occurred in the Florisil or C18 fraction, respectively. The pterin-like fraction fluoresced blue and was well-resolved into individual components using SCX-HPLC. The flavin-like fraction fluoresced yellow and was best-resolved using IP-HPLC. An aliquot of sterile Asn-2S media showed no fluorescent compounds in either the C18 or the Florisil fractions. The designation for unknown compounds from marine clones begin with M, followed by "Flor" or "C18" to designate the Sep-Pak fraction in which they were found, and then by a number indicating elution order on one of the HPLC systems used.

A. FLAVINS AND FLAVIN-LIKE COMPOUNDS

HPLC retention times. The absolute and relative retention times for flavin standards varied significantly using three different HPLC systems tested during the methods development phase of the project. This provided us with valuable information regarding the identities of the unknown compounds. The first system used to separate components of the C18 fraction of pigments from marine clones was SCX-HPLC. The composition of the C18 fraction showed less variability than did the Florisil fraction, however the clones did show variation in relative concentrations of the components. Since retention times for flavins did not change markedly over the time during which these samples were run, relative retention times were not needed to make preliminary

identifications. Typical chromatograms for each HPLC system are shown in Figs. 2.01 - 2.03. Additional chromatograms for other clones examined may be found in Appendix A.

Four major peaks were found in all the cultures tested (Fig. 2.01). Based on retention times these can be identified as MC18-1 = flavin mononucleotide (FMN), MC18-2 = flavin adenine dinucleotide (FAD), MC18-3 = riboflavin (rbfl), and MC18-4 = lumichrome (lcr). Lumiflavin (lfl) was not present in these samples.

The second type of HPLC separation used was ion-pairing (IP-HPLC) under two different mobile phase compositions, 60% and 70% aqueous phase. IP-HPLC resulted in a change in the order of elution of the peaks. With a 60% aqueous phase, riboflavin eluted first followed by FAD and FMN which eluted in a combined peak (Fig. 2.02). The lumichrome peak was the last to elute. Culture filtrates again showed peaks corresponding to rbfl, FMN, FAD, and lcr. Small amounts of lumiflavin (MC18-5) may have been present in the sample. An additional unknown component (MC18-6) corresponded to a new peak in the standard which eluted between rbfl and FMN.

When the mobile phase composition of IP-HPLC was changed to 70% aqueous, riboflavin eluted first (Fig. 2.03). FMN and FAD eluted fourth and fifth, and were resolved into two separate peaks (FMN was not present in the standard shown). MC18-6 was found again, and has been attributed to an impurity in the FAD standard as obtained from the chemical supply house. Several compounds have been reported to be present in so-called "pure" FAD, most of which are flavins of one type or another. We labelled this one "Unknown 1" (Unk1). It was found in

Figure 2.01. Comparison of flavin reference compounds (A) with bacterial flavins from the C18 fraction of mixed cultures MD1 (B) and D91gA (C) using SCX-HPLC. Chromatographic conditions as follows: 4.5 x 250 mm Partisil-10 SCX column with mobile phase = 1mM ammonium dihydrogen phosphate, pH 2.8 - 7% methanol - 5% acetonitrile at 1.5 ml/min. Fluorescence detection at >418 nm with excitation at 250 nm.

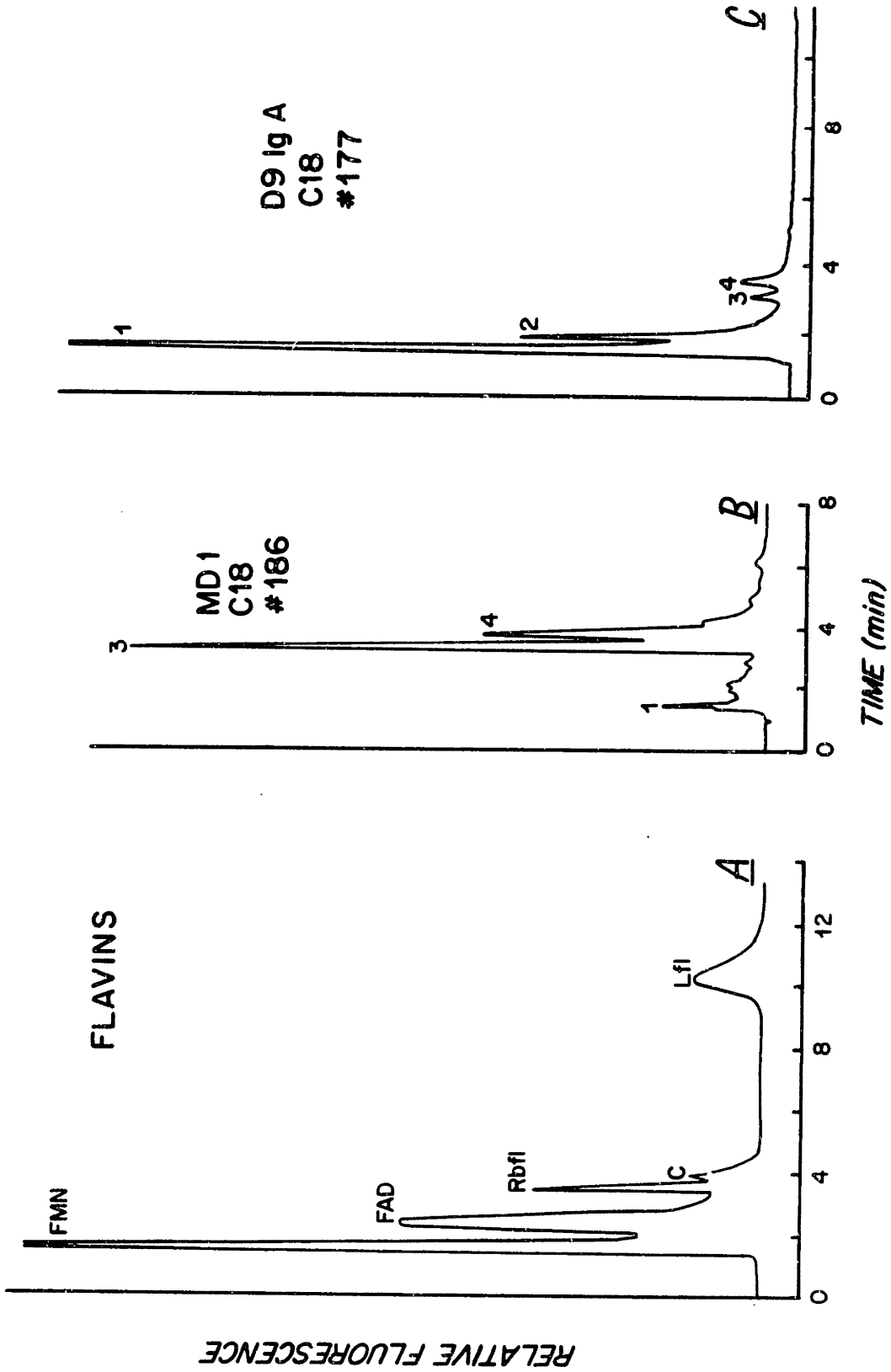

FLAVINS
FMN
FAD
RbfI
C
LfI
A
0
4
8
12
MD1
C18
#186
3
4
1
B
0
4
8
D9 Ig A
C18
#177
1
2
3
4
C
0
4
8
TIME (min)
RELATIVE FLUORESCENCE

Figure 2.02. Comparison of flavin reference compounds (upper) with bacterial flavins from the C18 fraction of clone D9 (lower) using ion-pairing chromatography with 60% aqueous phase. An Adsorbosphere-HS C18 column was used with mobile phase = 60:40 aqueous PIC A (Waters) : methanol at 2.0 ml/min. Fluorescence detection at >418 nm with excitation at 250 nm.

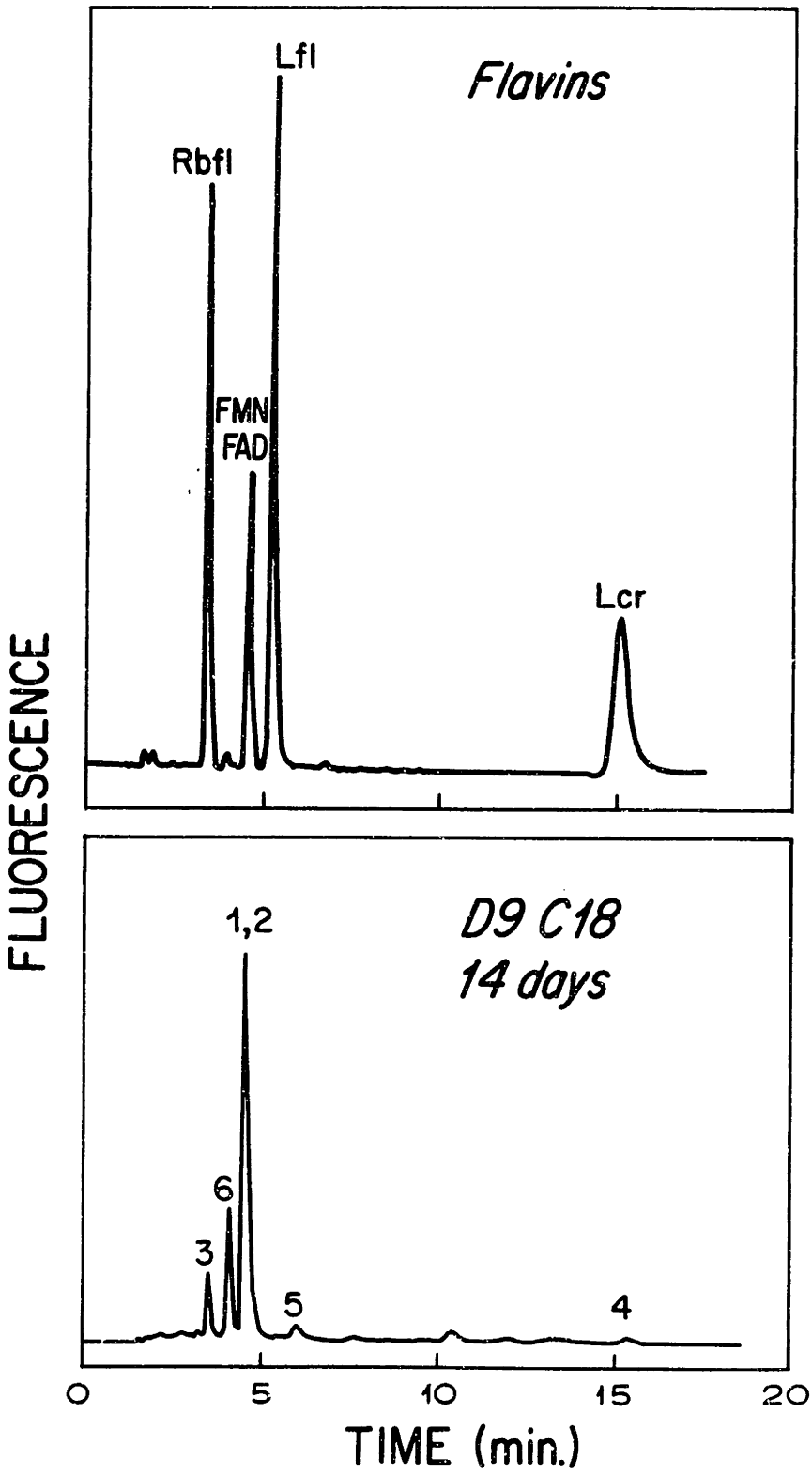
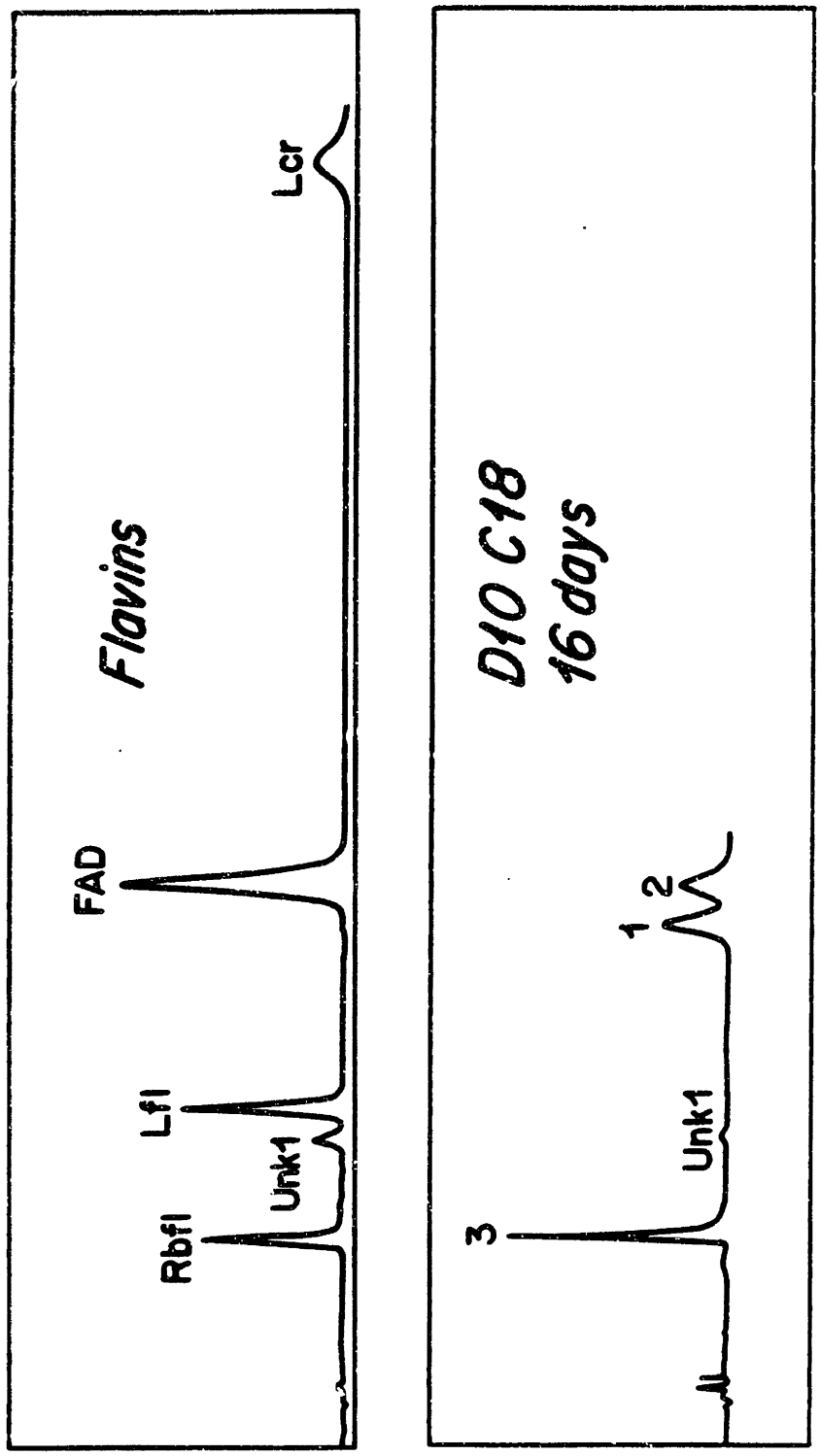

Flavins
Lfl
Rbfl
FMN
FAD
Lcr
D9 C18
14 days
1,2
6
3
5
4
FLUORESCENCE
0
5
10
15
20
TIME (min.)

Figure 2.03. Comparison of flavin reference compounds (upper) with bacterial flavins from the C18 fraction of clone D10 (lower) using ion-pairing chromatography with 70% aqueous phase. An Adsorbosphere-HS C18 column was used with mobile phase = 70:30 aqueous PIC A (Waters) : methanol at 2.0 ml/min. Fluorescence detection at >418 nm with excitation at 250 nm.


Flavins
FAD
Lcr
Lfl
Rbfl
Unk1
D10 C18
16 days
1
2
3
Unk1
TIME
RELATIVE FLUORESCENCE

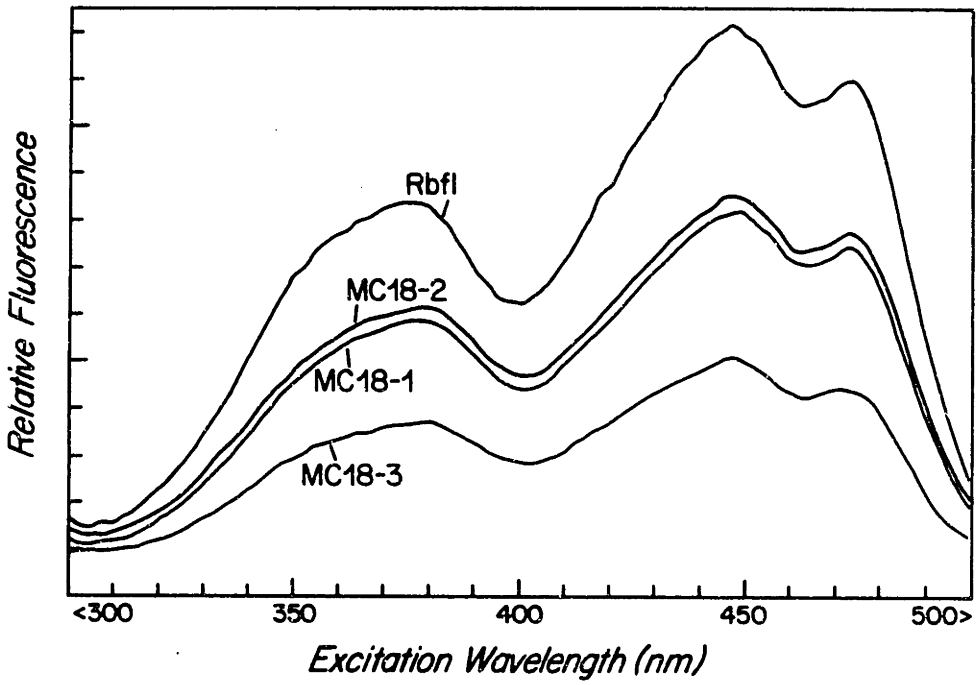
cultures and in seawater as well as in the standard, and its fluorescence properties will be discussed later.

Under the conditions of all three HPLC systems used in this study, components of the C18 fraction of bacterial pigment elute at the same time as the standards. The correspondence in retention times in all systems suggests that the compounds produced by these bacteria are indeed riboflavin, FAD, and FMN. Although lumichrome and lumiflavin were present in most of the early pigment preparations, they were not found in later samples, when greater precautions were taken to avoid exposure to light. It is therefore unlikely that any of the cultures synthesized lumichrome or lumiflavin, and their presence in these samples is probably the result of photodegradation of riboflavin.

To obtain further structural information, fluorescence spectra of standards and unknowns were compared. Both the excitation and emission spectra for compounds MC18-1, -2, and -3 are identical to those for rbf1 (Fig. 2.04). The fluorescence spectra of FAD and FMN are also identical to those of rbf1 because the fluorophore is the same. The fluorescence spectra for Unk 1 are not shown in Fig. 2.04, but they were identical to those for the other flavins. Fluorescence excitation spectra correspone to absorbance spectra, so this actually provides two pieces of evidence consistent with identification of UNK 1 as a flavin.

MC18-3 (Riboflavin). The EI spectra for acetylated riboflavin and MC18-3 are shown in Fig. 2.05. The riboflavin forms a tetraacetate with a molecular weight of 544. Loss of successive acetyls results in ions at 501, 459, and 417. Loss of acetic acid from the molecular

Figure 2.04. Top: Comparison of fluorescence excitation spectra of riboflavin and peaks MC18-1, MC18-2, and MC18-3 from the C18 fraction of clone D9. Emission wavelength was 530 nm. Bottom: Comparison of fluorescence emission spectra of riboflavin and peaks MC18-1, MC18-2, and MC18-3 from the C18 fraction of clone D9. Excitation wavelength was 360 nm. All are corrected spectra run on an SLM-Aminco SPF-500C spectrofluorometer.


Relative Fluorescence
Rbfl
MC18-2
MC18-1
MC18-3
<300
350
400
450
500>
Excitation Wavelength (nm)

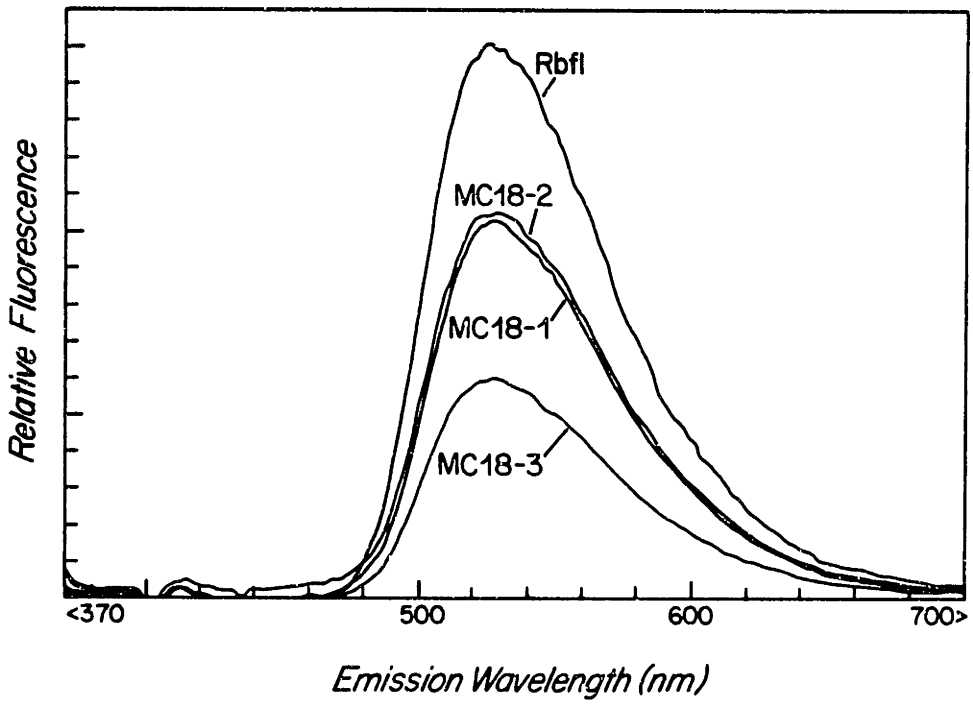
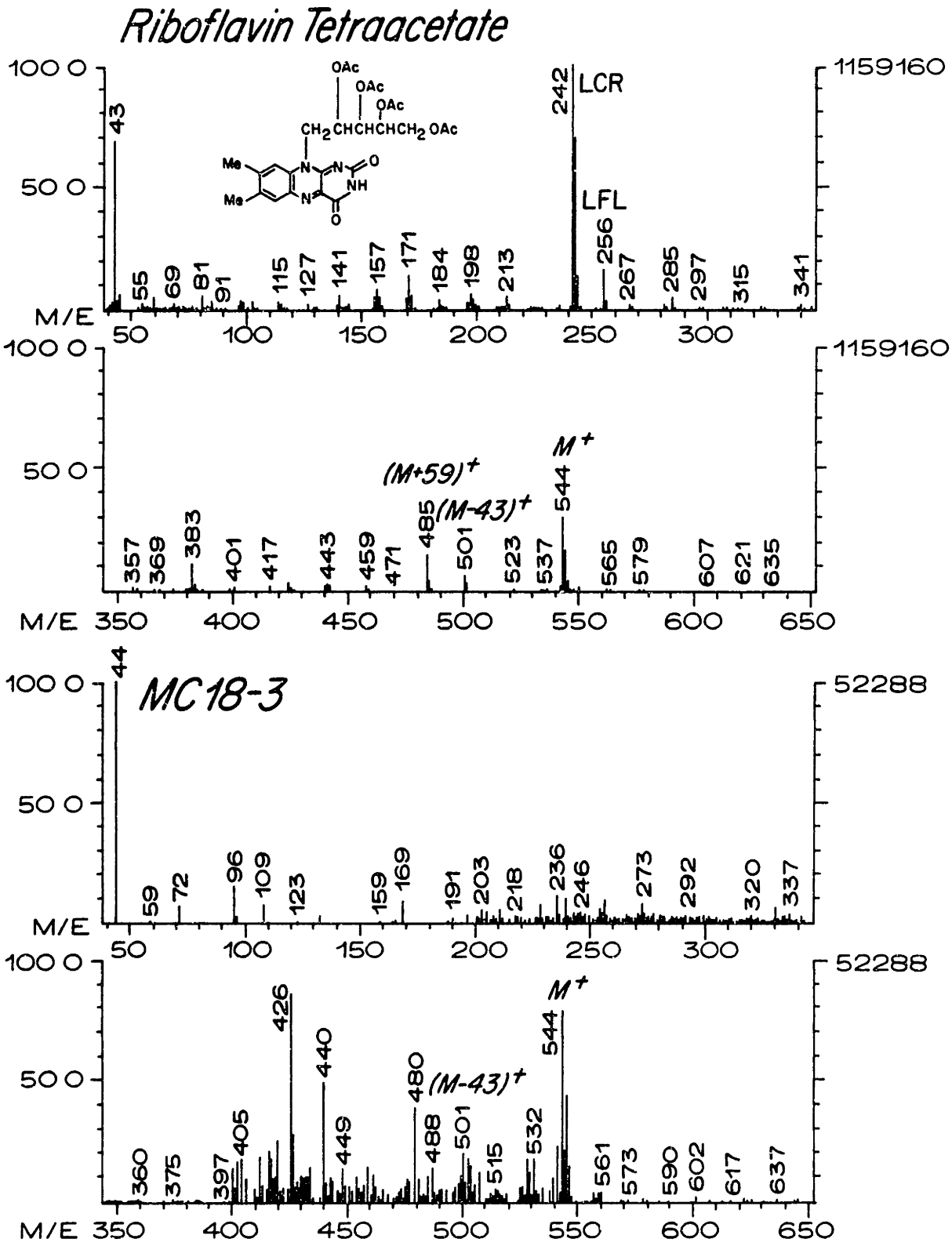

Relative Fluorescence
Rbfl
MC18-2
MC18-1
MC18-3
<370
500
600
700>
Emission Wavelength (nm)

Figure 2.05. EI spectra for acetate derivatives of riboflavin (top) and MC18-3 (bottom).


Riboflavin Tetraacetate
100 0
50 0
1159160
LCR
LFL
242
256
43
55
69
81
91
115
127
141
157
171
184
198
213
267
285
297
315
341
M/E
50
100
150
200
250
300
(M+59)+
M+
544
(M-43)+
485
357
369
383
401
417
443
459
471
501
523
537
565
579
607
621
635
350
400
450
500
550
600
650
MC18-3
52288
44
59
72
96
109
123
159
169
191
203
218
236
246
273
292
320
337
426
440
480
405
397
360
375
449
488
501
515
532
561
573
590
602
617
637

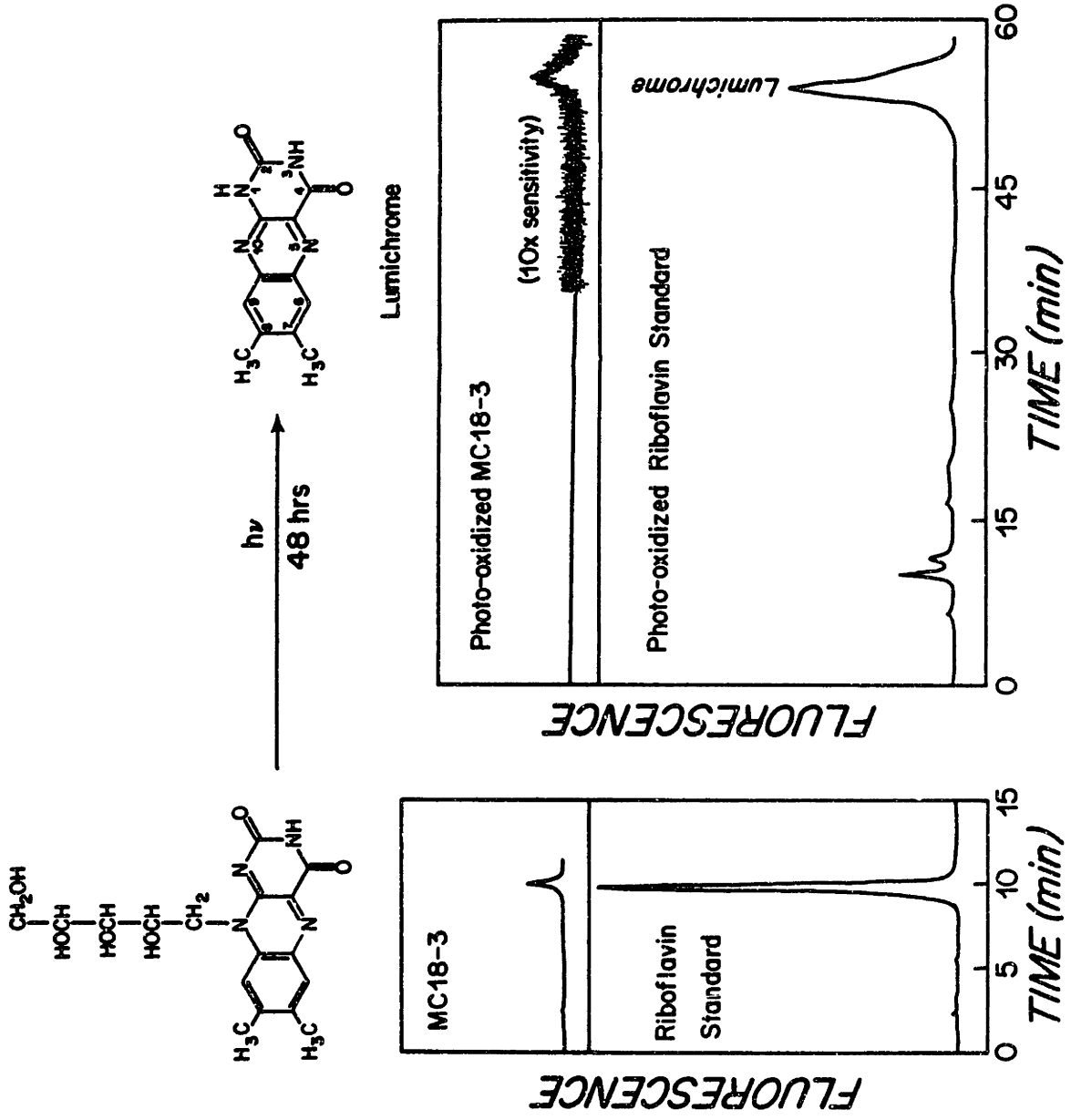
ion followed by loss of successive acetyls results in ions at 485, 443, and 401. The large peaks at 242 and 256 are lumichrome and lumiflavin, respectively, and result from cleavage of the ribityl chain. The spectrum for MC18-3 is consistent with a structure of riboflavin tetraacetate. Fragments representing the molecular ion at 544 and the ion resulting from loss of an acetyl at m/z = 501 were present. The major fragments from lumichrome at 242 and lumiflavin at 256 were absent in the corrected spectrum shown here. These fragments were present in the sample, but not at tlevels above background.

Analysis of riboflavin tetraecetate by gas chromatography - mass spectrometry (GC-MS) was unsuccessful, probably because the compound did not elute from the DB5 column used.

Further information regarding the structure of MC18-3 was obtained from comparison of photo-oxidation products of the unknown with those of riboflavin. Riboflavin is very unstable in light, and has a half-life of 11.8 s when exposed to surface sunlight radiation in seawater (Dunlap and Susic, 1986). The products of photodegradation are lcr (96%) and lfl (4%). The half-life is longer in distilled water, 3.19 min., and the photoproduct is 100% lumichrome. Half-lives for lumiflavin and lumichrome in seawater (12.2 and 226 h, respectively) are longer than in distilled water (6.64 and 32.0 h, respectively). Our reaction solution was the IP-HPLC mobile phase and light intensities were not measured. However, given the length of the experiment (48 hr), we would expect the product to be nearly 100% lumichrome.

Reaction scheme, and initial and final chromatograms are shown in Fig. 2.06. For the riboflavin standard, some starting material was

Figure 2.06. Outline of riboflavin photochemical degradation experiment. Top: Reaction scheme and conditions. Bottom left: IP chromatogram for MC18-3 (top) and riboflavin standard (bottom). Bottom right: IP chromatograms of photolysis products from MC18-3 (top) and standard (bottom).

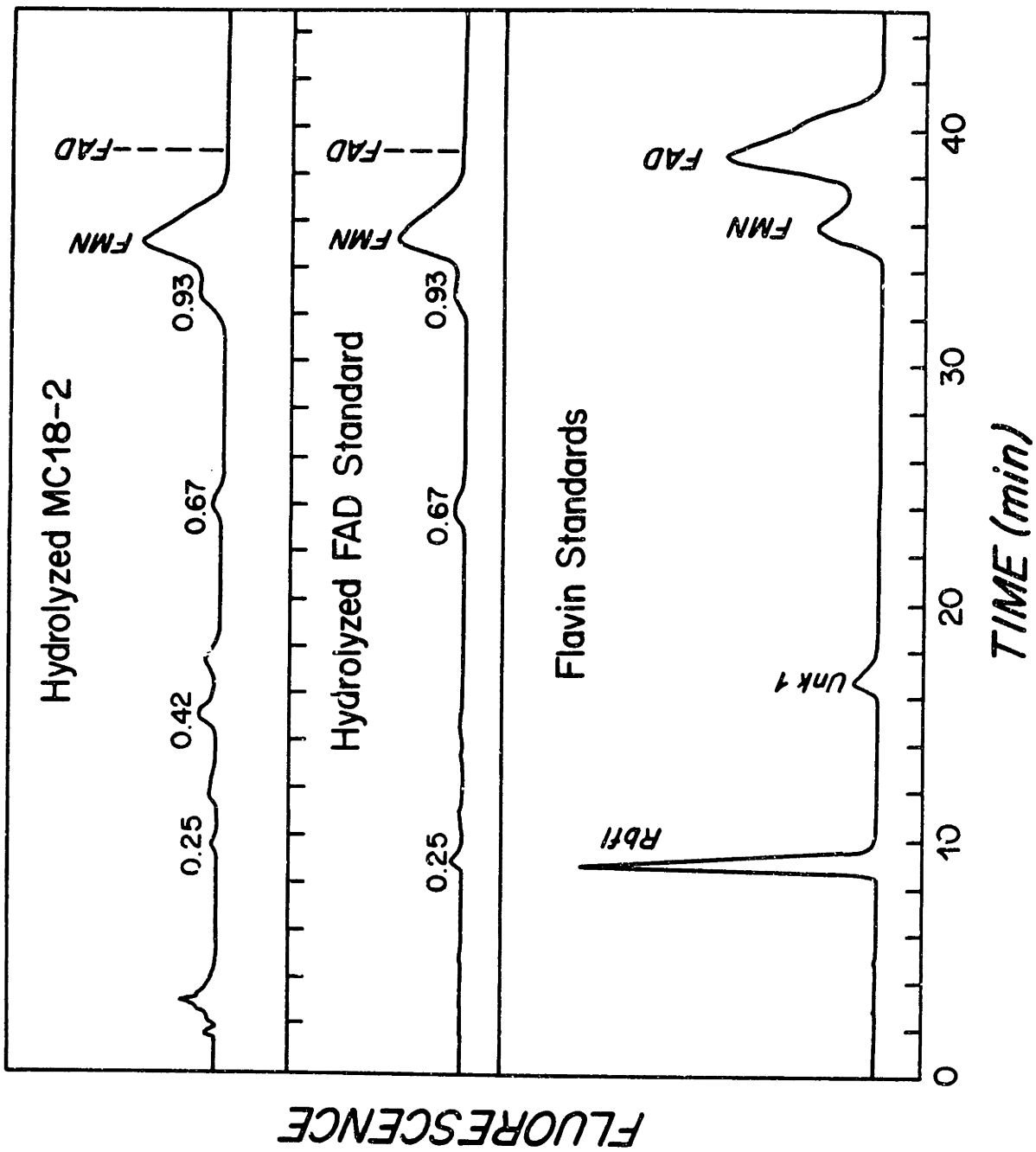

hν
48 hrs
Lumichrome
MC18-3
Riboflavin Standard
FLUORESCENCE
TIME (min)
0
5
10
15
Photo-oxidized MC18-3
(10x sensitivity)
Photo-oxidized Riboflavin Standard
Lumichrome
0
15
30
45
60
FLUORESCENCE
TIME (min)

left, but the major peak was lumichrome. The starting material of 497 pM rbfl yielded 283 pM lcr, as calibrated by rbfl standard. Using a response factor of 2.036 for lcr:rbfl as measured by our HPLC detector, this would give a result of 576 pM lcr for a calculated yield of 116%. Very small amounts of two minor components were also present. The unknown sample was much less concentrated, and the only reaction product produced in detectable quantities was lcr (Fig. 2.06). The starting material from MC18-3 of 98 pM rbfl produced 122 pM lcr for a yield of 125%. Yields for this experiment may be high due to errors associated with the lcr:rbfl ratio.

Results of this experiment are consistent with the identification of riboflavin as component MC18-3 of the bacterial pigment. Since the results of HPLC, fluorometry and mass spectrometry also support this identity, we can conclude that component MC18-3 is riboflavin.

MC18-2 (FAD). Further information regarding the structure of MC18-2 was not attempted by MS because the molecular weight of the acetylated derivative of FAD exceeds the useful mass range of 600 mμ for the presently available MS system. Instead, we used a simple hydrolysis scheme which selectively converts FAD to FMN. The hydrolysis product of FAD standard in distilled water and of MC18-2 showed the same pattern of fluorescent peaks (Fig. 2.07). The major component in both standard and unknown had the same retention time as FMN. No component was found to have the same retention time as FAD, and the reagent blank showed no fluorescent products. Co-injection of standard and sample did not result in any additional peaks (Fig. 2.07). Approximately 695 pM of MC18-2 were initially collected from a D10 filtrate sample. This yielded 284 pM of FMN after hydrolysis, or

Figure 2.07. Comparison of retention times for hydrolyzed MC18-2 and standard FAD. Top: IP chromatogram for the hydrolyzed unknown compound collected from a culture filtrate by HPLC. Middle: IP chromatogram for the hydrolyzed FAD standard. Bottom: IP chromatogram for unhydrolyzed standards. Notice that the starting material, FAD, is completely missing in top and middle panels. Numbers indicate retention times relative to FMN.

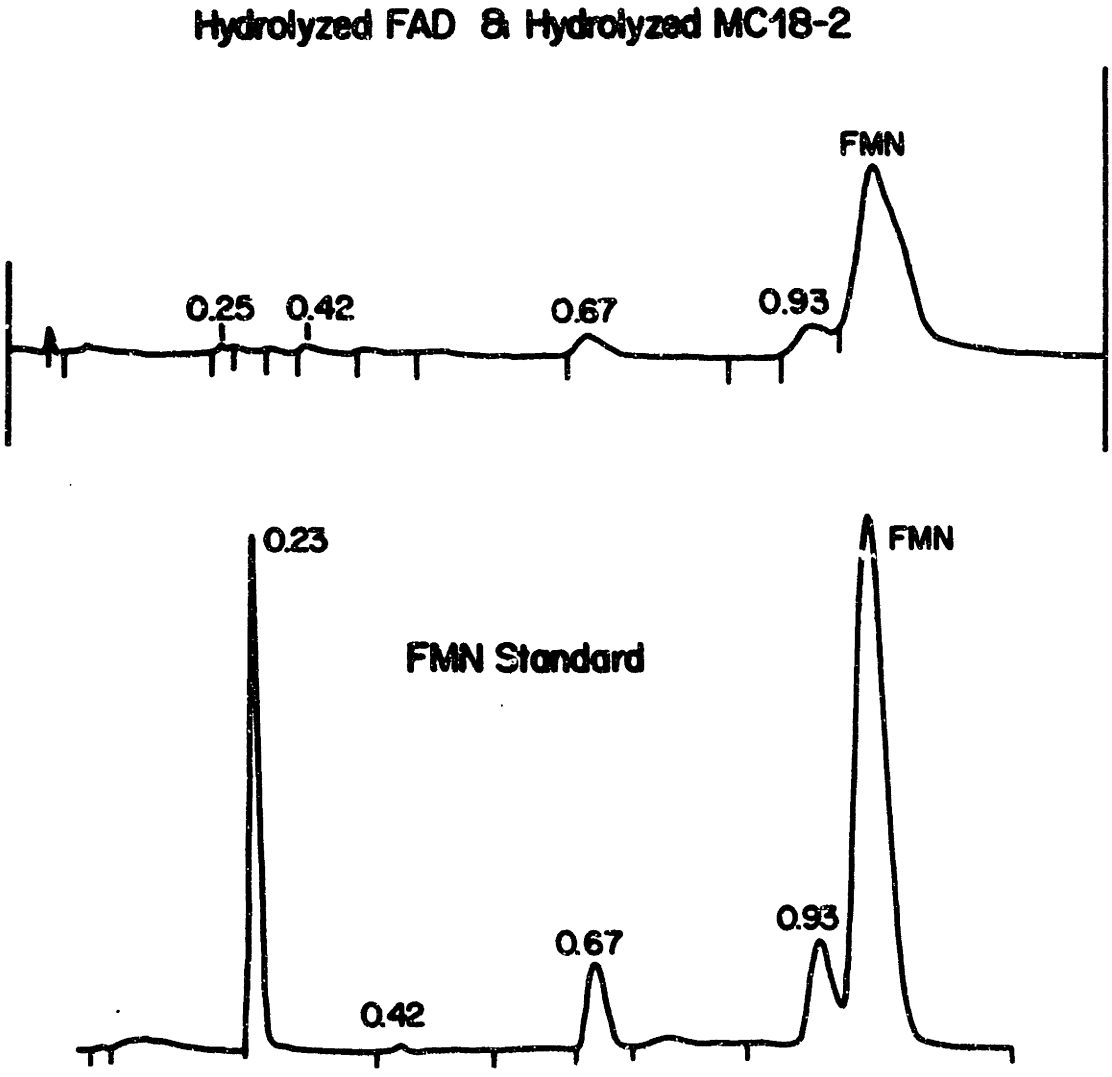

Hydrolyzed MC18-2
FAD
FMN
0.93
0.67
0.42
0.25
Hydrolyzed FAD Standard
FAD
FMN
0.93
0.67
0.25
Flavin Standards
FAD
FMN
Unk 1
Rbfl
0
10
20
30
40
TIME (min)
FLUORESCENCE

41%. This yield is significantly lower than that obtained originally for the FAD standard, and may represent losses resulting from concentration of the HPLC eluent.

The unidentified components in the chromatogram were not from the PIC A buffer used in the IP-HPLC mobile phase. Other potential products of the hydrolysis reaction include: AMP, ADP, and adenosine from the side-chain; and riboflavin, lumichrome, and lumiflavin from the fluorophore of the molecule. Adenosine and its phosphorylated derivatives are less fluorescent than the flavins, with very different excitation and emission maxima. The compound Unk 1 was present in the standard prior to hydrolysis and may have been the source of some of the unknown components found in the standard, however it was not initially present in the sample. Therefore, if these peaks result from Unk 1, we must postulate that Unk 1 itself is formed during the hydrolysis procedure from FAD.

Another possible source for the unidentified components is that they are isomers of FMN which formed because FAD hydrolysis proceeded beyond FMN. Isomerization of pure 5'FMN has been found to occur after a 15 min. exposure to 0.1 M HCl. It is possible that isomerization occurs under even milder conditions, since "pure" FMN (Sigma) shows the presence of 4 positional isomers of FMN and 3 isomers of riboflavin diphosphate, with an estimated purity for 5'FMN of 75% (Nielsen et al. 1986). Our FMN standard (Sigma) also contained four major fluorescent contaminants (Fig. 2.08). The components having relative retention times of 0.94, 0.66, 0.42, and 0.23 with respect to FMN would appear to be the same as four components of the hydrolysate (Fig. 2.08). It is very likely that the component which eluted just

Figure 2.08. Top: IP-HPLC chromatogram of coinjection of hydrolyzed FAD with hydrolyzed MC18-2. Bottom: IP-HPLC chromatogram for FMN standard from Sigma. Numbers are retention times relative to FMN.


Hydrolyzed FAD & Hydrolyzed MC18-2
FMN
0.25
0.42
0.67
0.93
0.23
FMN
FMN Standard
0.93
0.67
0.42

before 5'FMN is 4'FMN. Our relative retention time for this compound was 0.93 versus a relative retention time of 0.90 reported by Nielsen et al. (1986) for the 4' isomer under similar HPLC conditions.

The results of this experiment are consistent with the identification of FAD in the C18 fraction of bacterial pigment. Since results of HPLC and fluorometry are also in agreement with this identity, we conclude that compound MC18-2 is FAD.

B. PTERIN-LIKE COMPOUNDS

Several different patterns of pigment composition were observed using HPLC, two of which are shown in Fig. 2.09. (Chromatograms for all the other clones examined may be found in Appendix A.) Table 2.02 gives a summary of results of SCX-HPLC analysis of the Florisil fraction of all marine bacteria cultures tested. It can be seen that several of the peaks occurred in the majority of clones tested. Five of these can be tentatively identified based solely on relative retention times. They are: MFlor-3 (=isoxanthopterin), MFlor-5 (=pterin-6-carboxylic acid), MFlor-9 (=xanthopterin), MFlor-12 (=6-(hydroxymethyl)pterin), and MFlor-13 (=pterine). Unknown compounds MFlor-1 and MFlor-6 were found in most of the marine species. Eight unknown compounds were found in at least two of the samples tested. Most of the cultures tested differed in the composition of peaks eluting in the first six minutes. Three of these peaks had retention times similar to isoxanthopterin (iso), pterin-6-carboxylic acid (pt-6-COOH) and xanthopterin (xpt) in both MD10 and D91gA.

Table 2.02. Relative Retention Times for Florisil Fraction Unknowns (MFlor-1 - MFlor-13)

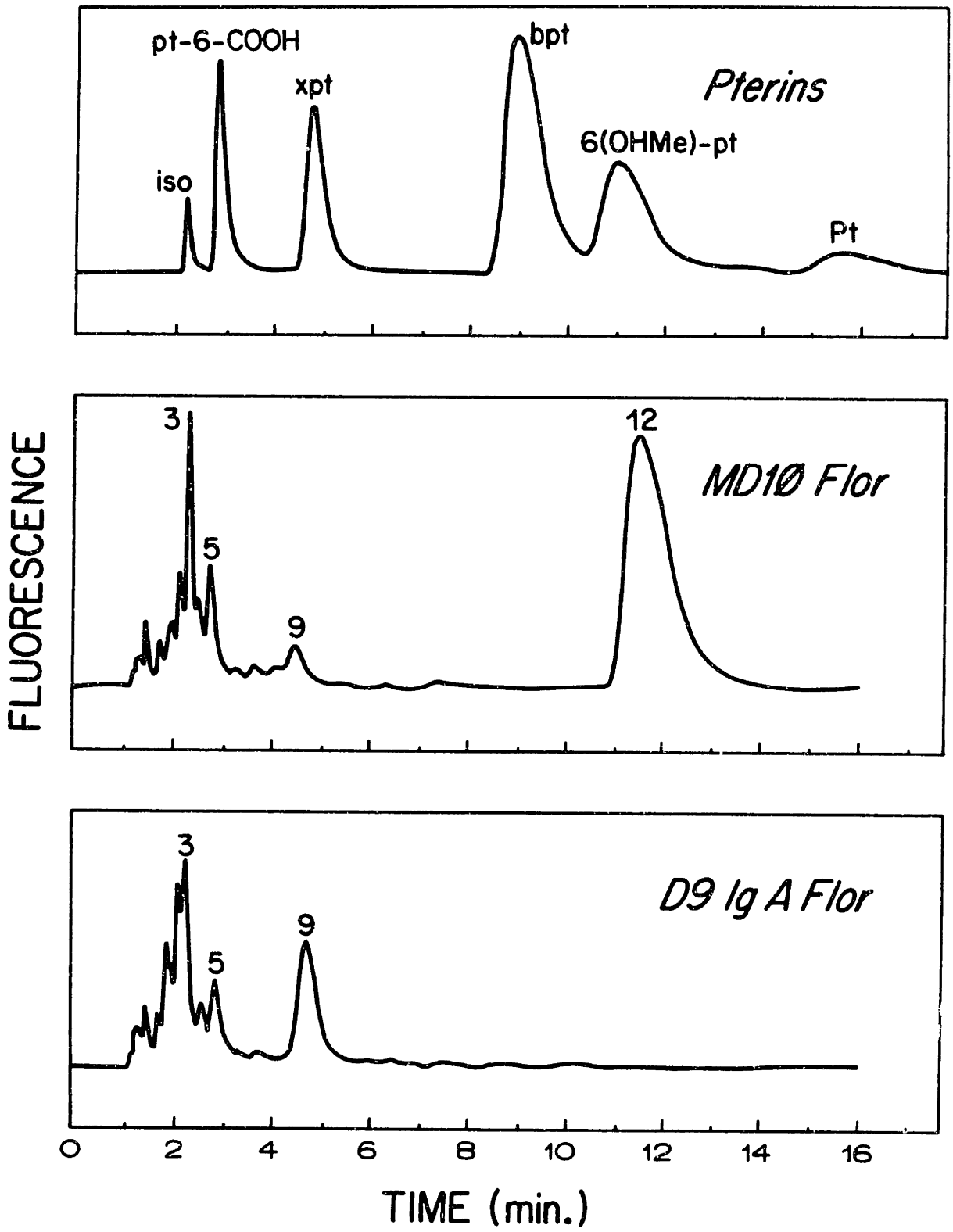
CLONE	RUN	MF-1	MF-2	MF-3	MF-4	MF-5	MF-6	MF-7	MF-8	MF-9	MF-10	MF-11	MF-12	MF-13
MC4	29	.18		.22		.28		.36	.44	.51				
C3A	157	.19		.22			.25			.49				1.86
C3A	168	.27		.31		.40	.34			.55	.68			1.64
MD1	158			.22		.28				.50				
MD1	164			.24						.48				
C21g	165	.22	.23	.25		.30	.27			.46				
MD10	167	.27	.29	.33	.31	.40	.36			.57			1.25	
MD2	169			.31		.38				.56		.83		
D9sm	170			.31		.38	.35			.53		.76		
MB34	171		.29	.32		.40	.35							
D91gA	163	.21		.24		.31	.27			.48				
"	333	.17		.22	.21	.29	.25	.35	.43	.51		.83		1.99
"	630	.25		.29	.28	.35	.33	.44	.51	.57	.77	.90	1.22	1.71

PTERIN STANDARD

iso			.21-.36											
pt6					.30-.46									
xpt														
6OHMept										.49-.62				
pt													1.21-1.34	
														1.61-2.09

MEANS Stds.														
Sample										.49-.62				
										.46-.57	.76-.90			1.6-2.0

Figure 2.09. Comparison of pterin reference compounds (upper) with the Florisil fraction of pigments from marine isolates MD10 (middle) and D91gA (bottom) using SCX-HPLC. Chromatographic conditions were as follows: 4.5 x 250 mm Partisil SCX column with 10 µm particle size; isocratic elution with ammonium phosphate (pH = 2.8), 7 % methanol, and 5 % acetonitrile at 1.5 ml/min.


pt-6-COOH
xpt
bpt
Pterins
6(OHMe)-pt
iso
Pt
3
12
MD10 Flor
5
9
FLUORESCENCE
3
9
D9 Ig A Flor
5
0
2
4
6
8
10
12
14
16
TIME (min.)

Clone D10 and mixed culture MD10, from which D10 was derived, were unlike all other clones tested in that the major pigment component (MFlor-12) had a retention time similar to that of 6-(hydroxymethyl)pterin (6-(OHMe)pt). Clone D91gA originally produced material eluting with a similar retention time, but this component disappeared from filtrates after several transfers.

MFlor-3. A comparison between the fluorescence spectra of MFlor-3 in buffer (pH = 2.8) and isoxanthopterin shows that both have the same excitation and emission maxima (Fig. 2.10). These fluorescence data give further confirmation that isoxanthopterin is one component of the bacterial pigment.

MFlor-12. Although MFlor-12 and 6-(OHMe)pt standard had similar HPLC retention times, their fluorescence spectra were not the same (Fig. 2.11). The data suggest that MFlor-12 is a mixture of two compounds, one with excitation/emission maxima at 315/400 nm, and one with excitation/ emission maxima at 330/470 nm. The mixture appears to be the same as 6-(OHMe)pt, however neither individual compound has the same fluorescence properties as the standard.

Further information pertaining to the identity of MFlor-12 was obtained using mass spectrometric (MS) analysis via chemical ionization (CI) with methane (Fig. 2.12,top). The sample shows what may be a molecular ion at 243, indicating the presence of a compound having a molecular weight of 242. The corresponding M+29 peak would be at m/z = 271. The other abundant peaks in the spectrum could be explained by the presence of a second compound in the same sample with a molecular weight of 256, having M+1 and M+29 peaks at 257 and 285, respectively.

Figure 2.10. Comparison of fluorescence properties of standard isoxanthopterin and MFlor-3. A. Uncorrected excitation spectra with emission at 410 nm for isoxanthopterin at pH = 2.8 (solid line) and pH 6.0 (dotted line), and emission spectrum with excitation at 355 nm (dashed line). B. Uncorrected spectra for MFlor-3 at pH = 2.8. Excitation with emission at 400 nm (solid line) and emission (dashed line) with excitation at 355 nm. All spectra were run on an Perkin Elmer spectrofluorometer Model LCI 100 with a xenon lamp.

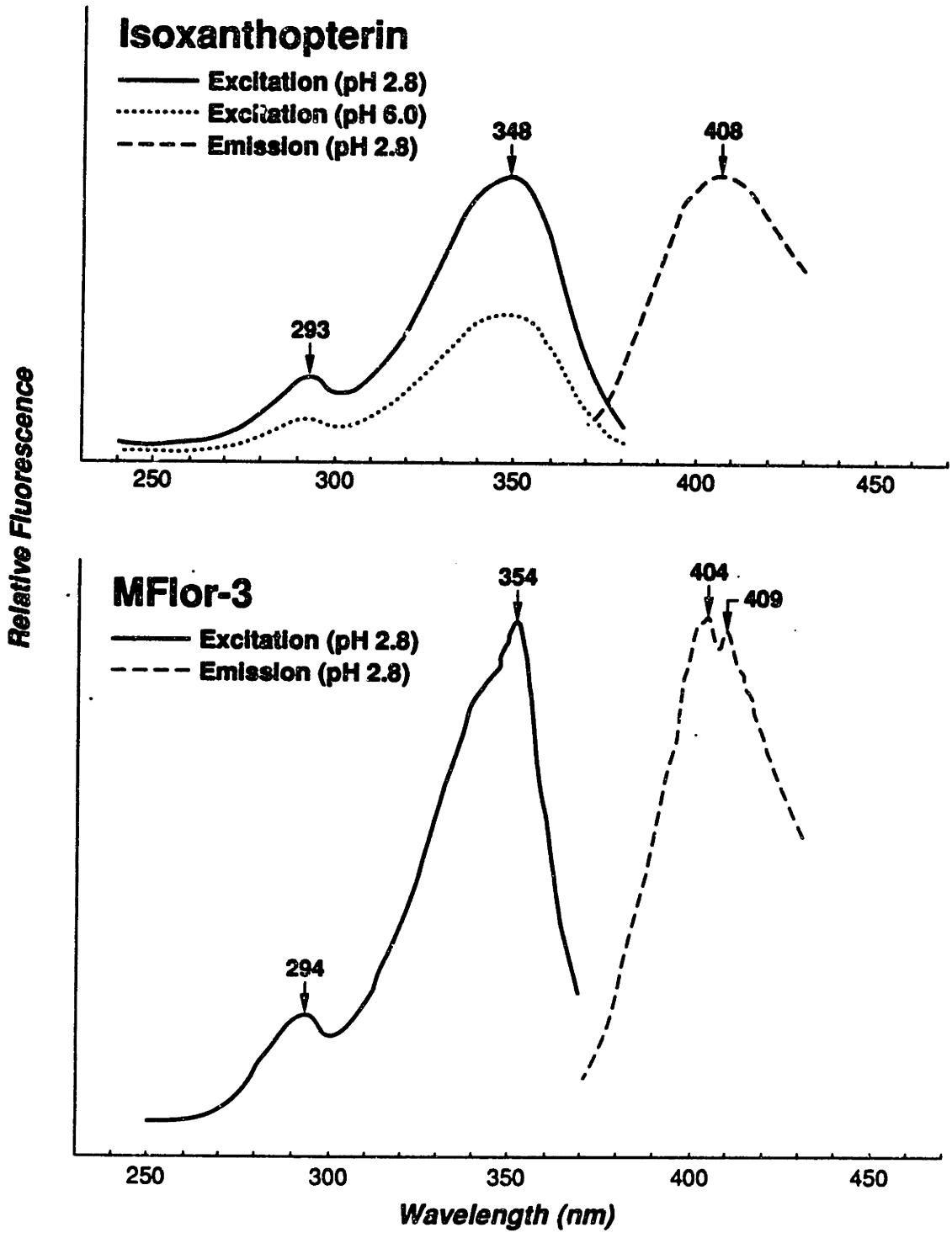

Isoxanthopterin
Excitation (pH 2.8)
Excitation (pH 6.0)
Emission (pH 2.8)
348
408
293
250
300
350
400
450
MFlor-3
Excitation (pH 2.8)
Emission (pH 2.8)
354
404
409
294
250
300
350
400
450
Relative Fluorescence
Wavelength (nm)

Figure 2.11. Top: Excitation spectrum of MFlor-12 with emission at 410 nm (solid line) and 490 nm (dotted line). Bottom: Emission spectra of MFlor-12 with excitation at 310 nm (solid line) and at 340 nm (dotted line). All spectra were run on an SLM-Aminco SPF-500C spectrofluorometer.

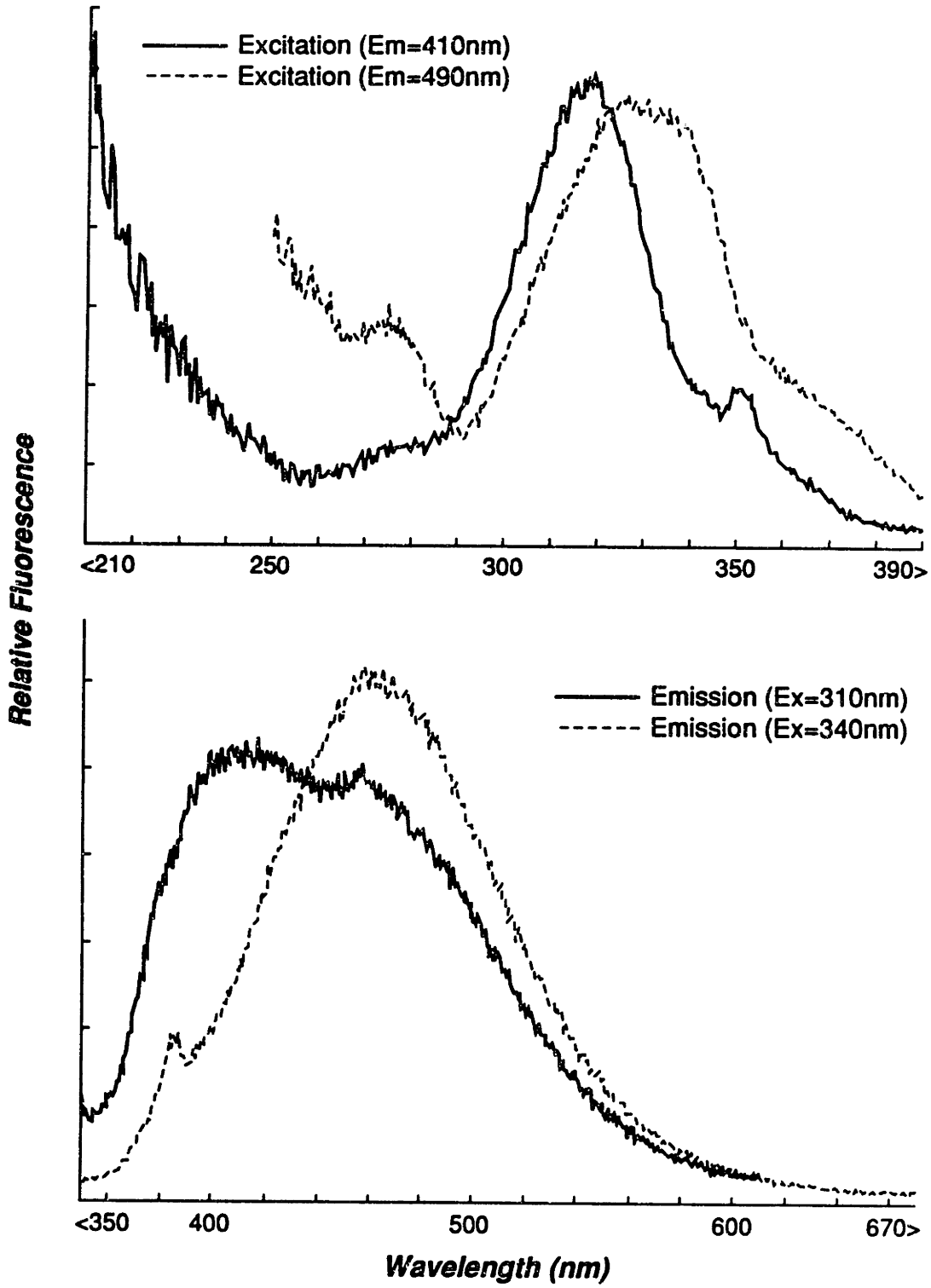
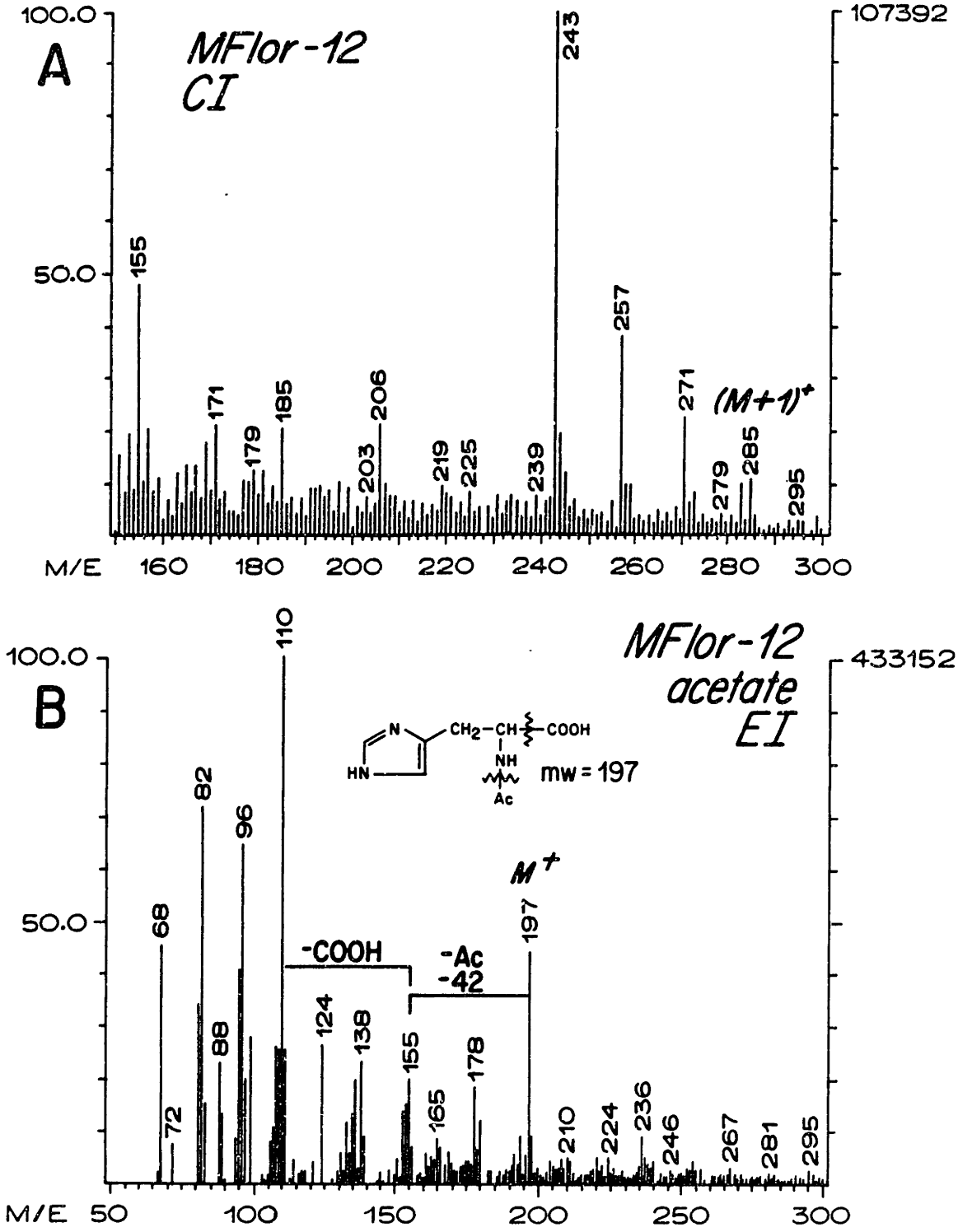

Excitation (Em=410nm)
Excitation (Em=490nm)
<210
250
300
350
390>
Relative Fluorescence
Emission (Ex=310nm)
Emission (Ex=340nm)
<350
400
500
600
670>
Wavelength (nm)

Figure 2.12. A. Chemical (methane) ionization mass spectrum for MFlor-12. B. EI spectrum for acetate derivative of MFlor-12.


A
MFlor-12
CI
100.0
50.0
107392
155
171
179
185
203
206
219
225
239
243
257
271
(M+1)+
279
285
295
M/E 160 180 200 220 240 260 280 300
B
MFlor-12
acetate
EI
100.0
50.0
433152
110
82
96
68
72
88
124
138
155
165
178
197
M+
-COOH
-Ac
-42
210
224
236
246
267
281
295
mw = 197
M/E 50 100 150 200 250 300

These ion peaks could also be explained by the presence of contaminating compounds, such as a homologous series of fatty acids. Peaks at 243, 257, 271, and 285 could represent the presence of C_{15}, C_{16}, C_{17}, and C_{18} fatty acids in the sample, or could represent fragmentation of octadecanoic acid. These compounds could be produced by the cultures, or octadecanoic acid could be a contaminant from the C18 Sep-Paks. None of the compounds are fluorescent, however, so they cannot explain the peak seen in the chromatogram.

In order to determine which ions in the MFlor-12 spectrum were due to compound and which to contamination, samples were acetylated and cleaned up using silica column chromatography prior to mass spectrometry. Electron impact (EI) MS was used this time, and the spectrum for acetylated MFlor-12 is shown in Fig. 2.12 (bottom). No traces of compounds having molecular weights of 242, 256, or 285 were found. This suggests that these were contaminants which were removed by the derivitization procedure. The fragmentation pattern is very similar to that published for histidine monoacetate, with a molecular ion at 197. The fragments at m/z = 155 and 110 could correspond to loss of acetate followed by loss of the carbonyl, respectively.

The combined results of HPLC, fluorometry and mass spectrometry for MFlor-12 do not lead to identification of this compound. SCX chromatography results suggest it is a pure compound similar to 6-(OHMe)pt, however, fluorometric analysis leads to the conclusion that it is a mixture of two fluorescent compounds, neither of which is 6-(OHMe)pt. Mass spectrometry results suggest that MFlor-12 may be histidine, but since histidine is not fluorescent, the MS results are not consistent with the fluorometric results. One possible

explanation is that MFlor-12 is a mixture of three compounds, one of which is histidine and two of which are fluorescent. More work is needed to resolve the problem.

DISCUSSION

We have shown here that marine bacteria synthesize riboflavin, FMN, and FAD, and that they are the major components of the C18-extractable dissolved fluorescence in spent media. Lumichrome and lumiflavin were found in spent media only after exposure to light. These compounds are photodegradation products of riboflavin and have not been found to be synthesized by biological organisms. In additions to flavins, other fluorescent compounds are also produced which pass through a C18 cartridge, but are retained on a Florisil cartridge. Components of this Florisil fraction have SCX-HPLC retention times similar to pterins, however we have not been able to make a positive identificatiion at this time.

The presence of flavins and pterins in spent media has been investigated for a few species of bacteria and phytoplankton. Excretion of riboflavin, FAD, and FMN has been demonstrated for many bacteria (Demain 1972), including some marine species. One indication of the widespread ability of bacteria to synthesize flavins is the finding of Burkholder (1963) that only one of the 1748 cultures of marine bacteria he tested required addition of riboflavin to the growth medium. Phytoplankton secrete a wide variety of organic compounds, including vitamins. Excretion of biopterin has been demonstrated for Poteriochromonas stipitata, Euglena gracilis, and Tetrahymena thermophila (Baker et al. 1981). Folates and riboflavin have been found in the spent growth medium of Ochromonas danica (Aaronson et al. 1971), P. stipitata, and E. gracilis (Baker et al. 1981). Marine bacteria unable to synthesize riboflavin have been shown to exhibit stimulated growth when cultured in proximity to a

colony of riboflavin-excreting bacteria or in proximity to Skeletonema costatum, thus demonstrating that riboflavin can be taken up by microorganisms which require it (Burkholder 1963).

Production of flavins and pterins by these bacteria is not surprising, since these compounds are constituents of coenzymes which are required by all living organisms. Since biopterin is the major precursor of folic acid, it is perhaps surprising that this pterin was not found in our culture filtrates. Lumichrome and lumiflavin have not been reported to be biosynthesized, but they are known to be photochemical degradation products of riboflavin, FMN, and FAD (Dunlap and Susic 1986).

The significance of results presented in this section lies not only in the actual identities of the compounds, but also in the demonstration that they are components of bacterial pigments produced by species isolated from the oceans and are sufficiently water-soluble to readily diffuse into surrounding in vitro or natural medium, i.e., seawater. This represents a first step towards characterization of fluorescent organic material derived from marine bacteria. A comparison between the pigments of these cultures and that of the classic fluorescent pigment of Pseudomonas fluorescens is presented in Chapter 3.

Until now, dissolved fluorescence was thought to be of two types: terrestrial humic material input from rivers, and marine humic material formed by condensation of individual molecules of various types of organics compounds (Kalle 1966). Results of this study imply that highly fluorescent material may also be produced by resident populations of bacteria. Indeed when we compare DFC in the Florisil

extract of seawater from the Cariaco Trench with the filtrate of a mixed culture of marine bacteria, the similarity is striking (Fig. 2.13). The fluorescence properties of individual pterins and flavins are also similar to those of dissolved fluorescence in the oceans. Furthermore, since pterins and flavins are so intensely fluorescent, they could provide a useful, albeit non-unique, signal for locating zones of intense microbial activity. Estimates of how important marine bacteria may be as a source of flavins and of fluorescence in the ocean are presented in Chapters 4 and 5.

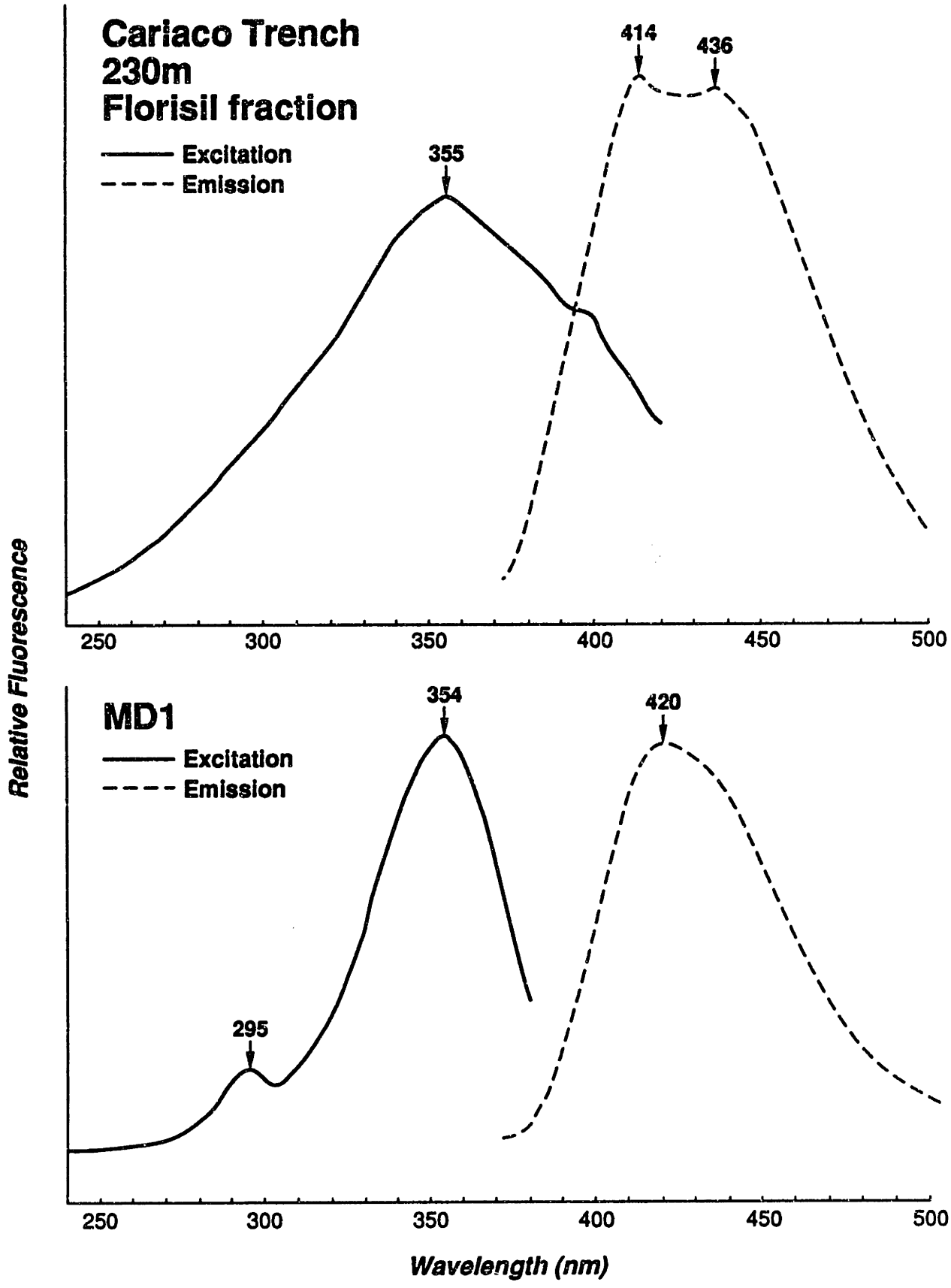
CONCLUSIONS

1. Among the variety of fluorescent compounds produced by nine isolates of marine bacteria, all have been found to produce riboflavin, FAD and FMN. Identifications are based on HPLC retention times under three separate conditions, fluorescence excitation/emission spectra, and HPLC analysis of degradation products. Concentrations up to 20 µg/l are reached in batch culture.

2. Based on SCX-HPLC retention times only, these same marine bacteria have also been found to produce isoxanthopterin, pterin-6-COOH, xanthopterin, 6-(hydroxymethyl)pterin, and pterin. Fluorescence excitation/emission spectra confirm the identification of isoxanthopterin, but show that 6-(hydroxymethyl)pterin is not present.

3. None of the compounds reported here, with the possible exception of MFlor-12 from clone D10, appeared to be unique enough to serve as a biomarker.

Figure 2.13. Comparison of excitation and emission spectra of DOM from seawater and from a culture of marine bacteria. Top: Florisil fraction of DOM collected at 230 m in the Cariaco Trench. Bottom: Whole filtrate from a mixed culture (MD1) of marine bacteria isolated from the Cariaco Trench.


Cariaco Trench
230m
Florisil fraction
Excitation
Emission
355
414
436
250
300
350
400
450
500
MD1
Excitation
Emission
354
420
295
250
300
350
400
450
500
Relative Fluorescence
Wavelength (nm)

4. Lumiflavin and lumichrome were not synthesized by the cultures tested. The presence of these compounds in culture filtrates was due to photodegradation.

REFERENCES

Aaronson, S., B. DeAngelis, O. Frank, and H. Baker. 1971. Secretion of vitamins and amino acids into the environment by Ochromonas danica. J. Phycol. 7: 215-218.

Anderson, J.J. 1982. The nitrite-oxygen interface at the top of the oxygen minimum zone in the eastern tropical North Pacific. Deep Sea Res. 29: 1193-1201.

Baker, E.R., J.J.A. McLaughlin, S.H. Hutner, B. DeAngelis, S. Feingold, O. Frank and H. Baker. 1981. Water-soluble vitamins in cells and spent culture supernatants of Poteriochromas stipitata, Euglena gracilis and Tetrahymena thermophila. Arch. Microbiol. 129: 310-313.

Birkhoffer, L. and A. Birkhoffer. 1948. Riboflavin, a component of "bacterial fluorescein." Zeits. Naturforschung 3b: 136.

Broenkow, W.W., A.J. Lewitus, M.A. Yarbrough, and R.T. Krenz. 1983. Particle fluorescence and bioluminescence distributions in the eastern tropical Pacifis. Nature 302: 329-331.

Burkholder, P.R. 1963. Some nutritional relationships among microbes of sea sediments and waters, pp. 133-150. In: Symposium on Marine Microbiology. C.H. Oppenheimer, ed. C.C. Thomas. Springfield.

Chakrabarty, A.M. and S.C. Roy. 1964. Characterization of a pigment from a Pseudomonad. Biochem. J. 93: 144-148.

Chappelle, E.W., and G.L. Picciolo. 1971. Assay of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) using the bacterial luciferase reaction. Meth. Enzymol. 18B: 381-385.

Demain, A.L. 1972. Riboflavin oversynthesis. Ann. Rev. Microbiol. 26: 369-388.

Dunlap, W.C., and M. Susic. 1985. Determination of pteridines and flavins in seawater by reverse-phase, high-performance liquid chromatography with fluorometric detection. Mar. Chem. 17: 185-198.

Dunlap, W.C., and M. Susic. 1986. Photochemical decomposition rates of pteridines and flavins in seawater exposed to surface solar radiation. Mar. Chem. 19: 99-107.

Forrest, H.S., C. Van Baalen, and J. Myers. 1957. Occurrence of pteridines in a blue-green alga. Sci. 125: 699-700.

Forrest, H.S., C. Van Baalen, and J. Myers. 1958. Isolation and identification of a new pteridine from a blue-green alga. Arch. Biochem. Biophys. 78: 95.

Forrest, H.S., C. Van Baalen, and J. Myers. 1959. Isolation and characterization of a yellow pteridine from the blue-green alga, Anacystis nidulans. Arch. Biochem. Biophys., 83: 508.

Giral, F. 1936. Sobre los liocromos characteristicos del grupo de bacterias fluorescentes. Anales de la sociedad espanola de fisica y quimica 34: 667-693.

Kalle, K. 1966. The problem of the gelbstoff in the sea. Oceanogr. Mar. Biol. Ann. Rev. 4: 91-104.

Keltjens, J.T., L. Daniels, H.G. Jannsen, P.J. Borm, and G.D. Vogels. 1983a. A novel 1-carbon carrier (carboxy-5,6,7,8-tetrahydromethanopterin) isolated from Methanobacterium thermoautotrophicum and derived from methanopterin. Eur. J. Biochem. 130: 545-52.

Keltjens, J.T., M.J. Huberts, W.H. Laarhoven, and G.D. Vogels. 1983b. Structural elements of methanopterin, a novel pterin present in Methanobacterium thermoautotrophicum. Eur. J. Biochem. 130: 537-544.

Kloepper, J.W., J. Leong, M. Teintze, and M.N. Schroth. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286: 885-886.

Kobayashi, K., and M. Goto. 1975. Mass spectra of acetylated and trimethylsilylated pteridines, pp. 57-70, In: W. Pfleiderer, ed. Chemistry and Biology of Pteridines, Proceedings of the 5th International Symposium. Walter de Gruyter. Berlin.

Lenhoff, H. 1963. An inverse relationship of the effects of oxygen and iron on the production of fluorescin and cytochrome c by Pseudomonas fluorescens. Nature 199: 601-2.

Lewitus, A. J., and W. W. Broenkow. 1985. Intermediate depth pigment maxima in oxygen minimum zones. Deep Sea Res. 32: 1101-1115.

MacLean, F. I., H. S. Forrest, and J. Myers. 1966. Characterization of the reduced pteridine in Anacystis nidulans. Arch. Biochem. Biophys. 114: 404.

Massey, V., and P. Hemmerich. 1980 Active-site probes of flavoproteins. Biochem. Soc. Trans. 8: 246-257.

Meyer, O. and K. V. Rajagopalan. 1984. Molybdopterin in carbon monoxide oxidase from carboxydotrophic bacteria. J. Bacteriol. 157: 643-8.

Nielsen, P., P. Rauschenbach, and A. Bacher. 1986. Preparation, properties, and separation by high-performance liquid chromatography of riboflavin phosphates. Meth. Enzymol. 122: 209-220.

Peel, J.L. 1958. Separation of flavins by paper electrophoresis and its application to examination of the flavin contents of micro-organisms. Biochem. J. 69: 403-416.

Peltzer, E.T., J.B. Alford, and R.B. Gagosian. 1984. Methodology for sampling and analysis of lipids in aerosols from the remote marine atmosphere. Woods Hole Oceanog. Inst. Tech. Rept. WHOI-84-9, 103 p.

Spinrad, R.W., H. Glover, B.B. Ward, L.A. Codispoti, and G. Kullenberg. 1989. Suspended praticle and bacterial maxima in Peruvian coastal waters during a cold water anomaly. Deep-Sea Res. 36: 715-734.

Stea, B., R.M. Halpern, B.C. Halpern, and R.A. Smith. 1980. Quantitative determination of pterins in biological fluids by high-performance liquid chromatography. J. Chromatogr. 188: 363-375.

Totter, J. R. and F. T. Moseley. 1953. Influence of the concentration of iron on the production of fluorescin by Pseudomonas aeruginosa. J. Bacteriol. 65: 45-7.

Van Haastert, P.J.M., R.J.W. DeWit, and T.M. Konijn. 1982. Antagonists of chemoattractants reveal separate receptors for cyclic AMP, folic acid and pterin in Dictyostelium discoideum. Exp. Cell Res. 140; 453-456.

Walsh, C. 1982. Scope of chemical redox transformations catalyzed by flavoenzymes, pp. 121-132, In: Flavins and Flavoproteins. V. Massey and C.H. Williams, eds. Elsevier North Holland, Inc. New York.

Ziegler, I. and R. Harmsen. 1969. The biology of pteridines in insects, pp. 139-203. In: Advances in Insect Physiology v. 6. J. W. L. Beament, J. E. Treherne and V, B, Wigglesworth, eds. Academic Press, London.

ZoBell, C.E. and C.B. Feltham. 1934. Preliminary studies on the distribution and characteristics of marine bacteria. Bull. Scripps Inst. Oceanogr., Tech. Rept. 3; 279-296.

ZoBell, C.E. and H.C. Upham. 1944. A list of marine bacteria including descriptions of sixty new species. Bull. Scripps Inst. Oceanogr. 5: 239-292.

CHAPTER 3

PRODUCTION OF PYOVERDINE AND OTHER FLUORESCENT METABOLITES BY *PSEUDOMONAS FLUORESCENS* AND MARINE BACTERIA

INTRODUCTION

It has long been recognized that certain species of pseudomonads produce yellow, water-soluble fluorescent pigments which can freely diffuse out of the cells into the surrounding growth medium. These pigments have been called "bacterial fluorescein" or "pyoverdine," but the chemical structure is still unknown, in part because a suitable method for isolation and purification has not yet been devised. The standard bacteriological procedure used to distinguish between pyoverdine and other yellow pigments involves examination of cultures grown on a solid defined, iron-deficient medium using a ultraviolet lamp (Palleroni 1984). Some pigments may be yellow or fluoresce under longer wavelength irradiation, but only pyoverdine fluoresces when viewed under short wavelength UV (254 nm) irradiation.

Pseudomonas fluorescens takes its name from the fact that it produces pyoverdine, and it is representative of the fluorescent pseudomonads. The pigment pyoverdine has a fluorescence emission maximum at around 470 nm and an excitation maximum near 400 nm (pH = 7). Changes in pH result in changes in the positions of these maxima. Various compounds such as pteridines, (Giral 1936; Chakrabarty and Roy 1964), flavins (Birkhofer and Birkhofer 1948), and pyrroles (Lenhoff 1963; Greppin and Gouda 1965) have been proposed as the fluorophore.

The pigment from P. fluorescens has been purified using a variety of methods, each of which gives slightly different results. Chakrabarty and Roy (1964) used paper chromatography and electrophoresis to separate the pigment into three fluorescent components. They reported a molecular weight of 210 for the

green-fluorescing component and suggested that it may be 2,4-dioxopterdine (lumazine) with a carboxyl or polyhydroxyl side chain. Meyer and Abdallah (1978) isolated the pigment by first complexing it with iron, in a manner similar to that used to isolate bacterial siderochromes. They reported a molecular weight of around 1500 and suggested that previous reports that pyoverdine consists of a mixture of compounds were due to chemical degradation of the whole pigment under alkaline conditions. Meyer (1977) suggested that the pigment contains a short aliphatic chain, a cyclic peptide, and a quinoline chromophore and that it is similar to hydroxymate siderophores produced by other bacteria, since one of the components of the peptide is N^5-hydroxyornithine. Askeland and Skogerboe (1987) purified the pigment using a procedure similar to that of Meyer and Abdallah (1978), but without making the iron-pigment complex. Their results indicate the presence of two fluorescent components.

A zinc porphyrin compound of molecular weight 920 has also been proposed for the structure of pyoverdine (Visconti et al. 1964). It has an absorbance maximum in ether at 405 nm, with smaller maxima at 537 and 573 nm. The authors suggest it may be a type of coproporphyrin (Visconti et al. 1964). Unfortunately, there is no estimate of absolute or relative quantities of these compounds produced, nor any estimate of what proportion of the total pigment they comprise.

In addition to pyoverdine, more than 70 other metabolites have been reported to be produced by the fluorescent pseudomonads (Leisinger and Margraff 1979). Among these are some fluorescent compounds including pterins, lumazines, porphyrins, and flavins.

These may have been mistaken for pigment components in the past, thereby contributing to the confusion as to the true identity of pyoverdine.

Our objectives in studying the Pseudomonas fluorescens yellow fluorescing pigment (PFYFP) were three-fold: to develop an improved method for purification and analysis of the pigment, to distinguish between pyoverdine and other fluorescent metabolites, and to compare the composition of dissolved fluorescence produced by P. fluorescens with that produced by cultures of marine bacteria isolated from the low-oxygen waters of the Cariaco Trench. In this chapter we will evaluate the effectiveness of electrophoresis, thin layer chromatography (TLC), and high performance liquid chromatography (HPLC) for resolving the fluorescent metabolites of P. fluorescens into individual components, including pyoverdine. We will compare the fluorescent metabolites of P. fluorescens with those produced by marine bacteria using HPLC, and compare conclusions based on HPLC analysis of pigments to those obtained using the standard test for pyoverdine production. Lastly, we will examine the production of fluorescent metabolites as a function of growth phase to determine if any show a precursor/product relationship.

METHODS

Culture conditions and test for fluorescent pigments. Pure freeze-dried cultures of P. fluorescens were obtained from the American Type Culture Collection (ATCC # 17559) and maintained on agar stab cultures at 4°C. Production of fluorescent pigments was evaluated on King's B medium (King et al. 1954) according to the procedure described by Jessen (1965), except that 20 gm/l sodium chloride was added to the medium used to test marine cultures. The King's B medium is fluorescent even before being inoculated with fluorescent bacteria due to components such as yeast extract and tryptone, which contain fluorescent amino acids. For this reason, all P. fluorescens cultures used for analysis of pigment composition were grown on non-fluorescent Asn-2 medium. This medium was the same as Asn-2S described in Chapter 2 Methods except that it contained no sodium chloride. Additional details of culture conditions and media are given in Chapter 2.

Sample preparation. Pyoverdine and other dissolved fluorescent compounds produced by P. fluorescens were collected from spent media by the method described in Chapter 2. Crude pigment preparations consisted of media which had been filtered through 0.2 um membrane or Whatman GF/F glass fiber filters and concentrated by rotary evaporation.

Zone electrophoresis. Samples and reference compounds were spotted or streaked onto microcrystalline cellulose (Avicell, 250 um thick) TLC plates, sprayed with 0.05 M acetic acid, and separated by zone electrophoresis in a variety of mobile phases and pH values

(Table 3.01). Fluorescent compounds were visualized under long UV radiation using a Mineralight.

Thin layer chromatography. The two-dimensional thin layer chromatography (2-D TLC) method of Wilson and Jacobson (1977) was used to separate the yellow pigment from filtrates of P. fluorescens. Cellulose plates were developed first in 1:1 isopropanol : 2% aqueous ammonium acetate (solvent system 1), dried at room temperature for 1 to 2 hours, then developed in 3 % aqueous ammonium chloride (solvent system 2). Pre-rinsing of plates in solvents followed by baking at 80 C for 30 min. was found to decrease fluorescence of samples, therefore no pre-treament of plates was used. Blank plates showed the presence of a yellow contaminant which moved with the solvent front, but it was not a serious contaminant. Fluorescent components were visualized under long UV light using a Mineralight.

Microscopy. A Zeiss epifluorescence microscope with illumination by 100 watt mercury lamp was used to examine bacteria for autofluorescence. The filter set used had the following specifications: BP 365/12, FT 395, LP 397. This combination results in an excitation wavelength of 365nm and an emission of >390 nm, and is routinely used for observation of cells stained with DAPI (4'6-diamidino-2-phenylindole), a DNA stain.

High performance liquid chromatography (HPLC). Methods used for HPLC analysis of pigment components are described in Chapter 2.

Growth studies. Aerobic batch cultures were grown in 1500 ml volumes of Asn-2 (P. fluorescens) or Asn-2S (marine species) media in 2800 ml Fernbach flasks and kept in the dark. The flasks were sampled periodically for cell density and dissolved fluorescent compound (DFC)

concentrations. Samples were collected via siphoning after 5 min. of vigorous stirring. Aliquots of 5 - 200 ml for DFC analysis were prepared as described in Chapter 2. Cell densities were measured by absorbance at 600 nm on 2.5% formalin-preserved samples.

RESULTS

A. CHROMATOGRAPHIC CHARACTERIZATION OF PYOVERDINE AND OTHER FLUORESCENT COMPOUNDS PRODUCED BY PSEUDOMONAS FLUORESCENS

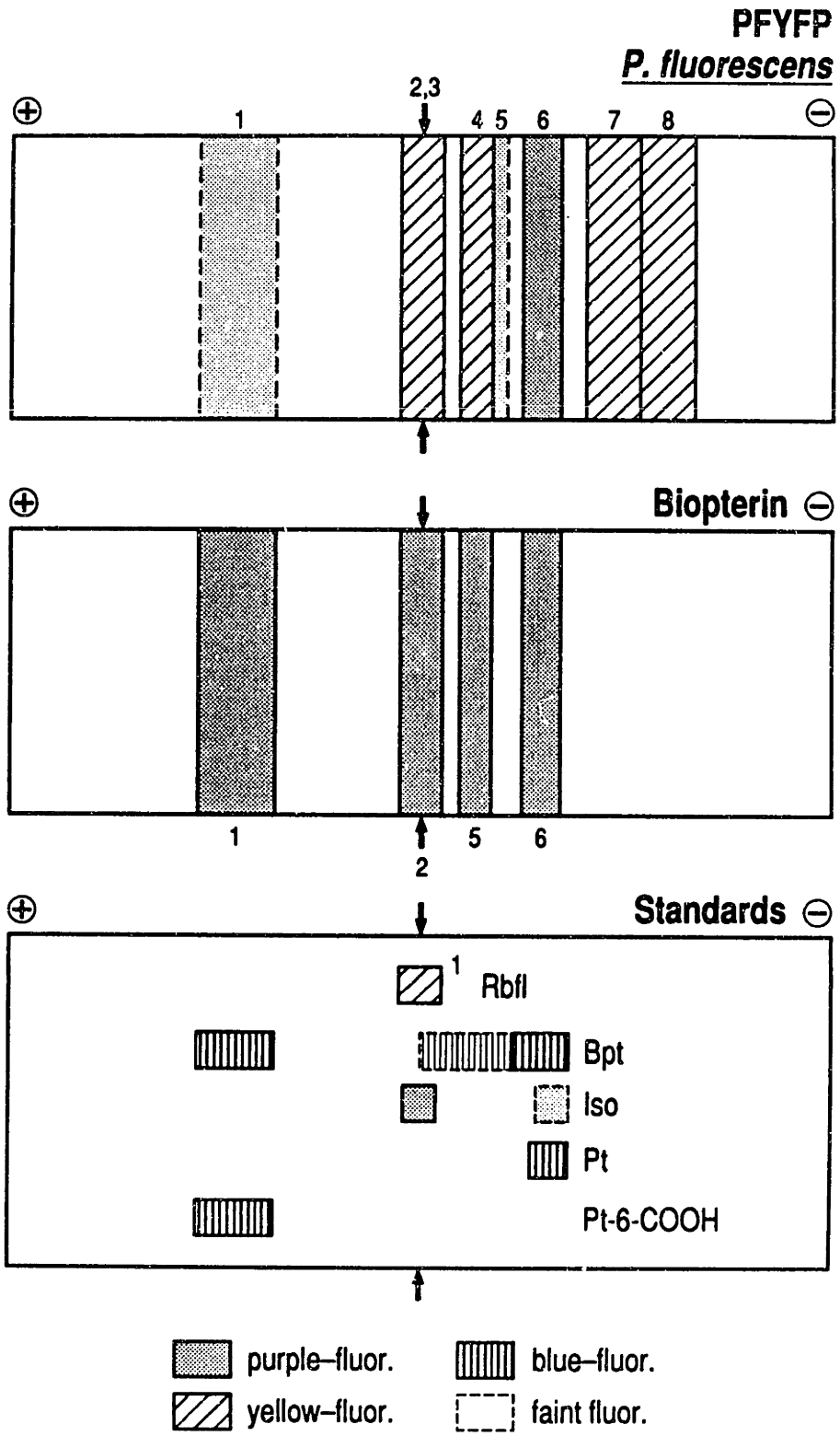
The first stage of our investigation of P. fluorescens was to devise a separation method which would resolve the mixture of fluorescent metabolites into as many components as possible. We will refer to this mixture, which includes pyoverdine, as P. fluorescens yellow fluorescent pigment, or PFYFP.

Electrophoresis. One technique frequently used to separate ionizable compounds is electrophoresis. The mobility of a given compound is a function of charge, size and shape. The net charge can be varied by varying the pH of the electrolyte, while altering the ionic strength of the electrolyte can change the mobility and resolution. Table 3.01 shows a summary of results for PFYFP and biopterin for all the electrophoresis conditions tested. Based on number of components resolved, the best separation of PFYFP was achieved in 0.05 M acetic acid. Increasing the strength of acetic acid to 0.8 M did not change the resolution. Examples of electrophoretic separation for PFYFP, crude biopterin (Sigma), and a mixture of pterin and flavin reference compounds are shown in Figure 3.01. The crude biopterin is composed of at least four compounds, three of which were also found in PFYFP. From the positions of the other standards (Fig. 3.01C), these would appear to be: pterin-6-COOH (band #1), isoxanthopterin (band #2), and pterine (band #6). The "pure" biopterin standard (CalBiochem, >98% purity) also may have been contaminated with these three compounds. The location of biopterin is

Table 3.01. Summary of results of zone electrophoresis separation of crude PFYFP and biopterin in various electrolyte systems. (+) = component travelled towards cathode; (-) = component travelled towards anode. Colors refer to color of fluorescence under UV light.

Electrolyte System	Compound			
	PFYFP		Biopterin	
	Description	Total #	Description	Total #
KCl/HCl 0.2M; pH 1.1	one band; +	1	one band; +	1
KCl/HCl 0.2M; pH 1.9	one band; -	1	one band; -	1
Acetic acid 0.05M	purple: 1 +, 2 - yellow: 1 neutral, 3 -	7	purple;1 +,1 neutral, 2 -	4
Acetic acid 0.8M	same as above	7	same as above	4
NaAc:HAc 0.2M; pH 4.0	purple: 1 + yellow: 1 neutral, 1 - blue: 2 -	5	not run	
NH_4OH 0.05M; pH 10.4	one band: + (yellow) to - (purple)	1	not run	
KCl/NaOH 0.05M; pH 12.8	one green band: - to +	1	blue: 2 +	2

Figure 3.01. Comparison of zone electrophoresis separation of PFYFP (top), crude biopterin (middle), and some other flavin and pterin standards (bottom). Cellulose plates were developed in 0.05 M acetic acid. Bands are keyed according to the color of fluorescence observed under long-wave UV radiation.

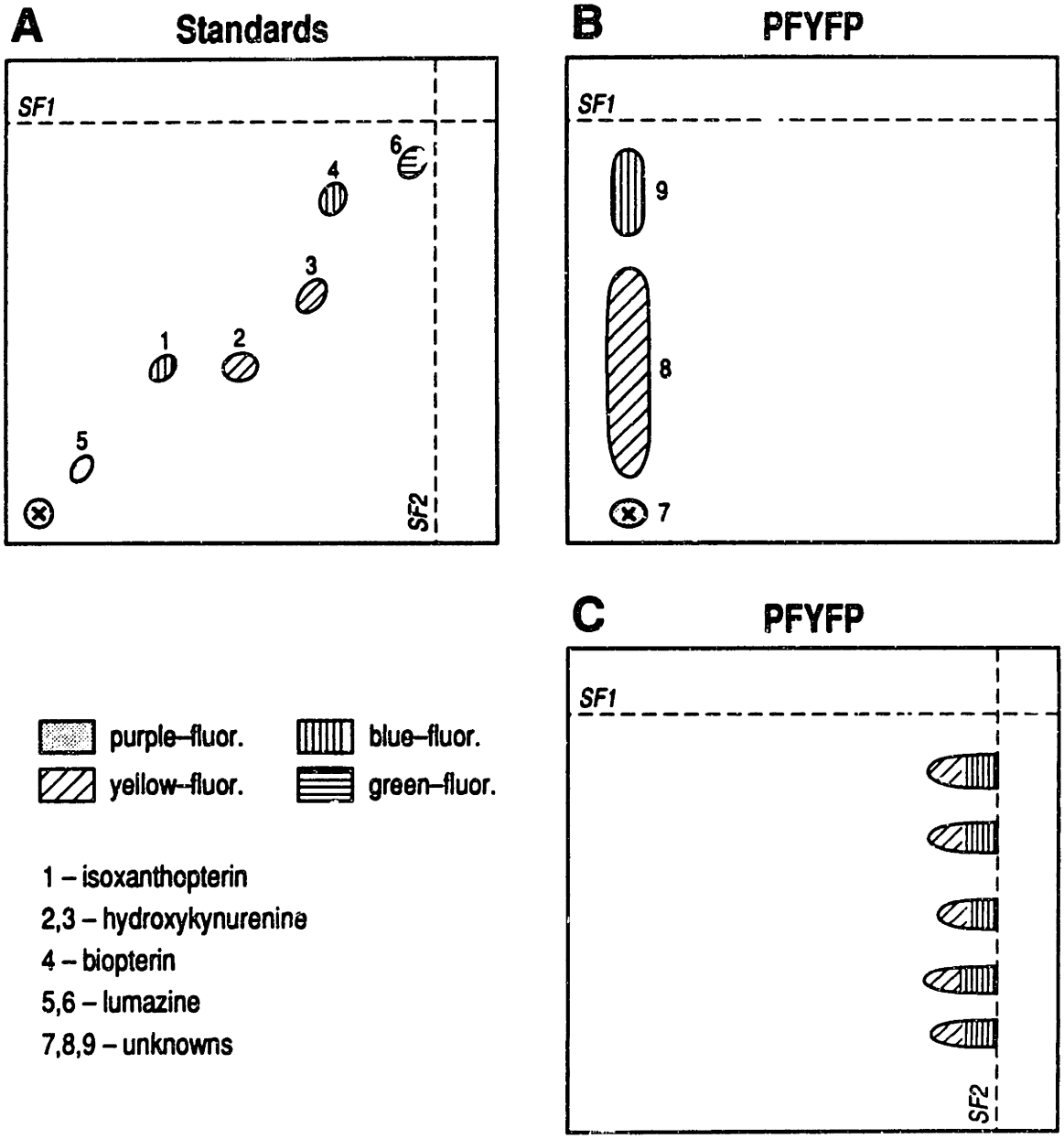

PFYFP
P. fluorescens
2,3
1
4 5 6 7 8
Biopterin
1
2
5
6
Standards
1
Rbfl
Bpt
Iso
Pt
Pt-6-COOH
purple–fluor.
blue–fluor.
yellow–fluor.
faint fluor.

therefore unclear, however it would appear to be present in PFYFP. The band at the origin (band #3) from PFYFP was yellow instead of the blue observed in biopterin. This may indicate that riboflavin was present in PFYFP instead of or in addition to isoxanthopterin. Three additional yellow bands (#4,7,8) which did not correspond to any of the reference compounds tested were observed in PFYFP. Overall, electrophoretic separation of PFYFP resulted in isolation of seven components and suggested the presence of pterin-6-carboxylic acid, biopterin, isoxanthopterin, pterine, and riboflavin.

Two-dimensional thin layer chromatography. Since results of electrophoresis indicated that PFYFP was composed of pteridines, we analyzed it using a two-dimensional thin layer chromatography (2-D TLC) method which has been used to resolve the pteridine components of Drosophila eye pigments (Wilson and Jacobson 1977). Figure 3.02 shows 2-D TLC results for PFYFP and for some reference compounds, selected on the basis of providing a broad range of Rf values using this method. The relative positions of the standards isoxanthopterin and biopterin compare well with results of Wilson and Jacobson (1977). Standard hydroxykynurenine should be located at spot #3, based on previously reported Rf values, and spot #2 is an impurity present in the standard obtained from CalBiochem. Spot #6 is lumazine, based on its characteristic green fluorescence and spot #5 must be an impurity in the standard obtained from Sigma.

The crude PFYFP was separated into two broad bands after development in the first solvent system, a fast moving blue-fluorescing band and a slower moving yellow-fluorescing band (Fig. 3.02B). A purple-fluorescing spot was observed at the origin.

Figure 3.02. Comparison of two-dimensional thin-layer chromatography for reference compounds and PFYFP. A. Reference compounds after development in both solvent systems. B. PFYFP after development in solvent system 1 only. C. PFYFP after development in both solvent systems. Solvent system 1 = 1:1 isopropanol : 2% ammonium acetate. "x" indicates original position of sample spots. Solvent system 2 = 3 % ammonium chloride. The positions of the first and second solvent fronts are indicated as SF 1 and SF 2, respectively. Spots are keyed according to the color of fluorescence observed under long-wave UV radiation.


A
Standards
SF1
SF2
1
2
3
4
5
6
B
PFYFP
SF1
7
8
9
C
PFYFP
SF1
SF2
purple–fluor.
blue–fluor.
yellow–fluor.
green–fluor.
1 – isoxanthopterin
2,3 – hydroxykynurenine
4 – biopterin
5,6 – lumazine
7,8,9 – unknowns

However, both the blue- (component 9) and yellow-fluorescing (component 8) bands migrated equally in solvent system 2 (Fig. 3.02C), and split further into blue- and yellow-fluorescing bands. Retention factors for PFYFP are within the range of standards tested for the first solvent system, but not for the second (Table 3.02). These results may have been caused by streaking or partial separation in the first solvent system, and two interpretations are possible regarding the number of components present in PFYFP. One explanation is that PFYFP contains two components, 8 and 9, each of which contained minor amounts of the other component. Because of streaking in the first development step, component 8 separated into 3 or 4 components during the second stage of development. Because component 8 was contaminated with small amounts of component 9, each spot derived from band 8 further split into yellow- and blue-fluorescing parts. The second possible explanation is that PFYFP is comprised of at least ten components, which were poorly separated and visualized after development in the first solvent system (solvent 1). All were much more soluble in the 100 % aqueous solvent system (solvent 2) than were pterins. In either case, the major pigment components are unlike any of the reference compounds tested. If flavins and pterins are constitutents of PFYFP, they must have been present at concentrations which were undetectable using this procedure.

High performance liquid chromatography. Crude PFYFP was analyzed using two types of HPLC: reverse phase (RP) and strong cation exchange (SCX). Using RP-HPLC, we were able to resolve crude PFYFP into seven UV-absorbing and two fluorescent components (Fig. 3.03). The fluorescent peak which eluted at 2.5 min. had a retention time

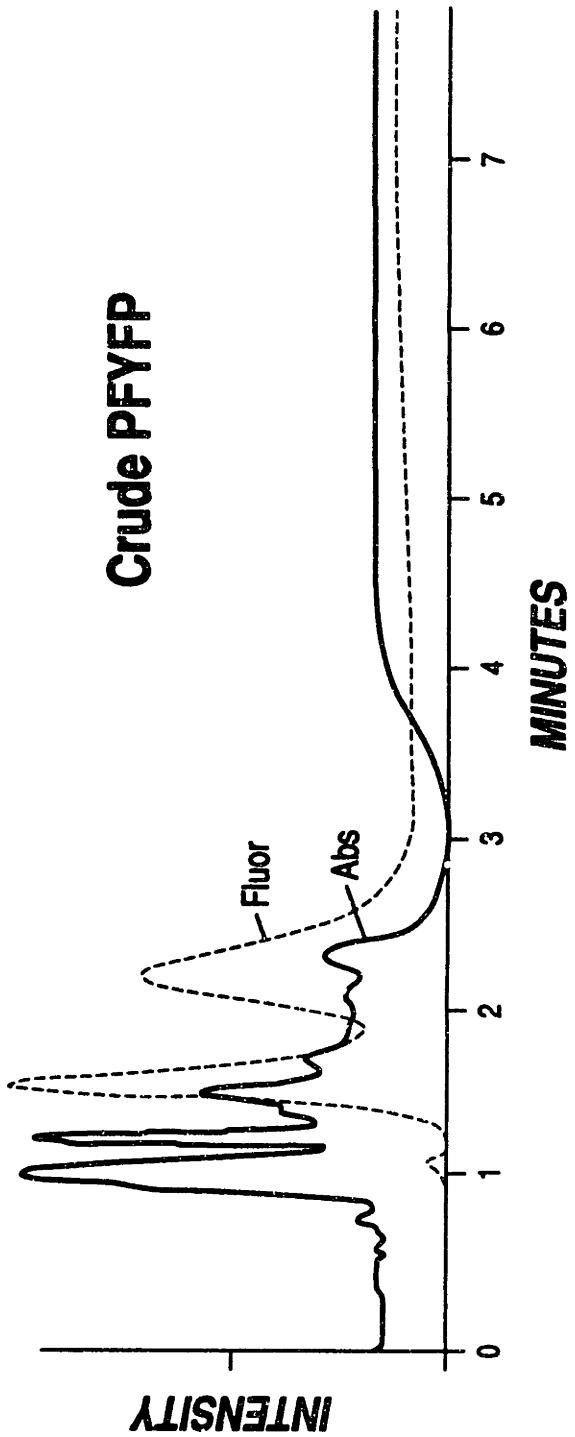
Table 3.02. Summary of two-dimensional TLC separation of PFYFP and various reference compounds. Spot # refers to numbers on Fig. 3.02, or letters in footnore below. Colors refer to color of fluorescence.

Compound	Spot #	R_f A	R_f B	Color
isoxanthopterin	1	0.15 - 0.26	0.36 - 0.44	blue
	a	0.355	0.45	
hydroxy-kynurenine	2	0.30 - 0.39	0.39 - 0.47	yellow
	3	0.39 - 0.52	0.44 - 0.56	yellow
	a	0.51	0.53	yellow
biopterin	4	0.51 - 0.61	0.53 - 0.64	blue
	a	0.6	0.745	
lumazine	5	0 - 0.05	0.03 - 0.10	purple
	6	0.53 - 0.67	0.53 - 0.67	green
P. fluor. YFP	7	0.28 - 0.34	0.79 - 1.00	
	8	0.41 - 0.66	0.79 - 1.00	yellow
	9	0.75 - 0.88	0.79 - 1.00	blue
L-erythro-biopterin	a	0.58	0.60	blue
riboflavin	b	0.5	0.25	

a = from Wilson and Jacobson (1977)

b = from Descimon and Barial (1966)

Figure 3.03. Separation of PFYFP using RP-HPLC with detection by absorbance at 254 nm (solid line) and by fluorescence (dashed line) (Ex=240; Em>470 nm). Vertical axis is not to scale. Chromatographic conditions: 4.6 x 250 mm x 10 µm Versapack C18; isocratic; 85:15 50mM ammonium acetate (pH 6.8):methanol.


Crude PFYFP
Fluor
Abs
INTENSITY
MINUTES
0
1
2
3
4
5
6
7

similar to that of biopterin. The chromatogram shown in Fig. 3.03 is similar to Fig. 2 in Askeland and Skogerboe (1987), which shows the composition of their purified pigment preparation using a reverse phase HPLC method similar to the one used here. Askeland and Skogerboe found only one fluorescent component because they had previously removed a second fluorescent fraction.

The use of SCX-HPLC resolved crude PFYFP into eight fluorescent components and the Florisil fraction into seven fluorescent components (Fig. 3.04). Peaks are numbered in order of elution for the Florisil fraction. Table 3.03 lists the retention times relative to biopterin for both samples and for some standard pterins and flavins. Based solely on retention times, it appears that isoxanthopterin and pterin-6-COOH are present in both samples. Unknown P-8 had the same relative retention time as riboflavin. No components corresponded to xanthopterin or lumichrome in either the whole or Florisil fraction of PFYFP. Unknowns PFlor-1, -4, and -6 corresponded to P-1, -4, and -6, respectively. P-9 and -10 were found only in crude PFYFP and therefore must have either been irreversibly bound on one of the Sep-Paks, or not retained at all by either type of Sep-Pak. PFlor-7 was only found in the Florisil fraction and therefore may be an artifact of the fractionation step.

The C18 fraction of PFYFP was not well-resolved by SCX-HPLC, however using IP-HPLC it can be shown to contain riboflavin, FMN, and FAD as minor constituents (Fig. 3.05). The majority of the fluorescent material elutes in two peaks (PC18-1 and PC18-2) before any of the flavins. By comparing peak areas relative to that of riboflavin in the chromatograms shown in Figs. 3.04C (peak #8) and

Figure 3.04. Comparison of SCX-HPLC separation of pterin reference compounds (top) with Florisil fraction of PFYFP (middle) and crude PFYFP (bottom) using detection by fluorescence (Ex=240; Em>470 nm). iso = isoxanthopterin, pt-6-COOH = pterin-6-carboxylic acid, xpt = xanthopterin, bpt = biopterin, and pt = pterine. Chromatographic conditions: 4.5 x 250 mm x 10 µm Partisil SCX column; isocratic; ammonium phosphate (pH = 2.8), 7 % methanol, and 5 % acetonitrile at 1.5 ml/min.

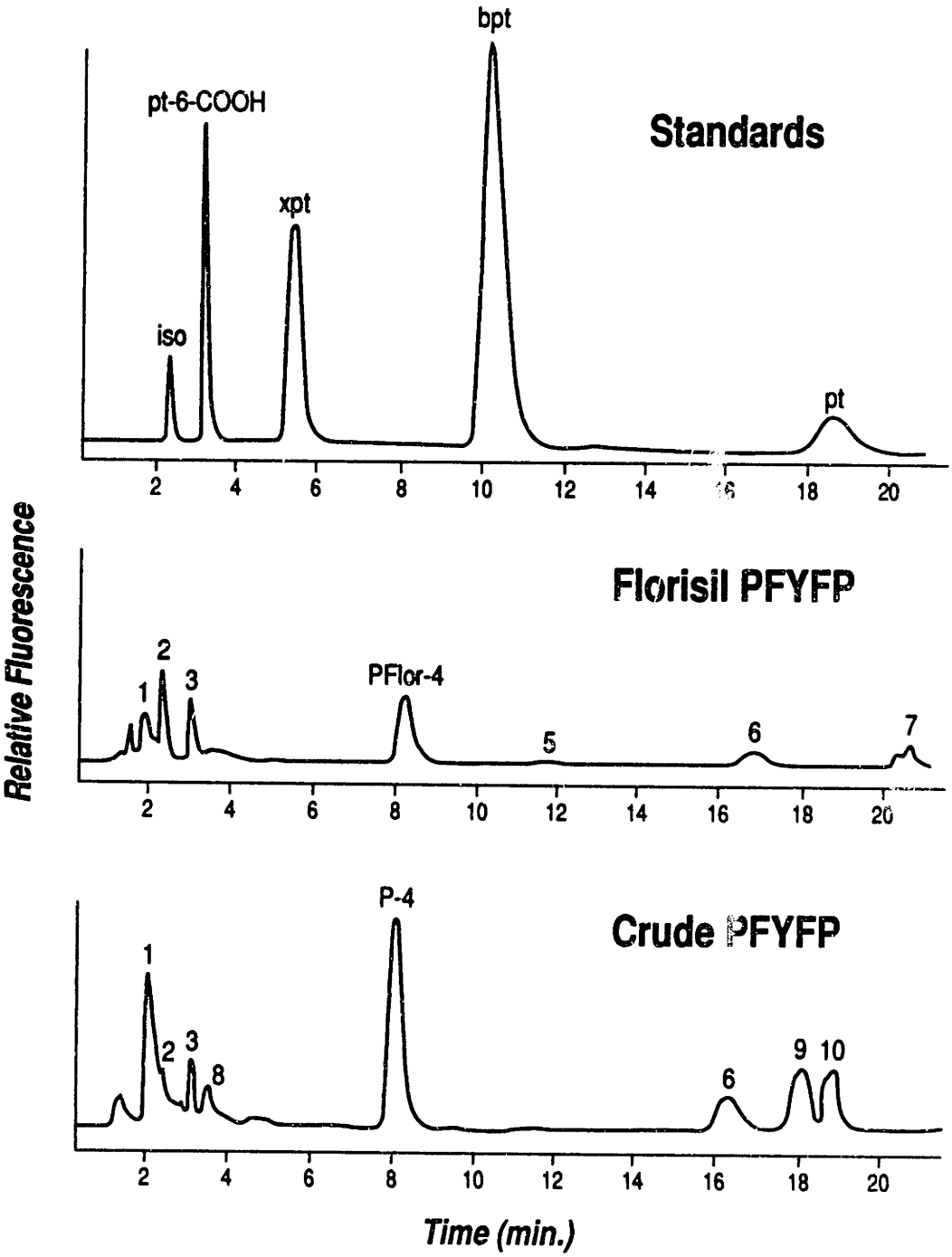
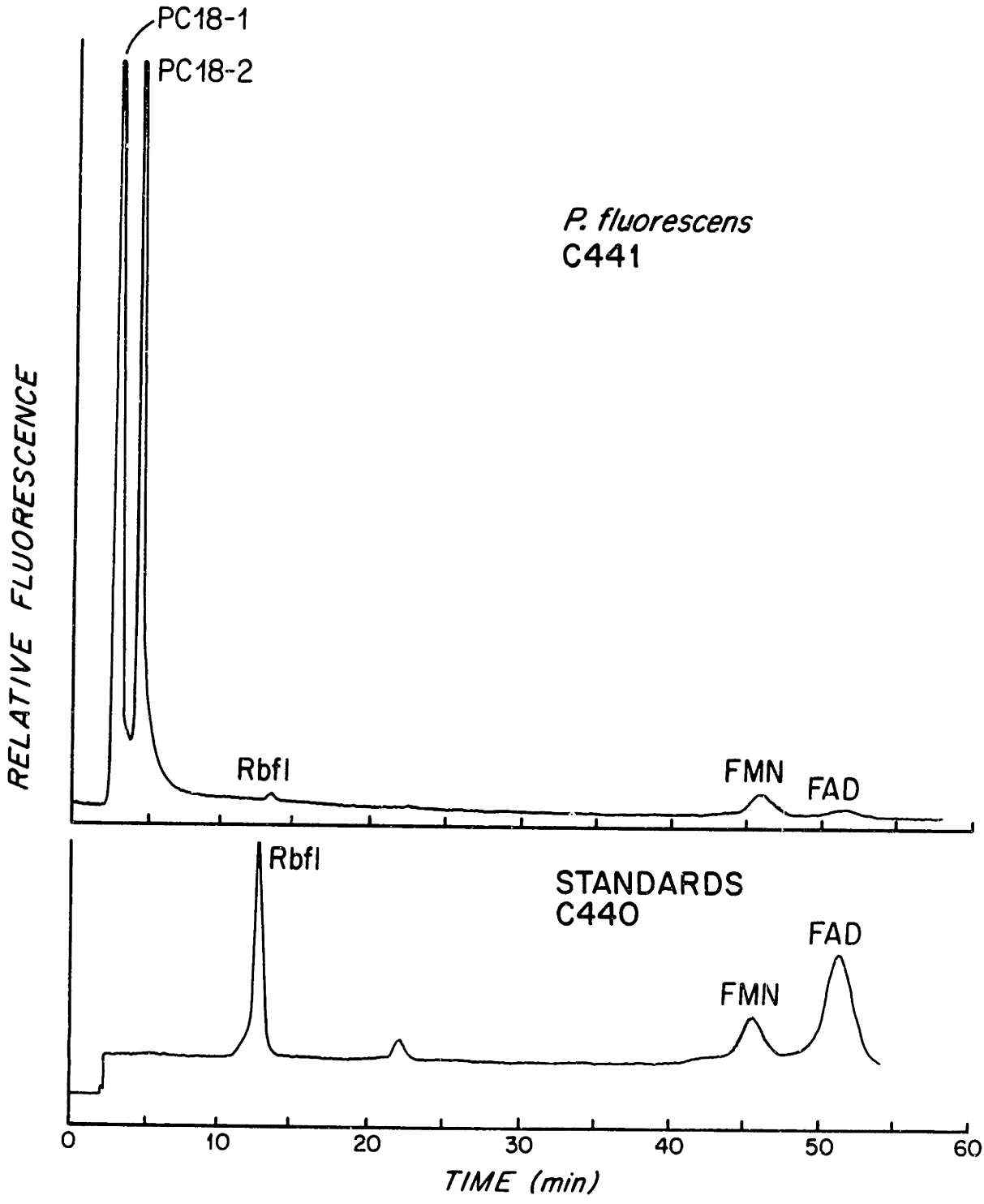

bpt
pt-6-COOH
Standards
xpt
iso
pt
2 4 6 8 10 12 14 16 18 20
Florisil PFYFP
1 2 3
PFlor-4
5
6
7
2 4 6 8 10 12 14 16 18 20
P-4
Crude PFYFP
1
2 3 8
6
9 10
2 4 6 8 10 12 14 16 18 20
Relative Fluorescence
Time (min.)

Table 3.03. Summary of relative retention times for crude PFYFP and the Florisil fraction of PFYFP using SCX-HPLC. Component names correspond to retention times relative to the retention time of biopterin. iso = isoxanthopterin; pt6COOH = pterin-6-carboxylic acid; xpt = xanthopterin; rbfl = riboflavin; lcr = lumichrome.

Component	.19	.24	.3	.34	.39	.53	.82	1.16	1.66	1.8	1.85	2.02
FLORISIL FRACTON												
PFlor-1	x											
-2		x										
-3			x									
-4							x					
-5								x				
-6									x			
-7												x
CRUDE PFYFP												
P-1	x											
-2		x										
-3			x									
-4							x					
-6									x			
-8				x								
-9										x		
-10											x	
STANDARDS												
iso		x										
pt6COOH			x									
xpt						x						
rbfl				x								
lcr					x							

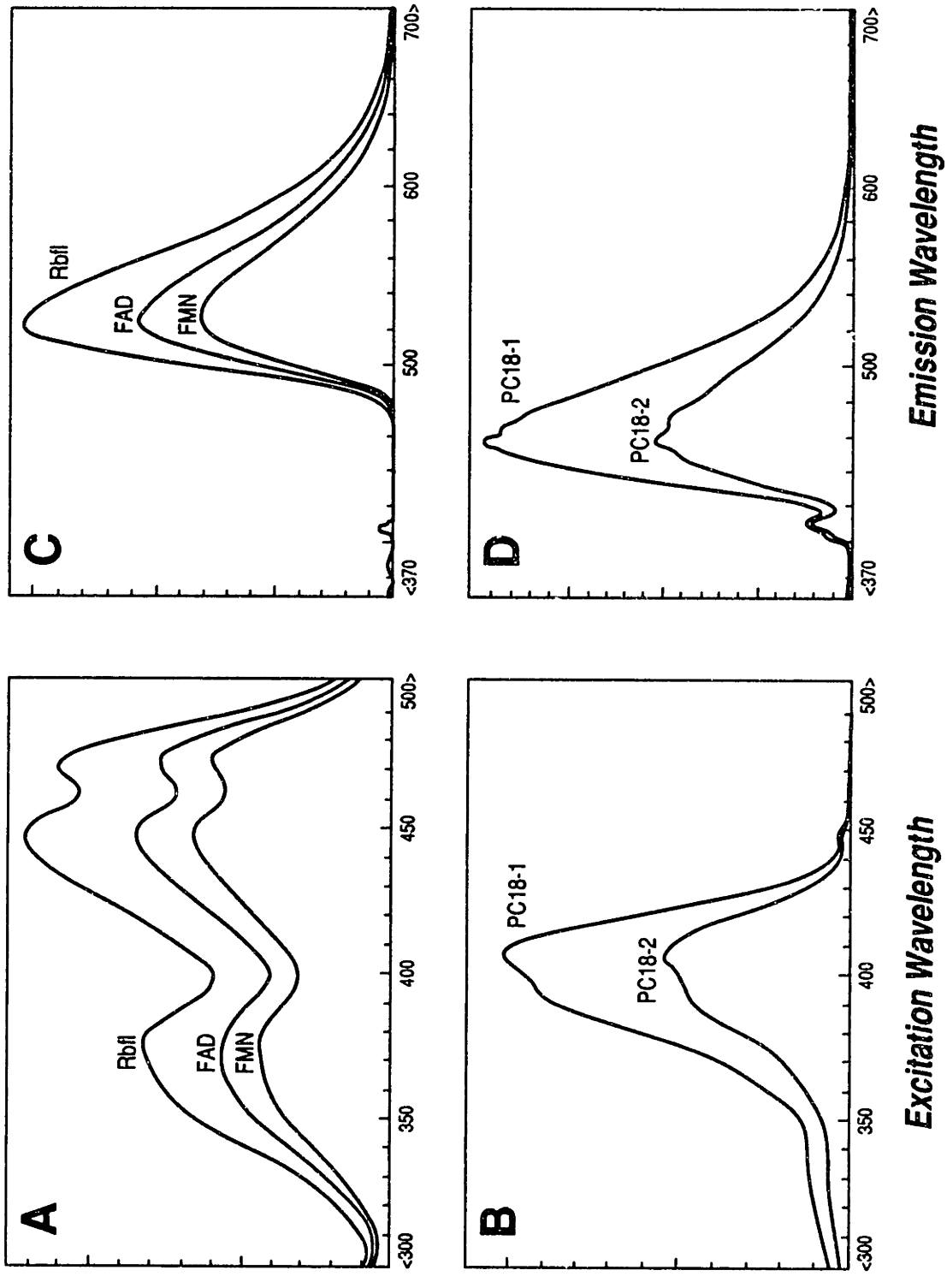
Figure 3.05. Comparison of IP-HPLC separation of the C18 fraction of PFYFP (top) with flavin standards (bottom). Chromatographic conditions: 4.5 x 250 mm x 7 µm Adsorbosphere-HS column; 70:30 PIC A (Waters): methanol at 1.2 ml/min. Detection by fluorescence at >418 nm with excitation at 250 nm.


PC18-1
PC18-2
P. fluorescens
C441
RELATIVE FLUORESCENCE
Rbfl
FMN
FAD
Rbfl
STANDARDS
C440
FAD
FMN
0
10
20
30
40
50
60
TIME (min)

3.05A (rbf1), it is clear that the flavin and pterin content of PFYFP is of the same order of magnitude, while the amount of material in peaks PC18-1 and -2 is an order of magnitude greater. If concentrations are calculated in riboflavin-equivalents, the ratio of flavins : pterins : (PC18-1 + PC18-2) is roughly 1:3:20. The material comprising unknowns PC18-1 and -2 did not appear in the SCX-HPLC chromatogram of crude PFYFP (Fig. 3.04C), nor was it found in the C18 fraction of PFYFP run by SCX-HPLC (not shown). We can state this with some certainty because no peaks of sufficient magnitude relative to riboflavin were observed. This suggests that PC18-1 and -2 was totally retained by the SCX column packing material. In the case of the RP column, it is possible that both of the fluorescent peaks in Fig. 3.03 are due these unknown compounds, which appear to be the two major components of PFYFP.

To determine whether or not components PC18-1 and -2 were in fact pyoverdine, the material was collected from the HPLC eluent and analyzed by fluorescence spectroscopy. A comparison of excitation and emission spectra of PFYFP with those of flavins (Fig. 3.06) clearly demonstrates that these unknown pigment components are not flavins. The single, broad excitation maximum for PFYFP is at 400 nm, whereas the excitation spectra of flavins have triple maxima at 375, 446, and 473 nm. The emission maximum for PFYFP is at 460 nm as compared to 525 nm for flavins. Although different from flavins, these fluorescence properties are similar to the absorbance and fluorescence properties previously reported for pyoverdine form P. fluorescens (Chakrabarty and Roy 1964; Meyer and Adballah 1978; Askeland and Skogerboe 1987).

Figure 3.06. Fluorescence spectra for flavin standards and unknowns PC18-1 and PC18-2 from PFYFP. A: Excitation spectra of flavins with emmission at 530 nm. B: Excitation spectra of PC18-1 and -2 with emission at 530 nm. C. Emission spectra of flavins with excitation at 360 nm. D. Emission spectra of PC18-1 and -2 with excitation at 360 nm. All spectra were run on SLM-Aminco SPF-500C.


A
Rbfl
FAD
FMN
<300
350
400
450
500>
B
PC18-1
PC18-2
C
Rbfl
FAD
FMN
<370
500
600
700>
D
PC18-1
PC18-2
Relative Fluorescence
Excitation Wavelength
Emission Wavelength

Overall, results of the methods development phase show that a combination of selective pre-absorption on Sep-Paks and HPLC resolves PFYFP into the most components. All three methods used detection by fluorescence, however the instrument used in conjunction with HPLC was more sensitive than the human eye used with electrophoresis and 2D-TLC. A total of twelve components were separated using HPLC, including isoxanthopterin, pterin-6-COOH, and riboflavin. Isoxanthopterin, pterin-6-COOH, and riboflavin were also identified as components of PFYFP using electrophoresis and HPLC. The fact that these compounds were not found using 2D-TLC may be due to their low concentrations relative to that of pyoverdine. FAD and FMN were also found in PFYFP using HPLC.

B. COMPARISON OF COMPOUNDS FROM P. FLUORESCENS AND MARINE CLONES

Fluorescence and production of pyoverdine. Crude preparations of the P. fluorescens pigment were yellow and fluoresced blue green at neutral pH. At pH greater than 10, the yellow color was intensified and the fluorescence was green. Addition of acid resulted in loss of color and change to orange fluorescence around pH 1. Neutralization restored original color and color of fluorescence.

Whole, unfiltered samples of the marine cultures exhibited a range of visible color and of color of fluorescence from the greenish-white characteristic of P. fluorescens to yellow (Table 3.04). After removal of the cells, all filtrates fluoresced the same bluish-white color as P. fluorescens, but the intensity of the fluorescence was much less. As with PFYFP, the color of fluorescence

Table 3.04. Observations of visible color, color of fluorescence and intensity of fluorescence for five mixed cultures and four pure clones of marine bacteria. RF = relative fluorescence intensity using chl a filters (Ex 5-60;Em 2-64; lamp [illegible]T4-B. Cultures are listed in order of increasing intensity of visible color.

CLONE	pH	RF	VISIBLE COLOR	COLOR OF FLUORESCENCE
MD2	6.8	7	barely cloudy	faint greenish white
MD10	7.6	5	slightly pink	greenish white
MB34		13	faintly yellow	pink
MC4	7.6	10	yellow	white
D91gA		4	yellow	green
MD1	8	25	yellow	peach
C3A	8	5		pink-violet
D9sm	7.2	1		yellowish white
C21g	7.2	2		orangish white
P. fluorescens			yellow	green

varied with pH, indicating that the fluorescent compounds produced by the marine species also displayed acid/base behavior.

To determine whether or not the pigments produced by the marine cultures could interfere with measurements of chlorophyll, the relative fluorescence intensity of filtrates was measured on a Turner Model 110 fluorometer using the lamp and filters routinely used to measure chlorophyll **a** fluorescence (Ex = CS 5-60, Em = CS 2-64, lamp F4T4-B). In general, intensities were fairly low, however seven out of nine samples showed fluorescence significantly higher than the blank (Table 3.04).

Ten mixed and pure cultures of marine bacteria were tested for production of pyoverdine using the routine bacteriological procedure of observing colonies with a UV lamp after growth on a defined solid medium (Jessen 1965; Palleroni 1984). The P. fluorescens culture turned the media yellow after a few days and by three weeks had turned the media a dark greenish-brown. The fluorescence was so intense that it appeared to be highly quenched. After one week, six out of ten of the marine cultures showed positive results when compared with blank media, although none fluoresced as intensely as did P. fluorescens. The four pure clones which gave positive test results can be ranked in decreasing order of production of UV-fluorescent compounds as follows: C7, C21g, D91gA, D2. Mixed isolate MD10, which gave a positive results and appeared to be a monoculture, was used to obtain pure clone D10.

Rapidly growing cultures of P. fluorescens were found to be autofluorescent when viewed with a epifluorescence microscope using the filter set routinely used for counting DAPI-stained cells (Ex =

365 nm, Em > 397 nm - see Methods). The marine denitrifier Pseudomonas perfectomarinus and the natural isolates from the Cariaco Trench were tested for comparison, but none displayed fluorescence when observed under the same conditions.

HPLC chromatograms. The Florisil fraction of pigments from P. fluorescens and the marine clones have been shown to contain isoxanthopterin, and pterin-6-COOH based on retention times alone. The other unknown components of the Florisil fraction were found exclusively in P. fluorescens or in the marine cultures, with two exceptions. A component having a relative retention time of 0.20 was found in P. fluorescens and in most marine cultures, however it was of minor importance. The major component of PFYFP retained on Florisil, PFlor-4 (Fig. 3.04B), was also found in crude PFYFP (P-4 in Fig. 3.04C), and may have been the same as unknown MFlor-11, which was produced in relatively minor amounts by Clone D91gA and two of the mixed marine cultures. This component had a relative retention time of 0.82, which is similar to that of neopterin reported by Stea et al. (1980). In summary, the marine species had three components in common with PFYFP, nine unique components, and P. fluorescens had three components not found in the marine species.

The C18 fraction from both the marine clones and P. fluorescens contained riboflavin, FAD, FMN, and Unk 1, an unidentified compound which is also found in the FAD standard. However, P. fluorescens also produced PC18-1 and -2 as the major components of PFYFP (Fig. 3.05). These two compounds have the fluorescence properties of pyoverdine, and were not found in any of the marine cultures analyzed by IP-HPLC.

C. GROWTH DEPENDENT CHANGES IN PIGMENT COMPOSITION

One explanation for the apparent species differences in the composition of the Florisil fraction of the pigment is that clones may have been sampled at different points in their growth cycle, and therefore the compounds observed were perhaps pre-cursors or decomposition products of flavins, many of which are also highly fluorescent (Plaut *et al.* 1974). To test this hypothesis, D10, D9 and *P. fluorescens* were grown in batch culture and sampled at intervals for cell density and DFC concentrations. The composition of the Florisil fraction of D10 pigment as a function of growth phase is shown in Fig. 3.07. Concentrations of minor components which elute in the first 5 min. were high during stationary phase and MFlor-12 was the only major component present during both exponential (day 4) and stationary phase (day 16).

Chromatograms for D9 (Fig. 3.08) and *P. fluorescens* (Fig. 3.09) at comparable time points lead to similar conclusions. Growth phase has no apparent effect on the type of compounds released by the cells into the surrounding media. Variations in the composition of fluorescent metabolites in these three species is not a function of growth phase, but rather seems to species-specific. This finding suggests that these are not flavin-precursors, and that one or more of these compounds could be specific enough to use as a biomarker compound.

Figure 3.07. Composition of Florisil fraction of fluorescent pigments from clone D10 as a function of growth stage. Top: at t = 4 days cells were in exponential growth phase. Bottom: at t = 16 days cells were in stationary growth phase. Numbers refer to component identifications and relative retention times listed in Chapter 2, Table 2.02. Chromatographic conditions: 4.5 x 250 mm x 10 µm Partisil SCX column; isocratic; ammonium phosphate (pH = 2.8), 7 % methanol, and 5 % acetonitrile at 1.5 ml/min. Detection by fluorescence at >418 nm with excitation at 250 nm. Inset shows growth curve as measured by absorbance at 600 nm.

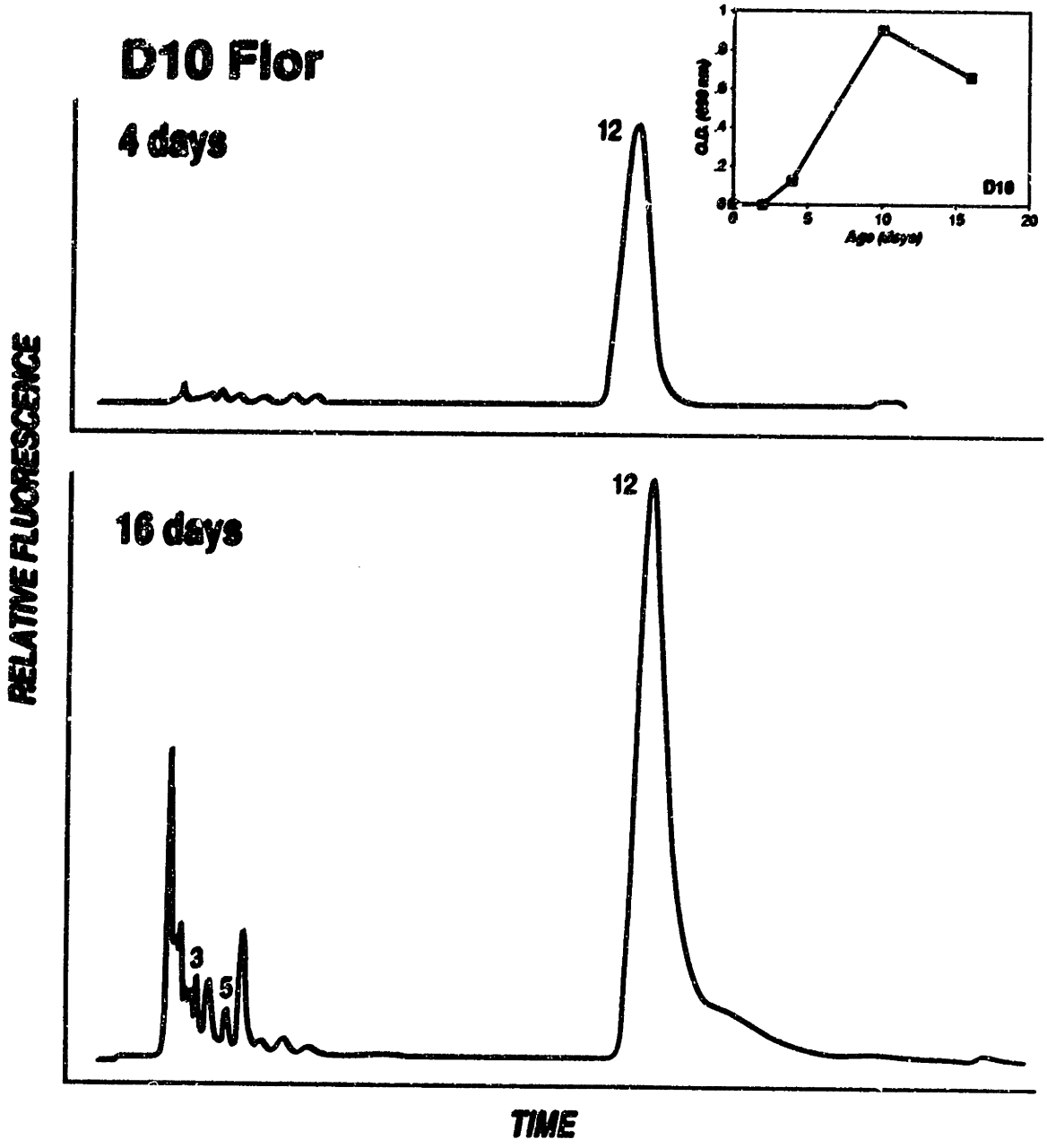

D10 Flor
4 days
12
16 days
12
3
5
RELATIVE FLUORESCENCE
TIME
D10
Age (days)
0
5
10
15
20

Figure 3.08. Composition of Florisil fraction of fluorescent pigments from clone D9 as a function of growth stage. Top: at t = 4 days cells were in exponential growth phase. Bottom: at t = 7 days cells were in stationary growth phase. The 4-day sample was roughly twice as concentrated as the 7-day sample. Numbers refer to component identifications and relative retention times listed in Chapter 2, Table 2.02. Chromatographic conditions: 4.5 x 250 mm x 10 µm Partisil SCX column; isocratic; ammonium phosphate (pH = 2.8), 7 % methanol, and 5 % acetonitrile at 1.5 ml/min. Detection by fluorescence at >418 nm with excitation at 250 nm. Inset shows growth curve as measured by absorbance at 600 nm.

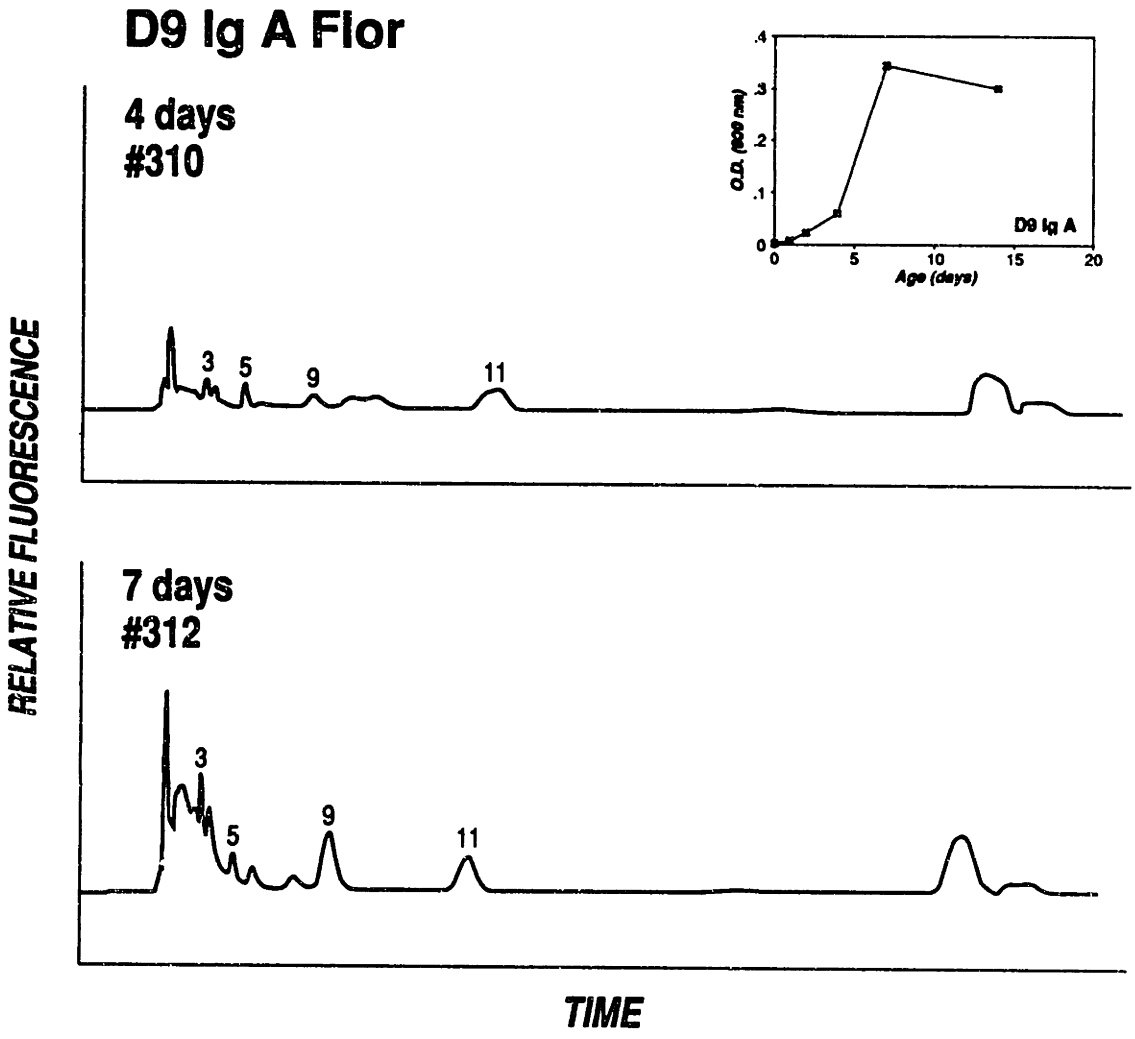
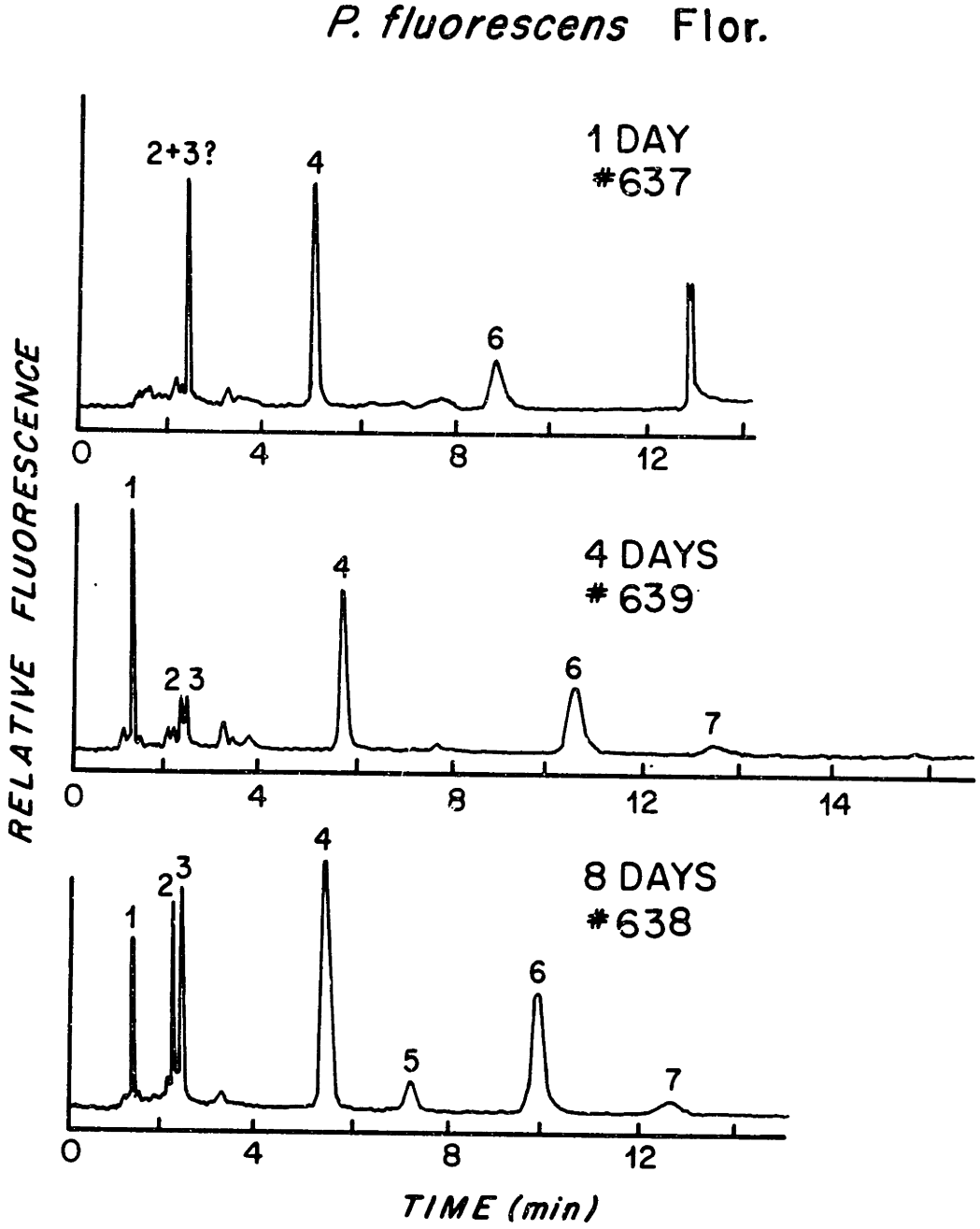

D9 Ig A Flor
4 days
#310
3
5
9
11
7 days
#312
3
5
9
11
RELATIVE FLUORESCENCE
TIME
O.D. (600 nm)
.4
.3
.2
.1
0
0
5
10
15
20
Age (days)
D9 Ig A

Figure 3.09. Composition of Florisil fraction of fluorescent pigments from P. fluorescens as a function of growth stage. Cultures at 1 day (top) and 4 days (middle) were in exponential growth phase. Cultures at 8 days (bottom) were in stationary growth phase. Numbers refer to component identifications and relative retention times listed in Table 3.03. Chromatographic conditions: 4.5 x 250 mm x 10 µm Partisil SCX column; isocratic; ammonium phosphate (pH = 2.8), 7 % methanol, and 5 % acetonitrile at 1.5 ml/min. Detection by fluorescence at >418 nm with excitation at 250 nm.


P. fluorescens Flor.
1 DAY
#637
2+3?
4
6
4 DAYS
#639
1
2 3
4
6
7
8 DAYS
#638
1
2
3
4
5
6
7
0
4
8
12
14
RELATIVE FLUORESCENCE
TIME (min)

DISCUSSION

A. PSEUDOMONAS FLUORESCENS PIGMENT

This work represents the most complete characterization to date of the entire suite of extracellular fluorescent metabolites produced by P. fluorescens. We have shown that HPLC analysis of spent media can be used to distinguish between pyoverdine and the other fluorescent metabolites. By demonstrating the identities and relative amounts of various flavin and pterin components of PFYFP, we can rule these out as principal components of the pigment. Furthermore, the use of HPLC analysis to test for production of fluorescent bacterial pigments eliminates subjective errors inherent in the standard King's B test and permits the pigment composition of various species to be compared directly.

Pyoverdine can be isolated from crude PFYFP on a C18 Sep-Pak. This purification step removes all contaminating fluorescent compounds except minor amounts of flavins. Evidence that unknowns PC18-1 and PC18-2 are pyoverdine is based on comparison of fluorescence excitation/emission spectra with those previously reported for pyoverdine (Meyer and Abdallah 1978). The fluorescence excitation spectrum is comparable to the absorbance spectrum, so this is actually two separate pieces of confirmatory data. Excitation and emission spectra of both unknown components were identical, therefore they must have chromophores (fluorophores) which are very similar, if not identical.

The material we isolate in the Florisil fraction is what some previous investigators describe as pyoverdine or as "degradation

products". The Florisil fraction represents roughly 10% of the total fluorescent material isolated by Sep-Paks which is consistent with the results of Meyer and Abdallah (1978) for the fraction they discarded. Since a final pH of 8.0 was observed in scenescent cultures of P. fluorescens, it is possible that some of the compounds reported here were degradation products which Meyer and Abdallah (1978) claim can form at pH values above 7.3.

Undoubtedly, some of the fluorescent compounds are produced by P. fluorescens independently of production of pyoverdine, and may have been mistaken for pyoverdine in early reports. For example, the first report that riboflavin was a component of bacterial "florescein", was in 1948 (Birkhofer and Birkhofer 1948). Later researchers refuted this result based on fluorescent spectral analysis of culture filtrates (Elliot 1958). They suggested that the Birkhofers used a different species of Pseudomonas which was a riboflavin producer. They comment that "separation of pyoverdine from riboflavin is not easy." Our results demonstrate that neither set of conclusions was entirely correct. Riboflavin is produced by P. fluorescens but it is distinct from pyoverdine. Elliot (1958) could not detect the presence of riboflavin because its concentration in PFYFP is low in comparison to pyoverdine. Birkhofer and Birkhofer (1948) may have isolated riboflavin instead of pyoverdine. Separation of riboflavin and pyoverdine is relatively easy using the method described in this study.

FAD, FMN, dihydropterin, (L-threo)neopterin, and 6-hydroxymethyl-pterin are produced by Pseudomonas roseus fluorescens (Visconti et al. 1964; Visconti and Frater-Schroeder 1968).

D-erythropterin and putidolumazine have been isolated from cultures of Pseudomonas putida (Suzuki and Goto 1971, 1972), and other pterins and ribityllumazines are also produced by fluorescent pseudomonads (Leisinger and Margraff 1979). Our results suggest that, as for riboflavin, these compounds are produced in small amounts as metabolites and may therefore be isolated from the spent media of fluorescent pseudomonads. They are not the same as the primary pigment component, pyoverdine.

B. OTHER WATER-SOLUBLE YELLOW AND/OR FLUORESCENT PIGMENTS

Perhaps the most important finding of this part of the research is demonstration of the large number and relative concentration of fluorescent pigment components to be found in species which test positive for fluorescent pigment production. Although a positive test result might lead to the interpretation that a given species was related to P. fluorescens, the components of bacterial fluorescence can be analyzed using HPLC and compared directly with pyoverdine, thereby eliminating errors due to interpretation of the King's B test. This points out the usefulness of HPLC analysis in the classification of bacteria based on pigment production.

None of the marine species produced nearly as much pigment as P. fluorescens. However, all species did release diffusible yellow compounds which fluoresced blue-green when grown on King's B agar. The color of the fluorescence of the marine clones appears to arise from the mixture of yellow- and blue-fluorescing components, which are retained on C18 and Florisil Sep-Paks, respectively. The yellow-fluorescing components are tentatively identified as flavins,

and the blue-fluorescing components are tentatively identified as pterins and some unidentified compounds. The color of fluorescence of the P. fluorescens pigment arises from that of pyoverdine itself, since flavins and pterin-like compounds are minor components.

The fluorescent pigments produced by our marine cultures may be more similar to the fluorescent pigment described by Morris and Roberts (1959) for Pseudomonas cepacia (formerly Pseudomonas multivorans). It is a water-soluble, diffusible, yellow pigment produced in asparagine media which displays "...a violet fluorescence in ultra-violet light easily distinguishable from the bluish-green fluorescence of the normal fluorescent pseudomonads..." (Morris and Roberts, 1959). Since the organism does not give a positive test on King's B media, the pigment it produces is classified as "non-fluorescent" for taxonomic purposes. Other closely-related species of the same genus which are thought to produce the same yellow pigment are P. marginata, now known as P. gladioli, and P. caryophylli (Buchanan and Gibbons 1974). All known isolates are from diseased plants or soil. The nature of the pigment is unknown. Pseudomonas caryophylli is capable of denitrification, while P. cepacia is able to reduce nitrate to nitrite. Pseudomonas caryophylli and P. gladioli colonies turn brown with age. It would be necessary to obtain cultures of these species for HPLC pigment analysis to determine any similarities to pigment from the marine clones. It is interesting to note that some of our marine isolates were also found to turn brown and discolor the agar with age.

Yellow pigments are also produced by non-pseudomonad bacteria. Some members of the family Azotobacteraceae, which are nitrogen

fixers, make water-soluble pigments, which fluoresce green or white (Tchan 1984). The Nitrobacteraceae (a family of nitrogen-oxidizers) are rich in cytochromes which reportedly turn cell suspensions yellow. Some members of the family Vibrionaceae and of the genus Flavobacterium are yellow in color, but do not produce water-soluble, diffusible pigments. One other species which produces a water-soluble yellow pigment is Beijerinckia derxii. Colonies of Xanthomonas are yellow due to the presence of a carotenoid pigment (Starr and Stephens 1964). The nature of these pigments is unknown, but analysis by HPLC should readily determine whether or not they are similar to pigments produced by other bacteria.

Marine bacteria have also been reported to produce yellow and fluorescent pigments (ZoBell and Feltham 1934; ZoBell and Upham 1944). However, since this work pre-dated that of King et al. (1954), their methods for determining production of fluorescent compounds cannot be duplicated. Furthermore, since they were unable to preserve their fluorescent species, no comparisons can be made with pyoverdine from the green fluorescent pseudomonads (Jessen 1965). In a review of taxonomy of marine aerobic bacteria found in nearshore waters of Hawaii, Baumann et al. (1972) did use the King's B test for pyoverdine production and found that none of the species they tested produced that pigment or any other yellow, cell associated pigment. However, since these species were from a limited geographical area, they are not necessarily representative of the oceans. The results of our study corroborate the reports of fluorescent marine bacteria made by Zobell and co-workers and represent the first recent documentation of such species.

One of the objectives of this study was to evaluate bacterial pigments as potential biomarkers of low-oxygen environments. It was thought that if marine species contained the same compound as P. fluorescens it would be a useful biomarker. The marine species tested so far do not contain the same pigment, and do not make nearly as much fluorescent material as does P. fluorescens. However, one of the pigment components isolated in this study would be worth pursuing as a biomarker. This is the compound MFlor-12 found in D10 (see Chapter 2). It appears to be unique to this clone and is produced in significant amounts in proportion to all other components. Further study of its structure may show it to be a compound which is already known, but if it is produced by a limited type of organism in the ocean it may still be useful.

CONCLUSIONS

1. An improved method for the isolation of pyoverdine from other water-soluble fluorescent compounds produced by P. fluorescens is reported. Analysis of all fluorescent components, including pyoverdine, using HPLC clearly shows that flavin and pteridine components are not the same as pyoverdine and are relatively minor constituents of PFYFP.

2. This study confirms the presence of riboflavin, FAD, and FMN in P. fluorescens, and adds isoxanthopterin, pterin-6-COOH, and xanthopterin to the list of components. 6-hydroxymethyl-pterin was not present. Four other components were found but not identified. Pyoverdine concentration in the spent media, as estimated by

fluorescence intensity, was roughly an order of magnitude higher than was total flavin or "pterin" concentration.

3. Several species of marine bacteria which give a positive test result for production of fluorescent pigments do not produce pyoverdine when analyzed using HPLC. The method reported here represents a significant improvement over the current King's B test for pyoverdine production.

4. These results corroborate early reports by Zobell and Feltham (1934) of marine bacteria which produce yellow diffusible fluorescent pigments. Pigments from isolates are different from the P. fluorescens group, but may be similar to the "non-fluorescent" pigments of other pseudomonads.

5. Variations in the composition of the Florisil pigment fraction is shown to be species-specific rather than a function of growth phase. Hence, the major components of the fluorescence are not simply precursors or degradation products of riboflavin synthesis.

REFERENCES

Askeland, R.A., and R.K. Skogerboe. 1987. Selective determination of iron by fluorescence quenching of a naturally occurring pigment. Anal. Chim. Acta 192: 133-43.

Baumann, L., P. Baumann, M. Mandel, and R.D. Allen. 1972. Taxonomy of aerobic marine bacteria. J. Bacteriol. 110: 402-429.

Birkhofer, L. and A. Birkhofer. 1948. Riboflavin, a component of "bacterial fluorescein". Zeits. Naturforschung 3b: 136.

Buchanan, R.E., and N.E. Gibbons. 1974. Bergey's manual of determinative bacteriology. 8th edition. The Williams and Wilkins Co. Baltimore.

Chakrabarty, A.M. and S.C. Roy. 1964. Characterization of a pigment from a Pseudomonad. Biochem. J. 93: 144-148.

Descimon, H., and M. Barail. 1966. Application de la chromatographie sur couche mince de cellulose a la separation des pterines naturelles. J. Chromatog. 25: 391-397.

Elliott, R.P. 1958. Some properties of pyoverdine, the water-soluble fluorescent pigment of the pseudomonads. Appl. Microbiol. 6: 241-246.

Giral, F. 1936. Sobre los liocromos characteristicos del grupo de bacterias fluorescentes. Anales de la sociedad espanola de fisica y quimica 34: 667-693.

Greppin, H., and S. Gouda. 1965. Action de la lumiere sur le pigment de Pseudomonas fluorescens Migula. Archives des sciences, Geneve 18: 721-25.

Jessen, O. 1965. Pseudomonas aeruginosa and other green fluorescing pseudomonads. A taxonomic study. Copenhagen: Munksgaard.

King, E.O., M.K. Ward, and D.E. Raney. 1954. Two single media for the demonstration of pyocyanin and fluorescein. J. Labor. Clin. Med. 44: 301-307.

Leisinger, T., and R. Margraf. 1979. Secondary metabolites of the fluorescent pseudomonads. Microbiol. Rev. 43: 422-442.

Lenhoff, H. 1963. An inverse relationship of the effects of oxygen and iron on the production of fluorescin and cytochrome c by Pseudomonas fluorescens. Nature 199: 601-2.

Meyer, J.M. 1977. These d'Etat. Univeristy Louis Pasteur, Strasbourge, France.

Meyer, J. M., and M. A. Abdallah. 1978. The fluorescent pigment of Pseudomonas fluorescens: Biosynthesis, purification and physicochemical properties. J. Gen. Microbiol. 107: 319-28.

Morris, M.B., and J.B. Roberts. 1959. A group of pseudomonads able to synthesize poly-B-hydroxy-butyric acid. Nature (London) 183: 1538-9.

Palleroni, N.J. 1984. Genus I. Pseudomonas, pp. 141-199. In: Krieg, N.R., and J.G. Holt, eds. Bergeys's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore.

Plaut, G.W., C.M. Smith, and W.L. Alworth. 1974. Biosynthesis of water-soluble vitamins. Ann. Rev. Biochem. 43: 899-922.

Starr, M., and W.L. Stephens. 1964. Pigmentation and taxonomy of the genus Xanthomonas. J. Bacteriol. 87: 293-302.

Stea, B., R.M. Halpern, B.C. Halpern, and R.A. Smith. 1980. Quantitative determination of pterins in biological fluids by high-performance luquid chromatography. J. Chromatogr. 188: 363-375.

Suzuki, A., and M. Goto. 1971. Isolation and characterization of pteridines from Pseudomonas ovalis. Bull. Chem. Soc. Japan 44: 1869-1872.

Suzuki, A., and M. Goto. 1972. The structure of a new pteridine compound produced by Pseudomonas ovalis. Bull. Chem. Soc. Japan 45: 2198-2199.

Tchan, Y.-T. 1984. Family II. Azotobacteraceae. pp. 219-234, In: Krieg, N.R., and J.G. Holt, eds. Bergeys's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore.

Visconti, M., M. Pouteau-Thouvenot, R. Buhler-Moor, and M. Shroeder. 1964. Substances naturelles isolees de microorganisms: Nouvelle pteridines et nouvelles porphyrines obtenues a partir de Pseudomonas roseus fluorescens J. C. Marchal 1937. Helv. Chim. Acta 47: 1948-50.

Visconti, M., and M. Frater-Schroeder. 1968. Isolierung von 6-hydroxymethyl-pterin aus kulturen von Pseudomonas roseus fluorescens J.C. Marchal 1937. Helv. Chim. Acta 51: 1554-57.

Wilson, T.G., and K.B. Jacobson. 1977. Isolation and characterization of pteridines from heads of Drosophila melanogaster by a modified thin-layer chromatography procedure. Biochem. Genetics 15: 307-319.

ZoBell, C. E. and C. B. Feltham. 1934. Preliminary studies on the distribution and characteristics of marine bacteria. Bull. Scripps Inst. Oceanogr., Tech. Rept. 3; 279-296.

ZoBell, C. E. and H. C. Upham. 1944. A list of marine bacteria including descriptions of sixty new species. Bull. Scripps Inst. Oceanogr. 5: 239-292.

CHAPTER 4

DISSOLVED FLUORESCENCE IN THE BLACK SEA

DISSOLVED FLUORESCENCE IN THE BLACK SEA

by

Paula Garfield Coble[1]α, Robert B. Gagosian[1], L.A. Codispoti[2],
Gernot E. Friederich[2], and John P. Christensen[3]

Manuscript submitted to Deep Sea Research Special Volume on the Black Sea Expedition of 1988

June 1989

[1]Chemistry Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543

[2]Monterey Bay Aquarium Research Institute, 160 Central Ave., Pacific Grove, CA 93950-0020

[3]Bigelow Laboratory for Ocean Sciences, McKown Point, West Boothbay Harbor, ME 04575

αFormerly Paula C. Garfield

ABSTRACT

Subsurface in situ fluorescence maxima have previously been found to occur in low oxygen waters below the bottom of the mixed layer at several locations in the Pacific Ocean. These fluorescence maxima coincide with maxima in nitrite, suspended particles, bacterial biomass and microbial activity, but are not associated with a pycnocline. While some of the fluorescence signal may be due to chlorophyll or its degradation products, it has also been suggested that fluorescent substances may be produced by the resident microbial populations. Our preliminary experiments with cultures of marine bacteria isolated from the Cariaco Trench in March 1986 verify that they are capable of producing fluorescent pigments. These pigments could be part of the observed fluorescence signal in low oxygen waters and potentially could serve as "real time" indicators of bacterial aggregations in the water column.

We present here results from the Black Sea where we made continuous profiles of fluorescence in three channels (chlorophyll, dissolved organic matter (DOM), and flavin) simultaneously, using a pump profiling system. Other parameters measured on each pump cast included temperature, salinity, beam attenuation coefficient (c), nutrients, oxygen and hydrogen sulfide. Dissolved organic matter (DOM) and flavin fluorescence increased with depth, while chlorophyll fluorescence showed two distinct maxima, one at the bottom of the euphotic zone, and one at the depth of the sulfide interface. This deeper maximum was associated with a particle maximum and a maximum in microbial activity (ETS), and is attributed to the presence of photosynthetic bacteria of the genus Chlorobium.

INTRODUCTION

Subsurface maxima in in situ fluorescence have previously been found to occur in low oxygen waters below the bottom of the mixed layer at several locations in the Pacific Ocean (ANDERSON 1982; BROENKOW et al. 1983; LEWITUS and BROENKOW 1985). These fluorescence maxima coincide with maxima in nitrite (for example, BRANDHORST 1959; WOOSTER et al. 1965), suspended particles (PAK et al. 1980; KULLENBERG 1981, 1984; GARFIELD et al. 1983; BROENKOW et al. 1983), microbial activity (GARFIELD and PACKARD 1979; GARFIELD et al. 1983), bacterial biomass (SPINRAD et al. 1989), particulate protein (GARFIELD et al. 1979), and phaeopigment concentration (BLASCO et al. 1979), but are not associated with a pycnocline (BARBER and HUYER 1979; GARFIELD et al. 1983).

Significant correlation between the total attenuation coefficient and in situ fluorescence suggests that the particles are a major source of the observed fluorescence (BROENKOW et al. 1983; LEWITUS and BROENKOW 1985; SPINRAD et al. 1989), however dissolved fluorescent substances may also be important. Fluorescence measurements have been used for many years to measure dissolved organic matter (DOM) concentrations in seawater (KALLE 1949; DUURSMA 1974). Dissolved fluorescence shows an inverse correlation with salinity in estuaries and has been attributed to runoff of terrestrial humic material (DUURSMA 1974, LAANE 1981; BERGER et al. 1984; HAYASE et al. 1987). Vertical distributions in the open ocean generally show a monotonic increase in dissolved fluorescence with depth (IVANOV 1962; DUURSMA 1974; HAYASE et al. 1987, 1988; CHEN and BADA 1989), however increased DOM fluorescence at the surface has also been found in association with areas of high productivity (KARABASHEV 1977). The similarity between the excitation maximum (350 nm) and

emission maximum (460 nm) for dissolved fluorescence in the ocean and those for humics and fulvics has led to the assumption that the latter are the principal components of the fluorescence signal (LAANE and KOOLE 1982 and references therein).

Measurements of in situ fluorescence give total fluorescence due to particles and dissolved materials. To a first approximation, separation of the signal due to plant pigments from that of DOM can be achieved by selection of excitation and emission wavelengths. However, there are a number of potential interferences. Previous studies of in situ fluorescence at sea have been conducted primarily using fluorometers with broad wavelength bands for both excitation (336-572nm) and emission (645-750nm), which allow compounds other than chlorophyll a to be detected, provided they are present in sufficient concentrations. Other compounds which may contribute to the fluorescence signal include chlorophyll degradation products, phycobiliproteins, bacteriochlorophylls, cytochromes, and flavoproteins. Some of these may be present in both the particulate and the dissolved phases.

The location of subsurface fluorescence maxima within the oxygen minimum zone (oxygen < 0.5 ml/l) suggests that they may be produced by the indigenous microbial community. Our recent work has shown that marine bacteria isolated from the low oxygen waters of the Cariaco Trench are capable of producing a variety of water-soluble fluorescent compounds, including several flavin compounds, which can freely diffuse out of the cells and into the surrounding seawater (COBLE 1989). The fluorescence characteristics of this material are more similar to those of DOM and flavins than to those of chlorophyll, however under certain circumstances this material can be present in sufficient quantities to interfere with in vivo chlorophyll fluorescence measurements (VASTANO 1988). More importantly, it should definitely be concentrated enough

to measure using a different fluorescence channel and could provide a valuable tool for locating areas of dense microbial populations.

On a recent cruise to the Black Sea, we had an opportunity to assess the contribution of DOM and flavin fluorescence to the in situ chlorophyll fluorescence signal, and to determine whether any of these three signals could be used to locate layers of high microbial activity below the euphotic zone. Samples for analysis of individual components of the "dissolved fluorescence" were also collected and those results will be published elsewhere.

METHODS

All samples were taken during Leg 5 of the 1988 Black Sea Expedition (R/V KNORR cruise 134-12) at a station located at approximately 43° 05.0' N and 34° 00.0' E. Continuous profiles of temperature, salinity, nutrients, sulfide, fluorescence and beam attenuation coefficient were obtained using a pumping system which was a modified version of the system described by FRIEDERICH and CODISPOTI (1987). A SeaBird CTD and Sea-Tech Inc. 25 cm beam transmissometer were attached to the same frame which held the pump. The frame was lowered by hydraulic winch at a rate of 6-10 m/min to a maximum depth of 380 m. A Hewlett Packard 85 computer collected data and controlled the Alpkem Rapid Flow Analysis system used to analyze nutrients via the methods of WHITLEDGE et al. (1981) with some slight modifications. The method for hydrogen sulfide was a slightly modified version of that printed in the Alpkem Operator's Manual, which is basically similar to that of CLINE (1969).

Profiles of in situ fluorescence were obtained by pumping water from depth through three on-board continuous flow fluorometers: a Turner 110 optimized for DOM fluorescence (Corning F4T4-B1 lamp, CS 7-60 excitation filter [T > 20% @ 320-390 nm] and Wratten 2A and 65A emission filters [T > 20% @ 475-530 nm]); a Turner 112 optimized for chlorophyll (Corning F4T4-B1 lamp, Wratten 47B excitation filter [T > 20% @ 420-470 nm] and CS 2-64 emission filter [T > 20% @ > 660 nm]) or for flavins (F4T4-B1 lamp, 47B excitation filter and an interference-type emission filter with peak at 527 +/- 8nm) and a Hitachi F1000 with xenon light source and dual monochrometers for chlorophyll (435/685 = Ex/Em) or flavins (450/525 = Ex/Em). The Hitachi had lower sensitivity to chlorophyll fluorescence than the Turner 112. Before

each cast, the baseline for surface water was set to zero for the DOM and flavin fluorometers and to 10% full scale for the chlorophyll fluorometer.

Flow rates of 3 - 5 liters per minute resulted in delay times of around 4 minutes for passage of water through the entire system from the pump head to the fluorometers. Delay times for pump cast data were collected for each cast by holding the pump at one depth until a constant signal was obtained, rapidly moving the pump to another depth, and determining the time required for a constant value to be re-established. This can be done directly for the CTD readouts and fluorometers, however for nutrients the analysis lag time must be added to the lag time due to flow rate. The values for lag time from the various channels agreed to within one minute. All tubing was protected from light to prevent photodegradation of organic compounds.

Attempts to calibrate the continuous-flow fluorometers directly with external standards were unsuccessful due to the large volume of the system. The broad band emission filter on the DOM channel did overlap slightly with the flavin channel. To determine the amount of overlap we measured fluorescence of quinine sulfate and riboflavin standards in both channels and found that quinine sulfate fluorescence in the flavin channel was reduced to 3% of its intensity in the DOM channel, while riboflavin fluorescence in the DOM channel was reduced to less than 15% of its intensity in the flavin channel.

Samples for dissolved oxygen and extracted chlorophyll were collected from the pump effluent at discrete depth intervals. Oxygen concentrations were measured by a modification of the Winkler method at high concentrations (CARPENTER 1965) or by the colorimetric "micro-oxygen" method of BROENKOW and CLINE (1969) at low concentrations. Chlorophyll samples were filtered through Whatman GF/F filters and returned to the lab frozen. Filters were thawed,

homogenized with a teflon/glass tissue grinder in 90% acetone, and allowed to extract for two hours in the dark at 4°C. Homogenates were clarified by centrifugation and the fluorescence of the supernatant was measured before and after acidification using a Turner Model 112 fluorometer configured for chlorophyll as described above. Concentrations of chlorophyll a (chl a) and phaeopigments (phaeo a) were calculated according to STRICKLAND AND PARSONS (1972). The fluorometer was calibrated with chl a from spinach.

Bacteriochlorophyll e (bchl e) and bacteriophaeopigment e (bphaeo e) concentrations were determined using an adaptation of the fluorometric chl a method. The fluorometer was configured as for chlorophyll a, but calibrated using pure bchl e extracted from material collected from the anoxic water column of Salt Pond in Woods Hole, MA. Crude pigment was extracted in acetone and purified using reverse phase thin layer chromatography (RPTLC) with 100% methanol as the solvent. Bchl e concentration of the standard was determined by absorption using a molar extinction coefficient of 10^5 at 466nm (REPETA, personal communication). A molecular weight of 822 corresponding to that of the 4-ethylfarnesyl isomer was used to convert concentrations to µg/l. This isomer was found to be the predominant one in Black Sea samples taken in May 1988 (REPETA et al. 1989). The ratio of fluorescence before and after acidification of pure bchl e was 0.583 +/- 0.057. This was used to modify the equations used for concentration calculations (STRICKLAND and PARSONS 1972) to:

$$\mu g \text{ bchl e/l} = F_D(-1.40)(R_B - R_A)v/V$$

$$\mu g \text{ bphaeopigments e/l} = F_D(-1.40)(0.583 \times R_A - R_B)v/V$$

where F_D is the bchl e calibration factor from standardization (µg bchl e /l/fluorescence unit), R_B and R_A are the sample fluorescence before and after

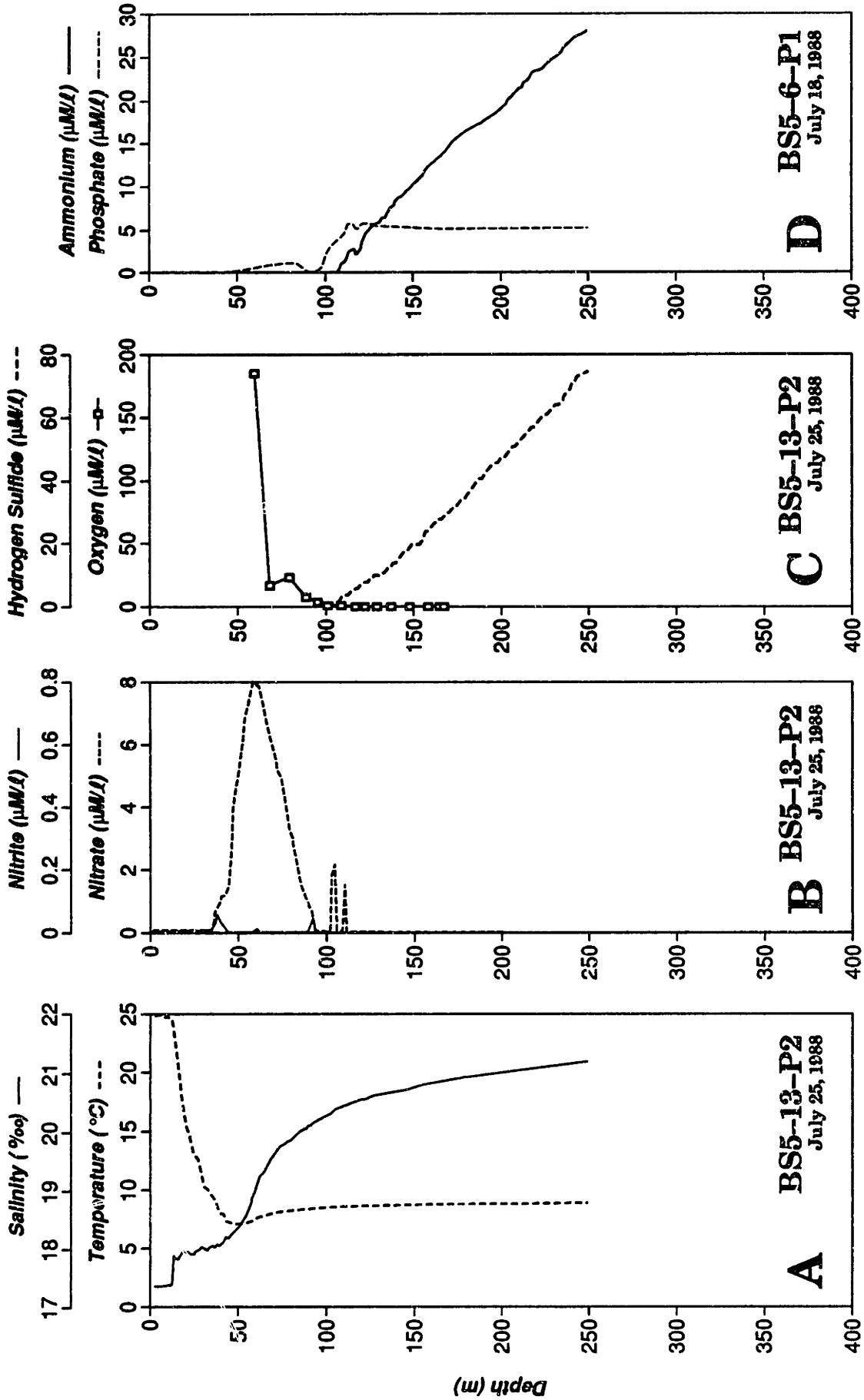
acidification, v is the extract volume in ml, and V is the sample volume in liters.

Electron transport system (ETS) activities were assayed using the method of KENNER and AHMED (1975), PACKARD (1985) and PACKARD et al. (1988) on samples of 2 - 4 liters collected using 30-liter Niskin or Go-Flo bottles on a rosette. A Seabird SBE-9/11 CTD and a Sea-Tech Inc. 25cm beam transmissometer mounted on the rosette provided hydrographic and light transmission data for these casts (WHITE et al. 1989). Water was filtered through 47 mm Whatman GF/F glass fiber filters at room temperature with a vacuum of less than 7 psi. Filters were rinsed with 50 ml of 0.2 um-filtered surface seawater to remove soluble sulfides. In addition to the substrate and turbidity blanks normally run, a chemical blank to correct for reduction of INT by particulate and soluble sulfides was also run (Torreton, personal communication). Samples were incubated at 15°C and activities were converted to in situ rates using the Arrhenius equation and an activation energy of 15 kcal/mole/day (PACKARD 1988). No attempt was made to extrapolate in situ respiration rates from ETS activities. Rather, the ETS profiles were used as a relative indicator of the distribution of planktonic micro-organisms.

RESULTS

Typical depth profiles of temperature and salinity are shown in Fig. 4.01A. A shallow mixed layer was present from surface to a depth of 10 m. A thermocline at 20 - 30 m and a halocline at 60 - 70 m separate the euphotic zone from the deep waters. Below 100 m, there is little temperature or salinity variability.

Fig. 4.01. A. Depth profiles of temperature and salinity for pump station 13-P2. B. Depth profiles of nitrate (____) and nitrite (---) for pump station 13-P2. All data are from continuous nutrient analyzer. C. Depth profiles of hydrogen sulfide (---) and oxygen (___) concentrations for pump station 13-P2. Sulfide data are from continuous nutrient analyzer. Oxygen concentrations were determined by micro Winkler titration on discrete samples collected from the pump effluent. D. Depth profiles of phosphate (---) and ammonium (___) for pump station 6-P1, taken one week prior to 13-P2 at the same location. All data are from continuous nutrient analyzer.

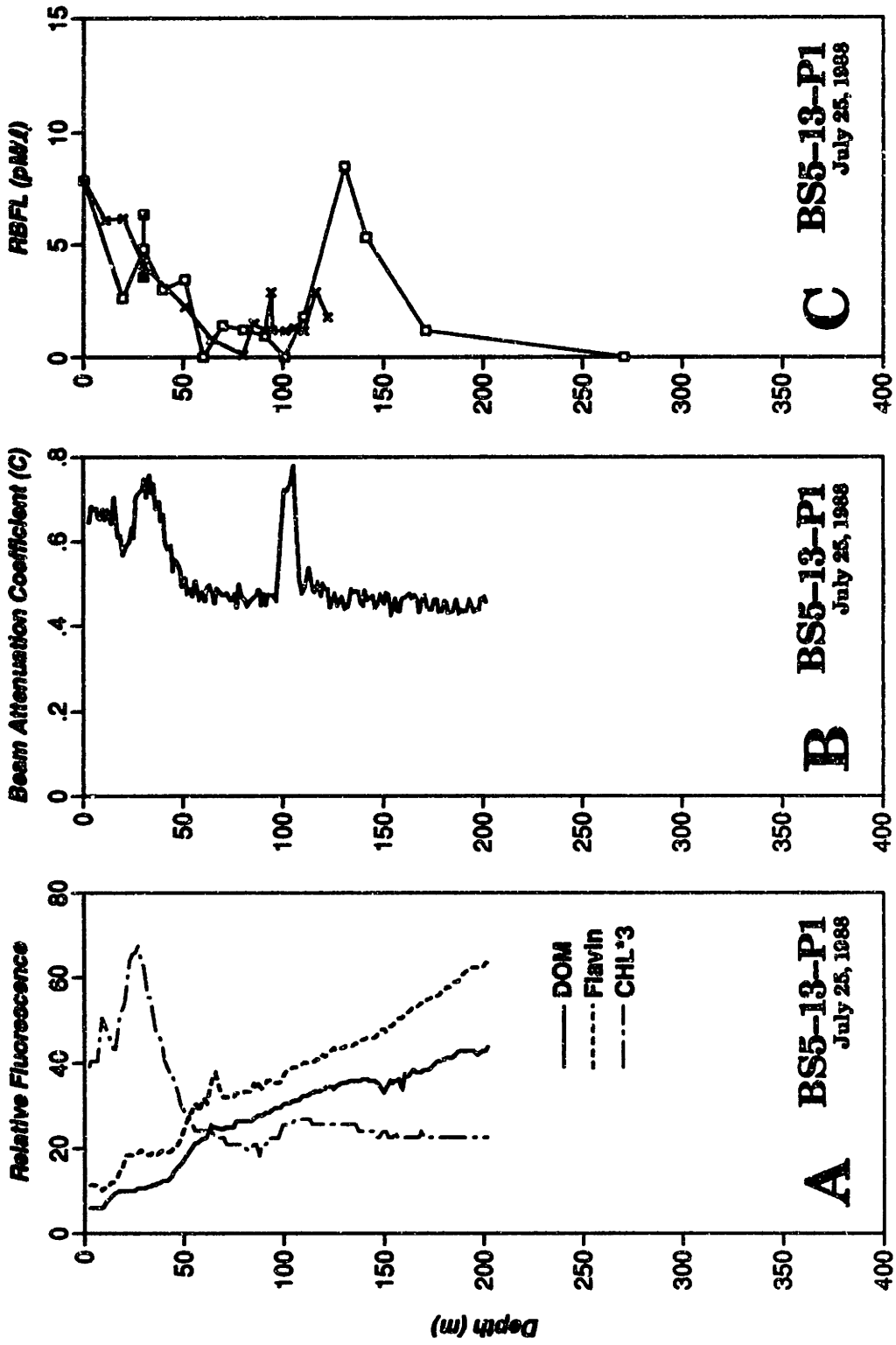

Depth (m)
A
BS5-13-P2
July 25, 1988
Salinity (‰) —
Temperature (°C) ---
B
BS5-13-P2
July 25, 1988
Nitrite (µM/l) —
Nitrate (µM/l) ----
C
BS5-13-P2
July 25, 1988
Hydrogen Sulfide (µM/l) ---
Oxygen (µM/l) -o-
D
BS5-6-P1
July 18, 1988
Ammonium (µM/l) ——
Phosphate (µM/l) ----

Nitrate concentrations were depleted in the surface mixed layer, but increased to maximum values of 8 μM in the region of the halocline (Fig. 4.01B). This was also the depth of the oxycline, where dissolved oxygen concentrations decreased rapidly. Below the oxycline, nitrate concentrations also decreased rapidly to zero. A primary nitrite maximum was observed at the top of the nitrate maximum and a secondary nitrite maximum was present at the bottom of the nitrate maximum, however nitrite concentrations were generally less than 0.3 μM. The disappearance of oxygen and appearance of sulfide in the water column were separated by a distance of 20 m.

Vertical distributions of nutrients and dissolved oxygen showed some temporal variability. The nitrate maximum and the sulfide interface were located about 20 m deeper in the water column at station 6-P1 (taken one week prior to station 13-P2 at the same location) and the maximum nitrite concentrations in the secondary nitrite maximum were three times higher.

Continuous in situ fluorescence profiles for chlorophyll, flavin, and DOM are shown along with beam attenuation coefficient (c) for two pump casts in Fig. 4.02A. The vertical distributions of DOM and flavin fluorescence are similar and show a gradual increase with depth. Two "steps" of increase in fluorescence were seen at the base of the surface mixed layer (10 m) and between the thermocline and the halocline (50 m). Flavin fluorescence showed some small peaks right at the depth of the halocline. This is also the depth of rapid decreases in nitrate and oxygen concentrations (Fig. 4.01C). Although these perturbations in the flavin fluorescence profile are small, they were observed repeatedly at this same depth.

Chlorophyll fluorescence was highest in the euphotic zone, with a primary maximum between the base of the thermocline and the top of the halocline. A small secondary maximum was observed at 100 - 110 m. Maxima in c corresponded


Relative Fluorescence
Depth (m)
DOM
Flavin
CHL*3
A
BS5-13-P1
July 25, 1988
Beam Attenuation Coefficient (C)
B
BS5-13-P1
July 25, 1988
RBFL (pM/l)
C
BS5-13-P1
July 25, 1988

to the depths of the primary and secondary chlorophyll maxima. Values for c were comparable in the two maxima, even though chlorophyll fluorescence was three times higher in the euphotic zone.

The secondary chlorophyll fluorescence maximum was more pronounced at station 6-P1 (Fig. 4.03A), taken one week earlier at the same location as 13-P1 and 13-P2. The subsurface particle maximum and secondary chlorophyll maximum were located just above the sulfide interface, which was about 10 m deeper than during cast 13-P2. The secondary particle maximum was not only deeper at station 6-P1, but also broader and somewhat more intense. This may have contributed to a more intense maximum in chlorophyll fluorescence. The greater sensitivity of the Turner 112 fluorometer (with associated greater noise) may also have contributed to the intensified signal. Extracted chl a concentrations uncorrected for phaeo a were well-correlated with in vivo chlorophyll fluorescence, and also showed a small maximum at the depth of the particle maximum.

Problems were encountered using the standard fluorometric technique for extracted chlorophyll samples taken between 100 and 150 m. Fluorescence after addition of acid increased about two-fold. Using the fluorometric chl a equations (STRICKLAND and PARSONS 1972), one would erroneously conclude that phaeo a concentrations were high in this secondary chlorophyll maximum zone. However, if this were the case, the fluorescence after acidification should decrease or remain unchanged. The fact that an increase was observed suggested the presence of some other type of pigment.

The nature of the chlorophyll in the secondary chlorophyll maximum was indeed distinctly different from that found in the primary chlorophyll maximum. Figure 4.04 shows a comparison of absorbance spectra for extracted samples taken from 40 m and 110 m at station 6-P. Chlorophyll a is the major

Fig. 4.03. A. Depth profiles of extracted in vitro chlorophyll (___), in situ chlorophyll fluorescence (---), and beam attenuation coefficient c (_._) for station 6-P1. Chlorophyll fluorescence was measured using a Turner 112 fluorometer and flavin fluorescence was measured using a Hitachi F1000 fluorometer. In vitro chlorophyll values are uncorrected for phaeopigments and are calibrated for chlorophyll a. B. Extracted pigment concentrations for chlorophyll a uncorrected for phaeo a (^), chl a corrected for phaeo a (#), and phaeo a (x) for pump station 6-P1. Determinations were made on discrete samples collected from pump effluent. Concentrations in the deep maximum are uncorrected for the presence of bchl e. C. Extracted pigment concentrations for bchl e (#) and bphaeo e (x) for pump station 6-P1. See text for possible errors associated with pigment concentrations. D. Depth profiles of ETS activities measured in the Black Sea during Leg 5.

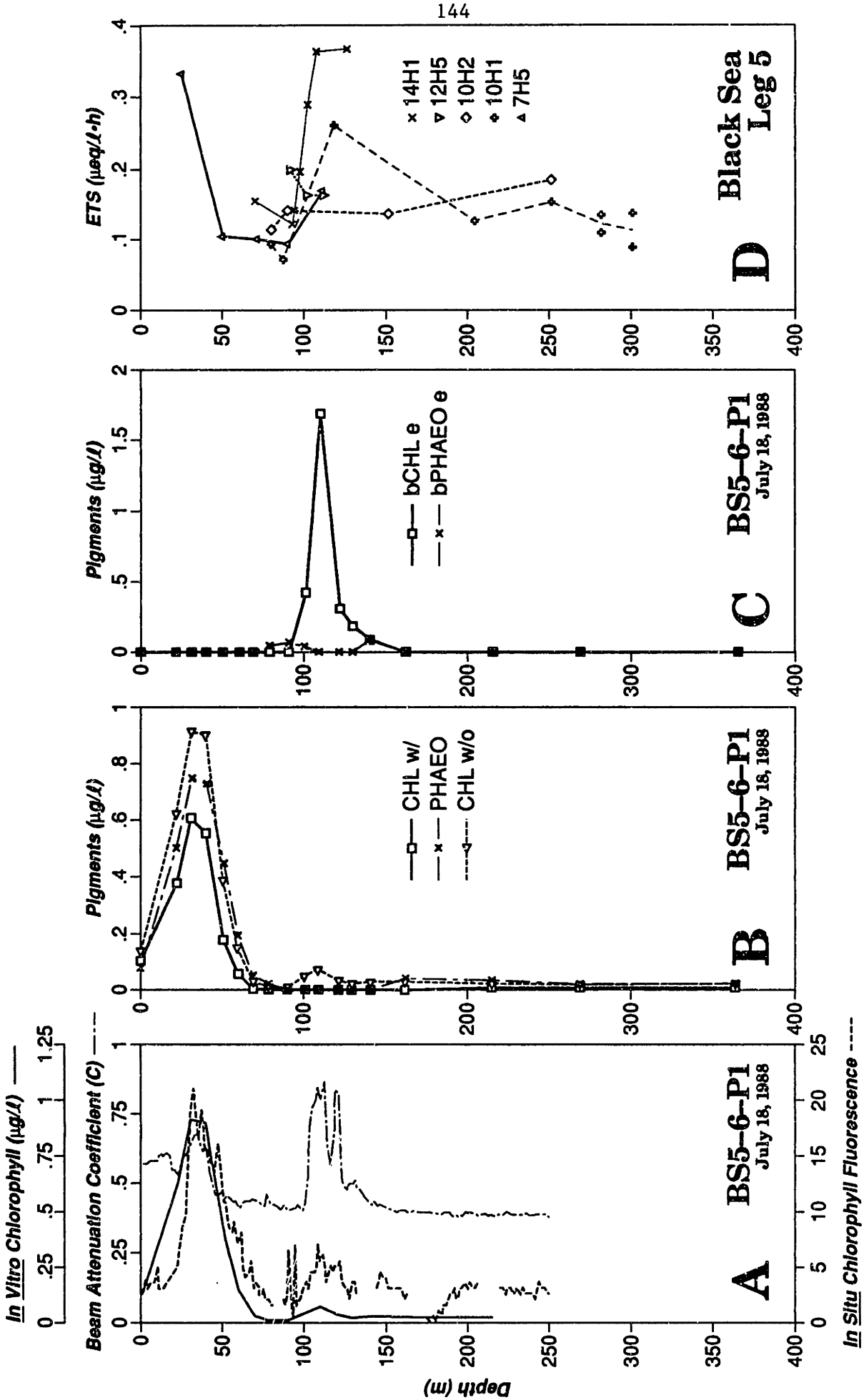
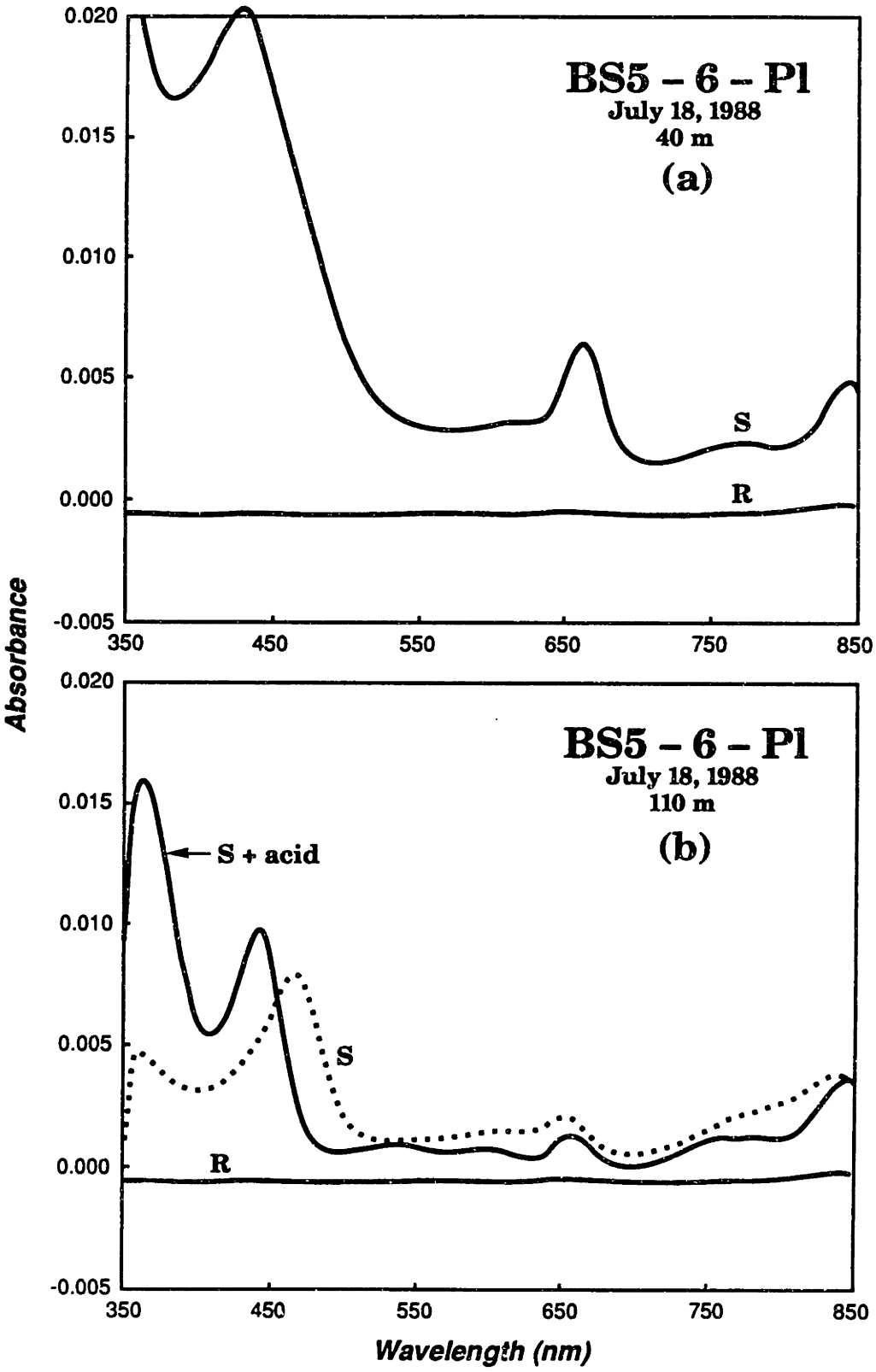

A
BS5-6-P1
July 18, 1988
B
BS5-6-P1
July 18, 1988
CHL w/
PHAEO
CHL w/o
C
BS5-6-P1
July 18, 1988
bCHL e
bPHAEO e
D
Black Sea
Leg 5
14H1
12H5
10H2
10H1
7H5
Depth (m)
Beam Attenuation Coefficient (C) ——
In Vitro Chlorophyll (µg/l) ——
In Situ Chlorophyll Fluorescence ----
Pigments (µg/l)
Pigments (µg/l)
ETS (µeq/l•h)

Fig. 4.04. A. Absorbance spectrum of extracted chlorophyll sample in 90% acetone taken in the primary maximum at 40m at station 6-P1. Sample (S) versus solvent blank (R). The spectrum is typical for chlorophyll a, with major peaks at 431 and 664 nm. B. Absorbance spectra of extracted chlorophyll sample in 90% acetone taken in the secondary maximum at 110m at station 6-P1. Sample before acid (S) and after acid (S + acid) versus solvent blank (R). The spectrum for the sample (S) is typical of that for bacteriochlorophyll e and has a major peak near 470 nm. After acidification (S + acid), this peak shifts to near 440 nm.


BS5 – 6 – Pl
July 18, 1988
40 m
(a)
S
R
BS5 – 6 – Pl
July 18, 1988
110 m
(b)
S + acid
S
R
Absorbance
Wavelength (nm)
0.020
0.015
0.010
0.005
0.000
-0.005
350
450
550
650
750
850

pigment present in the primary chlorophyll maximum (40 m), but the major pigment present in the secondary chlorophyll maximum (110 m) has spectral properties similar to those of bacteriochlorophyll-e (bchl e), a pigment present in two species of photosynthetic bacteria, Chlorobium phaeobacteroides and Chlorobium phaeovibrioides (GLOE et al. 1975). Although these organisms are grouped with the green sulfur bacteria, they are actually brown in color due to the presence of the carotenoid isorenieratene (TRUPER and PFENNIG 1981). The presence of bchl e and isorenieratene has been demonstrated in samples collected at the sulfide interface during Leg 2 of the 1988 Black Sea Expedition (REPETA et al. 1989).

The major absorbance peak of bchl e at 466 nm shifts to 443 nm after acidification (Fig. 4.04B). The excitation filter used for both in situ and in vitro chlorophyll measurements has its peak transmission at 435 nm, well below the wavelength at which maximum absorbance by bchl e occurs. This explains the underestimates of chlorophyll fluorescence and extracted chl a concentrations between 80 and 140 m. In contrast, the absorbance maximum of bphaeo e does fall within the wavelength band of the excitation filter, which explains the increased fluorescence observed after addition of acid to sample extracts and overestimates of phaeo a concentrations in samples containing bchl e.

Concentrations of bchl e and bphaeo e were calculated at all depths where the fluorescence after acidification either increased or remained unchanged (79 - 141 m). At 79 and 91 m, acidification had no significant effect on fluorescence. This indicates that all the pigment in these samples was degraded, but we cannot distinguish between phaeo a and bphaeo e. At 101 and 141 m, there was a slight increase after acidification, which indicates the presence of both bchl e and bphaeo e. For the depths in between, the ratio of

fluorescence before and after acidification was almost exactly the same as determined using pure bchl e. This is strong evidence that all the pigment at these depths was undegraded bchl e. There is a possibility that bchl e was present in samples taken above 71 m or below 141 m, however since fluorescence decreased after acidification we cannot discern its presence using this method and therefore we assumed bchl e concentrations were zero.

Several changes are apparent in the chlorophyll profile at station 6-P when corrections have been made for phaeo a, bchl e, and bphaeo e (Fig. 4.03B&C). Data for chl a without phaeo a or bchl e corrections (triangles) are the same as shown in Fig. 4.03A. Concentrations of phaeo a in the euphotic zone were nearly equal to corrected chl a concentrations. Below 150 m there is some low, non-zero level of both chl a and phaeo a. The amount of chlorophyll measured as bchl e in the secondary chlorophyll maximum is about 10 times greater than initially calculated as chl a. The maximum concentration was 1.6 µg/l, which is slightly higher than the maximum of 1 µg/l measured in Black Sea samples by REPETA et al. (1989) two months earlier using HPLC analysis.

A local maximum in ETS activities was also associated with the particle maximum (Fig. 4.03D). The depth and intensity of the particle maximum showed variability, but ETS activities from samples taken within the particle maximum were higher than from samples taken directly above or below and were approximately equal to activities measured in the euphotic zone. The particle layer was sometimes only 5 m thick and its position could vary by 5 to 10 m from the down cast to the up cast. For example, on cast 7-H5 the bottle at 110 m was tripped in the particle maximum on the up cast, even though this depth was above the maximum on the down cast. This shows the importance of having a real-time monitor for the feature one is trying to sample.

DISCUSSION

The Black Sea is an inland sea and we had, therefore, expected to find that terrestrially-derived fluorescent materials would be important. Riverine input of terrestrial humic materials is clearly the dominant source of fluorescence in estuarine areas. Since fluorescence behaves conservatively, the distribution can be explained by a simple mixing of freshwater with seawater. Results of this study showing an increase in the intensity of fluorescence due to DOM with depth are in agreement with previous reports for the Black Sea (KARABASHEV 1970) and for other open ocean areas (IVANOFF 1962; HAYASE et al. 1987, 1988; CHEN and BADA 1989). However, since salinity increases with depth in the Black Sea, our results are in direct contrast with previous studies of dissolved fluorescence in estuaries, where dissolved fluorescence and salinity show an inverse linear correlation (DUURSMA 1974; LAANE 1981; BERGER et al. 1984; HAYASE et al. 1987). The high salinity bottom waters of the Black Sea are derived from overflow of Mediterranean water through the Bosphorus and the low salinity surface waters are formed by mixing freshwater from rivers with saline deep waters. If fluorescence in the Black Sea is conservative and is primarily derived from riverine input, one would have to postulate that the intensity of fluorescence in the rivers entering the Mediterranean is much greater than in those entering the Black Sea. We have no data with which to evaluate this hypothesis.

A more likely explanation is that the fluorescence distribution in the Black Sea is not controlled by river runoff, but rather by the same processes which are thought to control distributions in open ocean areas. Destruction of fluorescence in the surface waters and production in deep waters would result in profiles similar to those shown in Fig. 4.02A. Photochemical

degradation of fluorescent compounds may be the primary pathway by which fluorescence is destroyed in surface waters. The intensity of fluorescence observed in deep water samples has been shown to decrease rapidly upon exposure to UV radiation (KRAMER 1979; HAYASE et al. 1988; CHEN and BADA 1989). Our results show sharp increases in DOM and flavin fluorescence at the base of the surface mixed layer and at the depth of the pycnocline. This distribution is consistent with photodegradation, since these boundaries cause increased isolation of water masses from the surface and therefore decreased exposure to full sunlight.

Production of fluorescent compounds in the waters below the pycnocline can be the result of several processes. One of these is in situ production of fluorescent compounds concomitant with nutrient regeneration. Fluorescence has been found to show a linear relationship with nitrate and phosphate in coastal and open ocean waters off Japan (HAYASE et al. 1987, 1988) and with ammonium ion concentrations in the anoxic sediments of the Santa Barbara Basin (CHEN and BADA 1989). In Black Sea, fluorescence cannot be simply correlated with phosphate, nitrate or ammonium, as the distributions of these nutrients are effected by the redox potential of the water column as well as by regeneration. Nitrate and phosphate show maxima between 40 and 100m (Figs. 4.01B&D), possibly as the result of regeneration or due to advection of water from the northwest part of the basin (MURRAY et al. 1989). Ammonium is not present in surface waters, but increases gradually with depth below 105 m. Although not apparent from Fig. 4.01D, phosphate concentrations also continue to increase slowly to the bottom due to anaerobic regeneration (FONSELIUS 1974). Our results are consistent with the hypothesis that fluorescence is produced during nutrient regeneration and we would therefore expect fluorescence to increase all the way to the bottom.

A second process which could produce fluorescence in the deep waters is the release of fluorescent material associated with solid mineral phases as a result of solubilization under anoxic conditions. This was postulated by CHEN and BADA (1989) to explain high fluorescence intensities observed in anoxic pore waters. Since metals have a quenching effect on the fluorescence of organic compounds, including natural fulvic and humic materials (WILLEY 1984 and references therein), solubilization should result in increased fluorescence. We did not observe an abrupt change in DOM or flavin fluorescence across the sulfide interface, which would seem to indicate a lack of involvement of fluorescent organic matter in the redox cycling of metals in the water column. Further profiles and experimental work are needed to verify this observation.

A third process which could cause high fluorescence of the deep waters is diffusion of fluorescent compounds out of the sediments. SCRANTON et al. (1987) have shown that the hydrogen sulfide in the water column of the Cariaco Trench can be attributed to upward diffusion from the bottom, with water column production having little importance. The implication for our study area is that processes occurring in the sediments can have a significant impact on the water column in deep, isolated anoxic basins. Diffusion of fluorescent compounds out of sediments was the proposed explanation for high fluorescence intensities in the bottom waters of the Santa Barbara Basin (CHEN and BADA 1989). Our profiles are consistent with this explanation, however none of our data were collected near the sediment water interface.

The remaining explanation for high DOM and flavin fluorescence in the intermediate waters of the Black Sea is that the lack of oxygen either prevents degradation of fluorescent compounds or favors organisms which produce unusually high amounts of this material. Further profiling in aerobic

water columns is needed before we can assess the potential effect of oxic vs. anoxic conditions on vertical distribution of fluorescence.

We had hoped to show that zones of high microbial biomass could be located by fluorescence peaks in either the DOM or flavin fluorescence channels. Previous results from flavin analysis in the Cariaco Trench led us to expect a large peak in fluorescence near the oxycline (COBLE 1989). Flavin fluorescence did show a spiky distribution at the bottom of the oxygenated waters (Fig. 4.02A), however the magnitude of these peaks was less than expected. Denitrification rates of <10 nmol N/l/hr were measured in this region of the water column during Leg 5 by acetylene reduction technique (BAZYLINSKI, HOWES, and JANNASCH 1989). These rates were much lower than measured during Leg 2 of this expedition and ETS activities show a minimum at 50-100m depth interval, so perhaps there was no significant elevation in bacterial biomass.

Previous explanations of maxima in suspended particulate matter in the Black Sea have been formulated based on vertical distributions of particulate iron, manganese and zinc (SPENCER et al. 1972). Three types of particles were found to be important: iron is associated with riverine input of detrital materials, soluble manganese diffusing out of the sulfide-bearing waters is re-oxidized and precipitated 30 - 50 m above the zero oxygen level, and zinc sulfides precipitate out at 35 m below the oxygen zero level. Our data indicate that a fourth process is also important, namely, in situ growth of photosynthetic bacteria. It is likely that other types of bacteria also contribute to the particle maximum at this depth. We cannot say how the ETS, beam attenuation coefficient, and bchl e maxima relate to the distribution of particulate metals from our data alone, however it may be possible to

construct a more complete description of particulates when results of other expedition participants are included.

Our report herein of bchl e in the water column of the Black Sea confirms the results of REPETA et al. (1989) and provides strong evidence for the presence of active populations of brown Chlorobium species at the depth of the sulfide interface. Chlorobium phaeovibrioides and C. phaeobacteroides are the only known species to contain bchl e (GLOE et al. 1975). They are unicellular, non-motile obligate phototrophs, are strictly anaerobic, and do not contain gas vacuoles (PFENNIG 1968; TRUPER and PFENNIG 1981). The only open ocean environment in which phototrophic sulfur bacteria have been found prior to the 1988 Black Sea Expedition is in the water column of the Black Sea below 500 m (KRISS and RUKINA 1953) and in Black Sea sediments (HASHAWA and TRUPER 1978). The latter authors suggested the bacteria at the bottom, although viable, were not actively growing and probably had been transported from their "home" in the estuaries and lagoons surrounding the Black Sea. Several previous attempts to isolate phototrophic bacteria above 500 m in the water column have failed (JANNASCH et al. 1974; HASHAWA and TRUPER 1978).

A Secchi depth of 14 m was measured during July 1988 (WARD, personal communication), so the light level at 100 m would be 0.0006% of total incident solar radiation at the surface. If we assume a value of 1.4 ly/min for a clear July day at 42°N, we would roughly estimate a value of 8×10^{-6} ly/min or about 4 lux at this depth (STRICKLAND 1958). The green sulfur bacteria are known to have the lowest minimum light requirements of any photosynthetic organism. Chlorobium phaeovibrioides has been shown to be capable of growth at 5 lux when grown in the presence of sulfate-reducing heterotrophic bacteria and with added acetate (BIEBL and PFENNIG 1978). The ability to grow at these extremely low light levels is enhanced in the presence of acetate (BERGSTEIN

et al. 1979). Based on what is known of the physiology of the brown Chlorobium and given the environmental conditions existing at 100 m in the Black Sea, it is highly probable that the presence of bchl e in the water column represents the presence of an active resident population of photosynthetic bacteria. The occurrence of a maximum in ETS activity at the depth of the secondary chlorophyll maximum is consistent with this conclusion, however since species of Chlorobium show strong syntrophy (PFENNIG 1981), we cannot rule out the possibility that other bacterial types are also present.

Our objective for this cruise was to test hypotheses based on observations in the eastern tropical Pacific Ocean, where particle maxima and associated features are found in oxygen-deficient waters. The major difference between those areas and this study site is that high concentrations of sulfide are present in the deep waters of the Black Sea. For this reason, the results presented here may be unique to anoxic basins. The presence of bchl e has never been demonstrated in the Pacific Ocean, and while possible, it is unlikely to be found in high enough concentrations to be responsible for the secondary fluorescence maximum there. The continuous increase in DOM and flavin fluorescence with depth reported here may likewise be related to features which are unique to the Black Sea, such as high sulfide bottom waters, diffusion out of the bottom sediments, or long residence time of the deep waters.

CONCLUSIONS

1. Vertical distributions of both DOM and flavin fluorescence are distinctly different from that of chlorophyll fluorescence. Flavin and DOM fluorescence do not appear to contribute to the secondary chlorophyll maximum observed at this study site.

2. DOM and flavin fluorescence vary directly with salinity and depth profiles are more similar to DOM profiles reported for open ocean areas than for coastal areas.

3. There is a local maximum in ETS activity at the depth of the subsurface particle maximum.

4. The secondary chlorophyll fluorescence maximum in the Black Sea is caused by a maximum in bacteriochlorophyll e.

5. The co-occurence of maxima in both bacteriochlorophyll e and ETS at the depth of the particle maximum lead us to conclude that bacteria are at least partially responsible for the maximum in beam attenuation coefficient.

6. The presence of the sulfide interface within 150 m of the surface makes this a unique marine environment and distributions reported here may not have general applicability to other marine environments.

ACKNOWLEDGEMENTS

This work was funded by NSF Contract No. OCE-8701386 to RBG and PGC. We thank Dr. W. Reeburgh, chief scientist, and the captain, crew and scientists on Leg 5 of the 1988 Black Sea Expedition, R/V Knorr 134-12. We also thank J. Doucette and D. Scheer for drafting the figures and D. Repeta for providing the Salt Pond material from which pure bchl e was obtained.

REFERENCES

ANDERSON J. J. (1982) The nitrite-oxygen interface at the top of the oxygen minimum zone in the eastern tropical North Pacific. Deep Sea Research, 29, 1193-1201.

BARBER R. T. and A. HUYER (1979) Nitrite and static stability in the coastal waters off Peru. Geophysical Research Letters, 6, 409-12.

BAZYLINSKI D. A., B. L. HOWES and H. W. JANNASCH (1989) Denitrification, nitrogen-fixation and nitrous oxide concentrations through the Black Sea oxic-anoxic interface. Deep-Sea Research, (this volume)

BERGER P., R. W. P. M. LAANE, A. G. ILAHUDE, M. EWALD and P. COURTOT (1984) Comparative study of dissolved fluorescent matter in four West-European estuaries. Oceanologica Acta, 7, 309-314.

BERGSTEIN T., Y. HENIS and B. Z. CAVARI (1979) Investigations on the photosynthetic sulfur bacterium Chlorobium phaeobacteroides causing seasonal blooms in Lake Kinneret. Canadian Journal of Microbiology, 25, 999-1007.

BLASCO D., J. PAUL and N. OCHOA-LOPEZ (1979) Chlorophyll and phaeopigment from the Peru Current, 1977. In: Biological data from JOINT-II, R/V Melville, Leg IV, May 1977, P.C. GARFIELD and T.T. PACKARD, editors, Coastal Upwelling Ecosystems Analysis (CUEA) Technical Reptort 53.

BRANDHORST W. (1959) Nitrification and denitrification in the eastern tropical North Pacific. Journal International pour l'Exploration de Mer, 25, 3-20.

BROENKOW W. W. and J. D. CLINE (1969) Colorimetric determination of dissolved oxygen at low concentrations. Limnology and Oceanography, 14, 450-454.

BROENKOW W. W., A. J. LEWITUS, M. A. YARBROUGH and R. T. KRENZ (1983) Particle fluorescence and bioluminescence distributions in the eastern tropical Pacific. Nature, 302, 329-331.

CARPENTER J. H. (1965) The Chesapeake Bay Institute technique for the Winkler dissolved oxygen method. Limnology and Oceanography, 10, 141-143.

CHEN R. F. and J. L. BADA (1989) Seawater and porewater fluorescence in the Santa Barbara Basin. (submitted to Geophysical Research Letters)

CLINE J. D. (1969) Spectrophotometric determination of hydrogen sulfide in natural waters. Limnology and Oceanography, 14, 454-458.

COBLE P. G. (1989) Marine bacteria as a source of dissolved fluorescence in the ocean. Ph.D. Thesis, Massachusetts Institute of Technology/Woods Hole Oceanographic Institution, (in press).

DUURSMA E. K. (1974) The fluorescence of dissolved organic matter in the sea. In: Optical Aspects of Oceanography, N. G. JERLOV and E. STEEMANN NIELSEN, editors, Academic Press, New York, pp. 237-256.

FONSELIUS S. H. (1974) Phosphorous in Black Sea. In: The Black Sea, E. T. DEGENS and D. A. ROSS, editors, American Association of Petroleum Geologists, Tulsa, Oklahoma, pp. 144-150.

FRIEDERICH G. E. and L. A. CODISPOTI (1987) An analysis of continuous vertical nutrient profiles taken during a cold-anomaly off Peru. Deep-Sea Research 34, 1049-1065.

GARFIELD P. C. and T. T. PACKARD (1979) Biological data from JOINT II R/V Melville, Leg IV. May 1977, Coastal Upwelling Ecosystems Analysis (CUEA) Technical Report 52, 186 p.

GARFIELD P. C., T. T. PACKARD and L. A. CODISPOTI (1979) Particulate protein in the Peru upwelling system. Deep-Sea Research, **26**, 623-639.

GARFIELD P. C., T. T. PACKARD, G. E. FRIEDERICH and L. A. CODISPOTI (1983) A subsurface particle maximum layer and enhanced microbial activity in the secondary nitrite maximum of the northeastern tropical Pacific Ocean. Journal of Marine Research, **41**, 747-768.

GLOE A., N. PFENNIG, H. BROCKMANN JR., AND W. TROWITZSCH (1975) A new bacteriochlorophyll from brown-colored Chlorobiaceae. Archives of Microbiology, **102**, 103-109.

HASHAWA F. A. and H. G. TRUPER (1978) Viable phototrophic sulfur bacteria from the Black-Sea bottom. Helgolunder wiss. Meeresunters **31**, 249-253.

HAYASE K., M. YAMAMOTO and H. TSUBOTA (1987) Behavior of natural fluorescence in Sagami Bay and Tokyo Bay, Japan - Vertical and lateral distributions. Marine Chemistry **20**, 265-276.

HAYASE K., H. TSUBOTA and I. SUNADA (1988) Vertical distribution of fluorescent organic matter in the North Pacific. Marine Chemistry, **25**, 373-381.

IVANOFF M. A. (1962) Au subjet de la fluorescence des eaux de mer. Comptes Rendu de Academe Sciences (Paris), **254**, 4190-4192.

JANNASCH H. W., H. G. TRUPER and J. H. TUTTLE (1974) Microbial sulfur cycle in Black Sea. In: The Black Sea - Geology, Chemistry, and Biology, E.T. DEGENS and D.A. ROSS, editors, American Association of Petroleum Geologists, Tulsa, Oklahoma, pp. 419-425

KALLE K. (1949) Fluoreszenz und gelbstoff im bottnischen und finnischen meerbusen. Dt. hydrogr. Z. **2**, 117-124.

KARABASHEV G. S. (1970) A method of studying the photoluminescence of sea water. Oceanology, **10**, 703-707.

KARABASHEV G. S. (1977) Characterization of the distribution of fluorescence and light scattering in the ocean during strong vertical mixing and upwelling. Oceanology, **17**, 200-204.

KENNER R. A. and S. I. AHMED (1975) Measurements of electron transport activities in marine phytoplankton. Marine Biology, **33**, 119-127.

KRAMER C. J. M. (1979) Degradation by sunlight of dissolved fluorescing substances in the upper layers of the eastern Atlantic Ocean. Netherlands Journal of Sea Research, **13**, 325-329.

KRISS A. E. and E. A. RUKINA (1953) Purple sulfur bacteria in deep sulfurous water of the Black Sea. Dolk. Akad. Nauk. SSSR **93**, 1107-1110.

KULLENBERG G. (1981) A comparison of distributions of suspended matter in the Peru and northwast African upwelling areas. In: Coastal Upwelling. F.A. RICHARDS, editor, American Geophysical Union, Washington, D.C., pp. 282-209.

KULLENBERG G. (1984) Observations of light scattering functions in two oceanic areas. Deep-Sea Research, **31**, 295-316.

LAANE R. W. P. M. (1981) Composition and distribution of dissolved fluorescent substances in the Ems-Dollart Estuary. Netherlands Journal of Sea Research, 15, 88-99.

LAANE R. W. P. M. and L. KOOLE (1982) The relation between fluorescence and dissolved organic carbon in the Ems-Dollart Estuary and the western Wadden Sea. Netherlands Journal of Sea Research, 15, 217-227.

LEWITUS A. J. and W. W. BROENKOW (1985) Intermediate depth pigment maxima in oxygen minimum zones. Deep-Sea Research, 32, 1101-1115.

MURRAY J. M. and E. IZDAR (1989) The 1988 Black Sea oceanographic expedition: Overview and new discoveries. Oceanography, 2(1), 15-21.

MURRAY J.M., H. W. JANNASCH, S. HONJO, R. F. ANDERSON, W. S. REEBURGH, Z. TOP, G. E. FRIEDERICH, L. A. CODISPOTI and E. IZDAR (1989) Unexpected changes in the oxic/anoxic interface in the Black Sea. Nature, 338, 411-413.

PACKARD T. T. (1985) Measurement of electron transport activity of microplankton. Advances in Aquatic Microbiology, 3, 207-261.

PACKARD T. T., M. DENIS, M. RODIER and P. GARFIELD (1988) Deep-ocean metabolic CO_2 production: Calculations from ETS activity. Deep-Sea Research, 35, 371-382.

PAK H., L. A. CODISPOTI and R. V. ZANEVELD (1980) On the intermediate particle maxima associated with oxygen-poor water off western South America. Deep-Sea Research, 27, 783-97.

PFENNIG N. (1968) Chlorobium phaeobacteroides nov. spec. und C. phaeovibrioides nov. spec., zwei neue Arten der grunen Schwefelbakterien. Archiv fur Mikrobiologie, 63, 224-226.

REPETA D. J., D. J. SIMPSON, B. B. JORGENSON AND H. W. JANNASCH (1989) The distribution of bacteriochlorophylls in the Black Sea: Evidence for anaerobic photosynthesis. Nature (submitted).

SCRANTON M. I., F. L. SAYLES, M. P. BACON AND P. G. BREWER (1987) Temporal changes in the hydrography and chemistry of the Cariaco Trench. Deep-Sea Research, 34, 945-963.

SPENCER D. W., P. G. BREWER AND P. L. SACHS (1972) Aspects of the distribution and composition of suspended matter in the Black Sea. Geochimica Cosmochimica Acta, 36, 71-86.

SPINRAD R. W., H. GLOVER, B. B. WARD, L. A. CODISPOTI and G. KULLENBERG (1989) Suspended praticle and bacterial maxima in Peruvian coastal waters during a cold water anomaly. Deep-Sea Research, 36, 715-734.

STRICKLAND J. D. H. (1958) Solar radiation penetrating the ocean. A review of requirements, data and methods of measurement, with particular reference to photosynthetic productivity. Journal of the Fisheries Research Board of Canada, 15, 453-493.

STRICKLAND, J. D. H. AND T. R. PARSONS (1972) A practical handbook of seawater analysis. Fisheries Research Board of Canada, Bulletin 167, 310p.

TRUPER H. G. and N. PFENNIG (1981) Characterization and identification of the anoxygenic phototrophic bacteria. In: The Procaryotes, M. P. STARR, H. STOLP,

H. G. TRUPER, A. BALOWS and H. G. SCHLEGEL, editors, Springer-Verlag, New York, pp. 299-312.

VASTANO S. E. (1988) Processes affecting the distribution of flavins in the ocean. MS Thesis, Univ. Miami, 67p.

WHITE G., M. REELANDER, J. POSTEL and J. W. Murray (1989) Hydrographic data from the 1988 Black Sea Ocenaographic Expedition. College of Ocean and Fishery Sciences Special Report # **109**, University of Washington, Seattle.

WHITLEDGE T. E., S. C. MALLOY, C. J. PATTON and C. O. WIRICK (1981) Automated nutrient analyses in seawater. Brookhaven National Laboratory Report 51398, 216pp.

WILLEY J. D. (1984) The effect of seawater magnesium on natural fluorescence during estuarine mixing and implications for tracer applications. Marine Chemistry, **15**, 19-45.

WOOSTER W. S., T. J. CHOW and J. BARRETT (1965) Nitrate distribution in Peru Current waters. Journal of Marine Research, **23**, 210-21.

CHAPTER 5

DISTRIBUTION OF FLAVINS AND BACTERIA IN TWO MARINE ANOXIC BASINS

INTRODUCTION

The first successful attempt to measure flavin concentrations in the ocean was reported by Momzikoff (1969a). Thousands of liters of seawater had to be extracted to obtain measurable quantities of riboflavin and lumichrome, which were found to be present in surface seawater at concentrations in the ng/l range. Subsequent reports that these same compounds were present in and excreted by marine copepods (Momzikoff 1969b; Momzikoff 1973; Momzikoff and Legrand 1973) and marine ascidians (Gaill and Momzikoff 1975; Gentien 1981) was taken as evidence that riboflavin and lumichrome had a biological origin in seawater. In the last five years an interest in the role of flavins as photosensitizers in the oceans and improvements in separation technology have spurred new research in this area.

A routine method for isolating flavins from seawater and analyzing them using HPLC has been described (Dunlap and Susic 1985) which requires only a few liters of sample. Concentrations of four flavins (riboflavin, flavin mononucleotide (FMN), lumichrome and lumiflavin) in surface waters surrounding the Great Barrier Reef in Australia were found to be in the ng/l range. Some of these compounds were also found to be excreted by one species of coral and one species of sponge living in the reef community, once again implicating a biological origin (Dunlap and Susic 1985). Similar concentrations have also been reported for Florida coastal waters (Vastano 1988) and the Cariaco Trench (Mopper and Kieber 1986).

Riboflavin, FMN, and flavin adenosine dinucleotide (FAD) appear to have a biological origin in seawater, however lumichrome and

lumiflavin have never been shown to be biosynthesized and must arise from photo-oxidation. Several recent studies have shown that the half-lives of riboflavin and FMN are less than a minute in the surface waters (Dunlap and Susic 1988; Mopper and Zika 1988).

Flavins play an important role in the photochemistry of the oceans. Riboflavin has been shown to act as a photosensitizing agent in seawater due to its efficiency in producing singlet oxygen (Momzikoff et al. 1983). Mopper and Zika (1987) have shown that riboflavin at natural seawater concentrations can account for 13 - 70% of the total photosensitizer activity in seawater. Riboflavin is also a significant producer of hydrogen peroxide, which is itself a strong oxidizing agent. It has been suggested that the photochemical degradation of organic matter in the surface waters may be an important pathway by which high molecular weight refractory dissolved organic matter (DOM) is recycled in the oceans (Mopper et al. 1987).

Although bacteria have not previously been proposed as a source of flavins in the water column, there is much evidence to suggest they are likely candidates. Some bacteria, yeast and molds produce in excess of 10 mg flavins per liter in culture and are therefore classified as "overproducers" (Demain 1972), and even "normal" bacteria produce relatively high amounts of extracellular flavins (Wilson and Pardee 1962).

We have demonstrated in a previous chapter (Chapter 2) that riboflavin, FAD, and FMN are released into spent media by several species of marine denitrifying bacteria. In this chapter, we will present vertical profiles of flavin concentrations from environments in which such bacteria are typically found: the Black Sea and the

Cariaco Trench. Flavin distributions will be related to distributions of temperature, salinity, nutrients, oxygen, fluorescence, beam attentuation coefficient, chlorophyll and microbial biomass in an attempt to determine whether or not flavin concentrations are indicative of bacterial population densities.

METHODS

A. HYDROGRAPHIC MEASUREMENTS AND NUTRIENT CONCENTRATIONS

Cariaco Trench. Samples were collected using either a rosette with 10 liter Niskin bottles and Neil Brown CTD, or with bottles attached to the hydrowire. Accepted depths were based on pressure measurements for samples collected on the rosette. For samples taken by hydrocasts, accepted depths were based on the mean pressure-salinity relationship measured in CTD casts 007 and 014.

Nitrate plus nitrite was measured using the chemiluminescent method of Yoshizumi et al. (1985) with some modifications (Hanley and Zafiriou 1986). Low-level oxygen concentrations were measured using the colorimetric method of Broenkow and Cline (1969) except that standardization was based on gas mixtures and oxygen-free water rather than on biiodate. At higher levels of oxygen, concentrations were measured using the Winkler titration method (Carpenter 1965). Sulfide concentrations were measured using the method of Cline (1969). A complete listing of the data presented here can be found in a report by Casso et al. (1988).

Black Sea. Details of methods for hydrographic and nutrient data collection and anaylsis are given in Chapter 4 (Coble et al. 1989).

B. MEASUREMENT OF FLAVINS IN SEAWATER

Samples consisted of one liter of seawater which was stored in amber bottles and filtered immediately through Whatman GF/F filters. Dissolved fluorescent compounds were isolated, concentrated and analyzed by IP-HPLC using the methods described in Chapter 2. The seawater samples contained a more complex mixture of components in the C18 fraction than did the culture filtrates, making the HPLC analysis more difficult.

Riboflavin, FAD, FMN, lumichrome, and lumiflavin were identified by comparison of retention times with those of authentic standards. Absolute retention times of standards varied slightly, probably due to the gradual accumulation of material on the stationary phase which was totally retained under the elution conditions of the method. Unknown compounds were identified based on their relative retention times with respect to that of riboflavin. A list of identifiable components is given in Table 5.01 along with ranges of relative retention times for samples and standards from Black Sea stations 6-P1 and 9-P1. The same known and unknown flavins were found in the samples from the Cariaco Trench, however, due to problems in quantitation of these samples, these data have not been tabulated.

Verification of the identities of flavins in the seawater samples was also made by co-injection of standard and sample. The presence of rbfl in the sample from 250 m in the Cariaco Trench (cast # C017) is shown in Figure 5.01. The presence of all six flavin standards in the sample from 0 m in the Black Sea is shown in Figure 5.02.

Table 5.01. Relative retention times of flavins identified in Black Sea samples. * in first column indicates that this peak was also found in samples from the Cariaco Trench.

NAME	STATION 6-P1	STATION 9-P1
.82	.81-.82	.83-.87
.93	.90-.93	.90-.94
RBFL	1.00	1.00
1.15	1.11-1.2	1.10-1.19
1.3	1.25-1.33	1.22-1.29
*1.4	1.37-1.43	1.37-1.43
1.5		1.50-1.54
LFL	1.50-1.73	1.54-1.65
UNK1	1.76-1.88	1.74-1.84
1.9	1.88-2.00	1.84-1.94
*2.05	2.04-2.12	2.01-2.09
2.2	2.06-2.18	2.14-2.26
2.45	2.40-2.49	2.38-2.49
2.55		2.52-2.68
2.8	2.73-2.86	2.68-2.86
3	2.92-3.11	2.92-3.09
FMN	3.59-3.82	3.41-3.50
FAD	3.74-4.13	3.75-3.85
*A	4.36-4.62	4.21-4.48
*4.6	4.58-4.92	4.55-4.93
5	4.96-5.22	4.97-5.03
LCR	5.6-5.81	5.61-5.90
*8.0		7.9-8.0

Figure 5.01. Evidence for verification of flavin peaks by co-injection of standards. A. Sample taken from 250 m in the Cariaco Trench. B. Cariaco Trench sample plus standards. C. Standards only. All chromatograms were run using IP-HPLC.

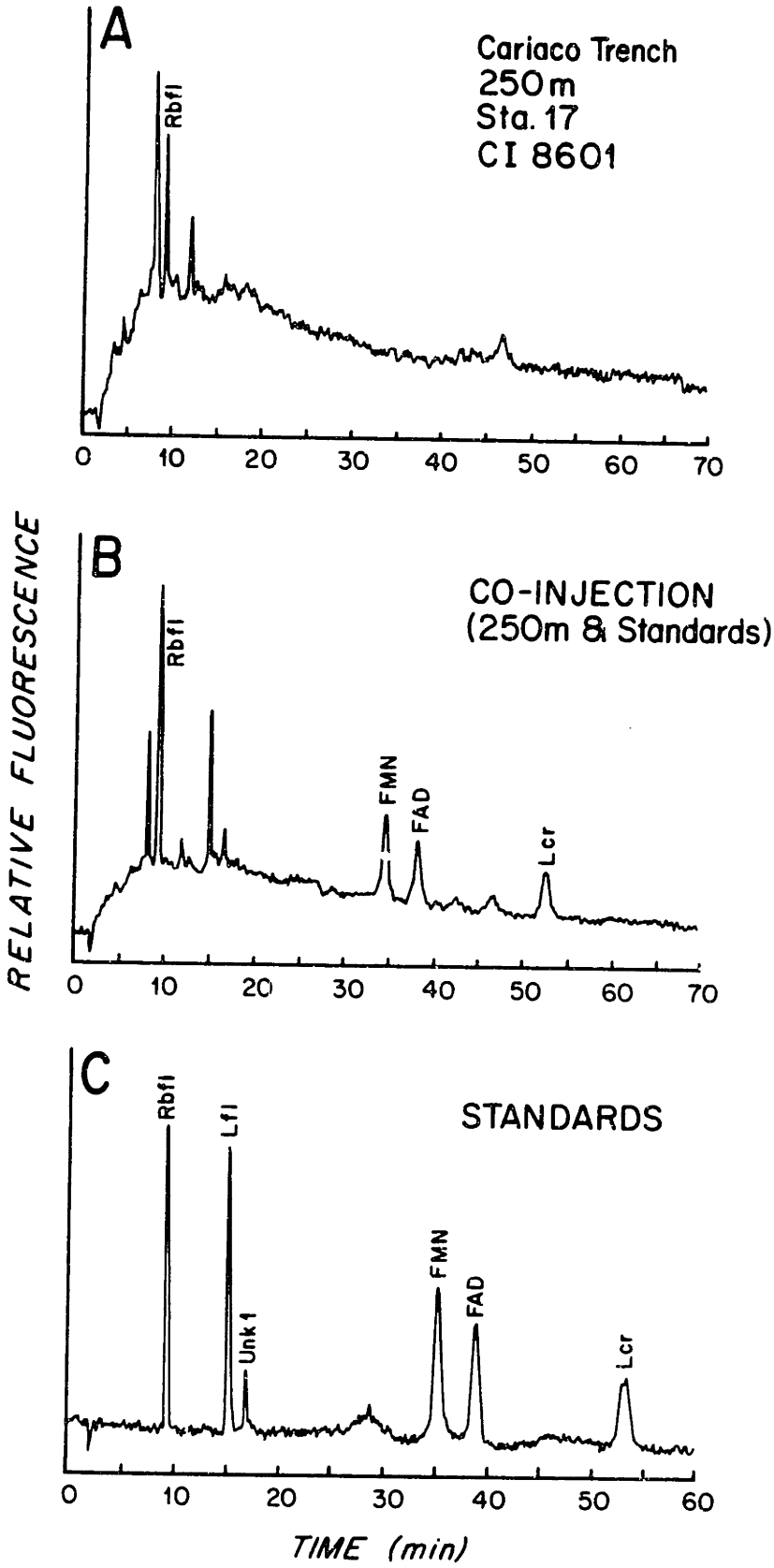
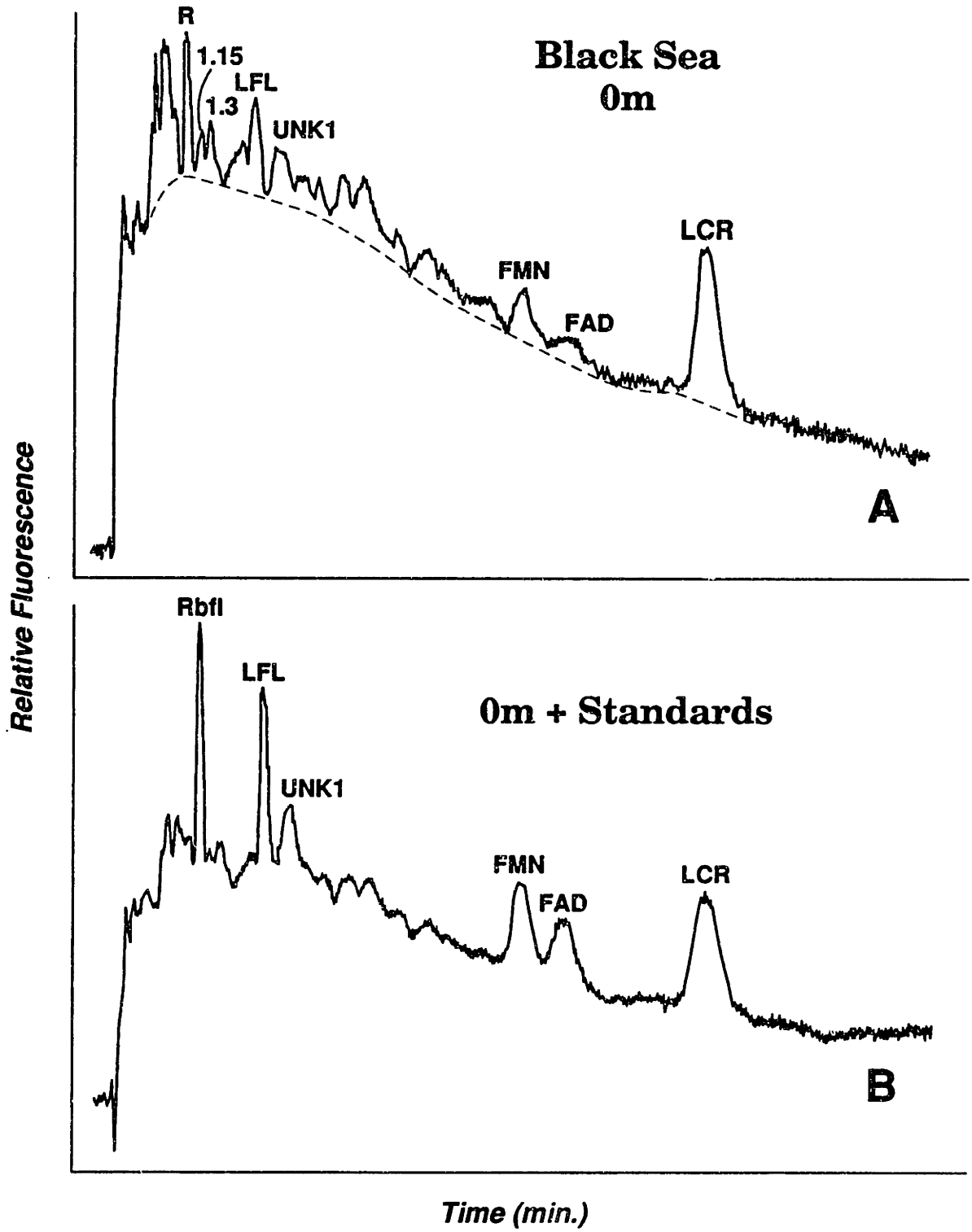

A
Cariaco Trench
250m
Sta. 17
CI 8601
Rbfl
0 10 20 30 40 50 60 70
B
CO-INJECTION
(250m & Standards)
Rbfl
FMN
FAD
Lcr
0 10 20 30 40 50 60 70
RELATIVE FLUORESCENCE
C
STANDARDS
Rbfl
Lfl
Unk1
FMN
FAD
Lcr
0 10 20 30 40 50 60
TIME (min)

Figure 5.02. Evidence for verification of flavin peaks by co-injection of standards. A. Sample taken from 0 m in the Black Sea. B. Black Sea sample plus standards. Both chromatograms were run using IP-HPLC.


R
1.15
LFL
1.3
UNK1
Black Sea
0m
LCR
FMN
FAD
A
Rbfl
LFL
0m + Standards
UNK1
FMN
FAD
LCR
B
Relative Fluorescence
Time (min.)

Concentration of standards coinjected with the sample were calculated to be from 70 to 106% of actual amounts added.

Quantitative mixed standards were run at the beginning of each day, followed by several injections of a quantitative riboflavin standard. Additional standards were run about every fifth sample. Daily calibration factors were compared to previous values obtained on the same column and were rejected if they differed by more than 10% from mean values. The calibration factor and molecular weight of riboflavin were used for quantification of unknown compounds as riboflavin-equivalents (rbfl-eq). Concentrations of all compounds were calculated using the following equation:

$$\text{flavin (pM/l)} = \frac{\text{peak area} * \text{RF} * \text{sample volume}}{\text{volume filtered} * \text{molecular wt.}}$$

The largest source of error in quantitation of seawater samples was in establishing a reliable baseline due to the presence of a "hump" of unresolved components in seawater samples. Integration of peak areas was based on a baseline drawn from valley to valley, as illustrated by the dotted line in Figure 5.02A. Samples from the Cariaco Trench were integrated by hand. Samples from station 9P1 in the Black Sea were integrated on a Perkin-Elmer LCI-100 integrator. Samples from station 6P1 in the Black Sea were integrated with using Waters Expert software after the baseline was set manually for each peak. These differences in data analysis resulted in mean concentrations for duplicate samples which differed from individual determinations by up to 40%.

Recoveries were determined by adding standards to distilled water and preparing as for samples, with and without separation onto Sep-Paks. Samples were stored frozen for three months prior to analysis. The samples not subjected to Sep-Pak treatment were concentrated by rotary evaporation prior to storage, while the others concentrated after storage. Recoveries are shown in Table 5.02. A loss of 20-50% resulted from the concentration step alone (without Sep-Pak). Slightly higher losses resulted from the combination of Sep-Pak treatment and concentration. Concentrations of Unknown 1 increased by a factor of three following Sep-Pak treatment in conjunction with a loss of FAD. Degradation of FAD to Unk 1 may be responsible for the low recoveries for FAD.

C. MOST PROBABLE NUMBER ESTIMATES (MPN)

Seawater was used to inoculate anaerobic enrichment media contained in sterile Hungate tubes (Hungate 1950) which had been purged with nitrogen and contained a gas collection tube. The media used was designed in conjunction with Dr. John Waterbury to select for denitrifying bacteria and consisted of: 1 g/l sodium lactate, 5 ml/l glycerol, 10 g/l potassium nitrate, 10 mg/l yeast extract, 54 mg/l ammonium chloride, 13.7 mg/l potassium phosphate (dibasic), 1 mg/l chelated iron (GEIGY), and 1 ml/l A-5 trace metal mix (contains trace amounts of B, Mn, Zn, Mo, Cu, and Co). A 4 x 6 series of these tubes was inoculated for estimation of most probable number (MPN) and all tubes were returned to the lab. Results were tabulated and MPN values with confidence intervals were computed using a micro-computer program

Table 5.02. Recovery of flavin standards.

Compound	without Sep-Pak	Sep-Pak
riboflavin	78%	66%
FMN	75%	50%
FAD	48%	57%

(Clarke and Owens, 1983). All tubes which gave a positive test for gas production also tested positive for nitrate reduction, as indicated by the presence of nitrite in the broth.

D. ELECTRON TRANSPORT SYSTEM (ETS) ACTIVITY MEASUREMENTS

Details of this method are given in Chapter 4 (Coble et al. 1989). This assay measures the rate of potential respiratory electron transport when the enyzmes are de-coupled from actual respiratory demand of the cells. It is valid for both eucaryotic and procaryotic cells, and for mitochondrial as well as microsomal electron transport systems (Packard 1985). Ratios of respiration rate to ETS activity vary with the physiological state of the organism, however ETS activity has been shown to be directly proportional to biomass for both phytoplankton (Blasco et al. 1982) and bacteria (Packard et al. 1983). Based on these studies, we have assumed that ETS is constitutive and use ETS activities here simply to estimate the standing stock of metabolically active organisms in the water column.

RESULTS

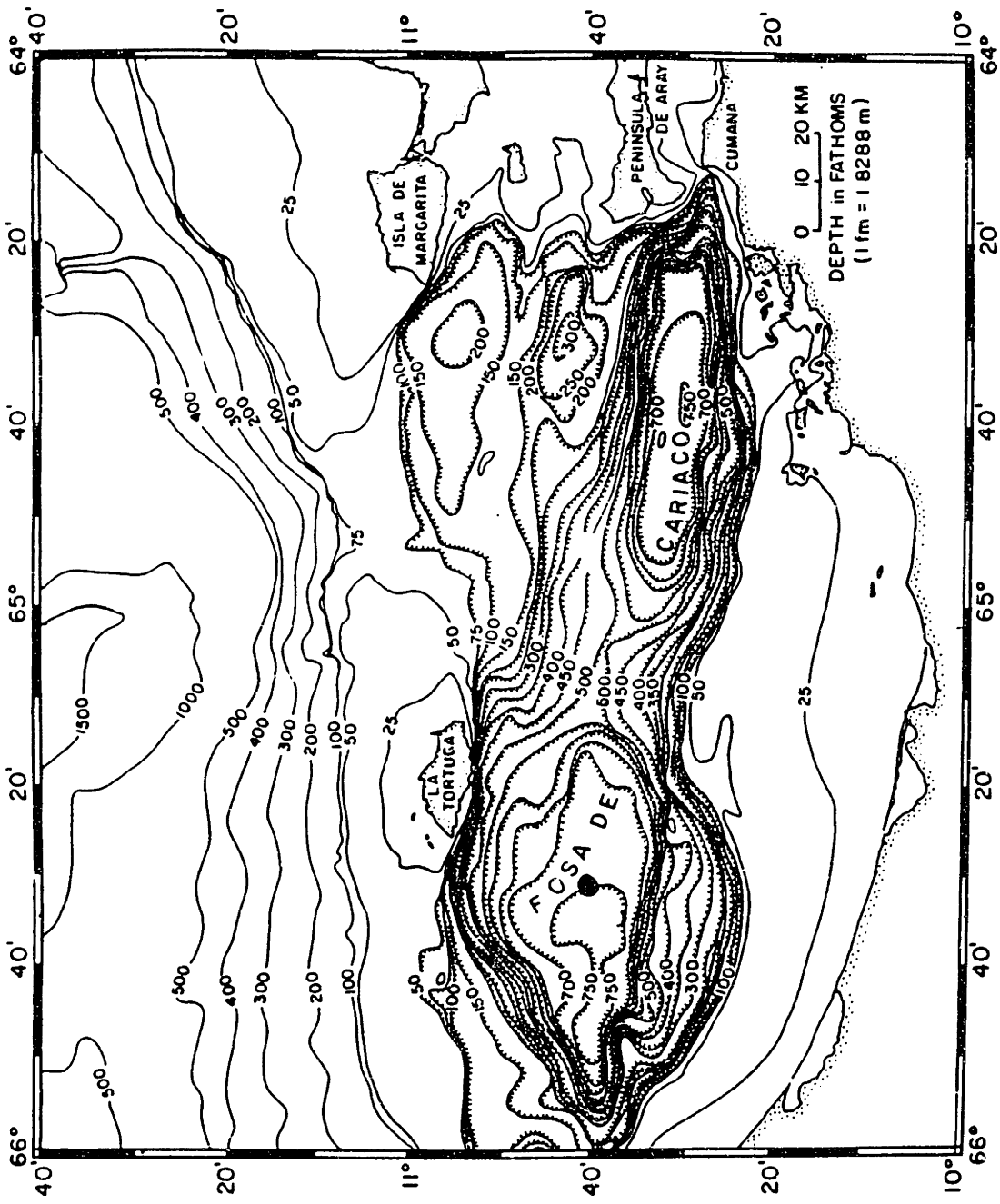
A. THE CARIACO TRENCH

The Cariaco Trench is located on the continental shelf just north of Venezuela (Fig. 5.03). It is the second largest anoxic basin in the world. The deepest point in the western basin is about 1370 m, and the sill which prevents free exchange with the nearby waters of the Carribean has a depth of near 150 m. Geological evidence indicates that the basin is a graben, formed by down-thrust of a block along fault lines (Ball et al. 1971). (see review by Richards 1975 for further descriptive details). All data were collected during cruise CI8601 of the R/V Columbus Iselin in Feb.-Mar. 1986. The sample site was located in the center of the western basin at 10° 40.0' N, 65° 30.0' W.

The vertical distributions of hydrographic and chemical parameters are shown in Figures 5.04 and 5.05. The anoxia of the bottom waters is perpetuated by the presence of a permanent pycnocline located above 200m (Fig. 5.04). Salinity values are highest at the surface due to excess evaporation over precipitation. A surface mixed layer 50 m thick corresponded to the euphotic zone, as evidenced by nitrate depletion (Fig. 5.05). Below a depth of about 300 m, temperature, salinity, and density are nearly constant.

The anoxic interface was located at a depth of around 250m, with dissolved oxygen present above this depth and hydrogen sulfide present below (Fig. 5.05A). Maximum hydrogen sulfide concentrations of near 60 µM were measured near the bottom at 1380 m (Casso et al. 1987). Low oxygen concentrations and rapidly decreasing nitrate concentrations

Figure 5.03. Map of the Cariaco Trench showing the study site located near the center of the western basin.


ISLA DE MARGARITA
PENINSULA DE ARAY
CUMANA
LA TORTUGA
CARIACO
FOSA DE
0 10 20 KM
DEPTH in FATHOMS
(1 fm = 1.8288 m)
64° 40'
20'
11°
40'
20'
10°
66°
65°

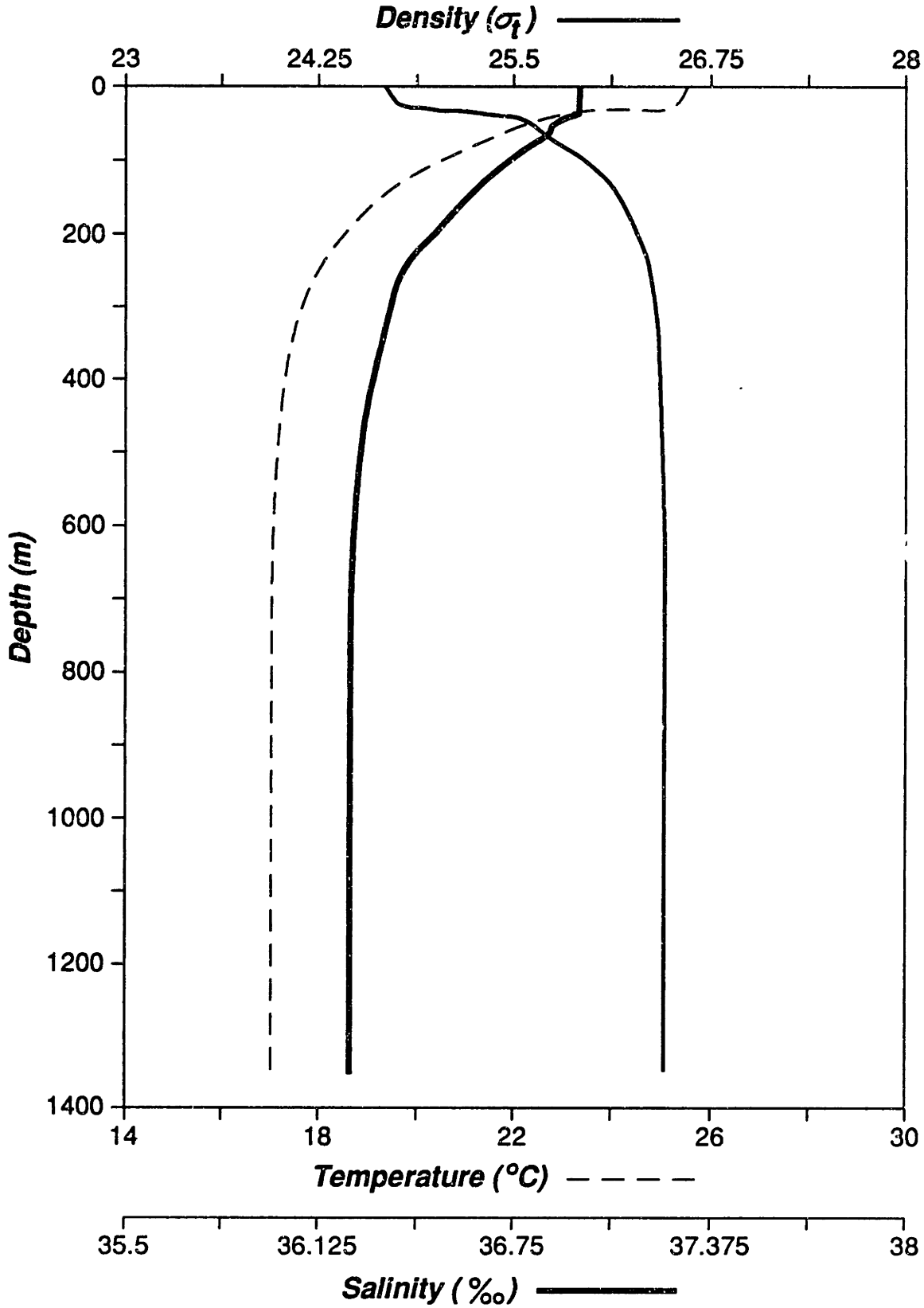
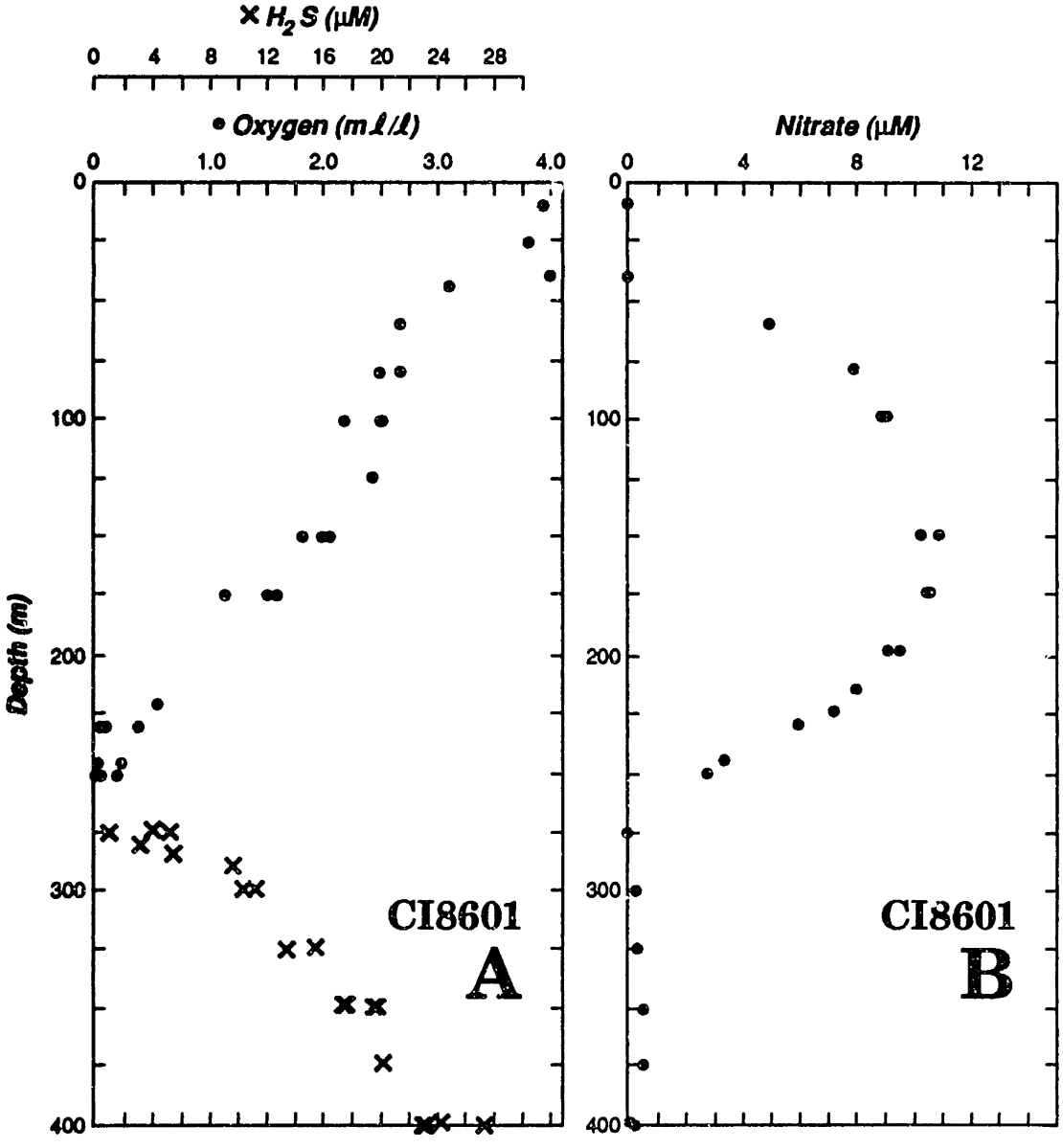


Figure 5.04. Depth profiles of temperature, salinity, and sigma-t in the Cariaco Trench collected by CTD.

Figure 5.05. Depth profiles of (A) oxygen concentration (dots) and hydrogen sulfide (x) and (B) nitrate plus nitrite concentration in the eastern basin of the Cariaco Trench durin March 1986. Results are a compilation of all samples collected from rosette Niskin bottles during Leg I of R/V Iselin cruise CI8601.

Cariaco Trench
March 1986



between 200 and 260 m suggest this is the zone of denitrification (Fig. 5.05). Data for nitrite concentrations were unreliable because they were not stored properly and the activities of nitrifying and denitrifying bacteria present in the samples significantly altered nitrite concentrations prior to analysis.

Flavin concentration data are listed in Table B.01 (Appendix B). Since there was little temporal or spatial variability in profiles of the hydrographic parameters during the study period, data from all casts during the study period were combined. Vertical profiles are shown in Figure 5.06. Riboflavin concentrations below the surface mixed layer were generally less than 5 pM/l, with the exception of a maximum at 220 m depth. This is in the region of low oxygen concentrations and where nitrate concentrations decrease sharply, presumably due to dissimilatory nitrate reduction by denitrifying bacteria. The riboflavin data are in agreement with data collected by others during this same expedition (Mopper and Kieber 1986; Vastano 1988) as to the magnitude, approximate depth of the riboflavin maximum, and overall shape of the profile. Vertical profiles of unknown flavins 0.84 and 0.93 also showed maxima at the depth of the oxic/anoxic interface (Fig. 5.06B).

Estimates of the abundance of denitrifying bacteria based on MPN measurements ranged from 2.3 to 3616 cells per ml (Table B.02, Fig. 5.07), with the highest abundance at a depth of 260 m. Previous estimates of 2 to 20 denitrifier cells per ml based on MPN determinations have been reported for the eastern basin of the Cariaco Trench (Morris et al. 1985). Our report here of a few higher values is probably due to the fact that we concentrated our sampling efforts

Figure 5.06. Depth profiles of riboflavin (A) and two unkown compounds (B) the Cariaco Trench.

Cariaco Trench
1986

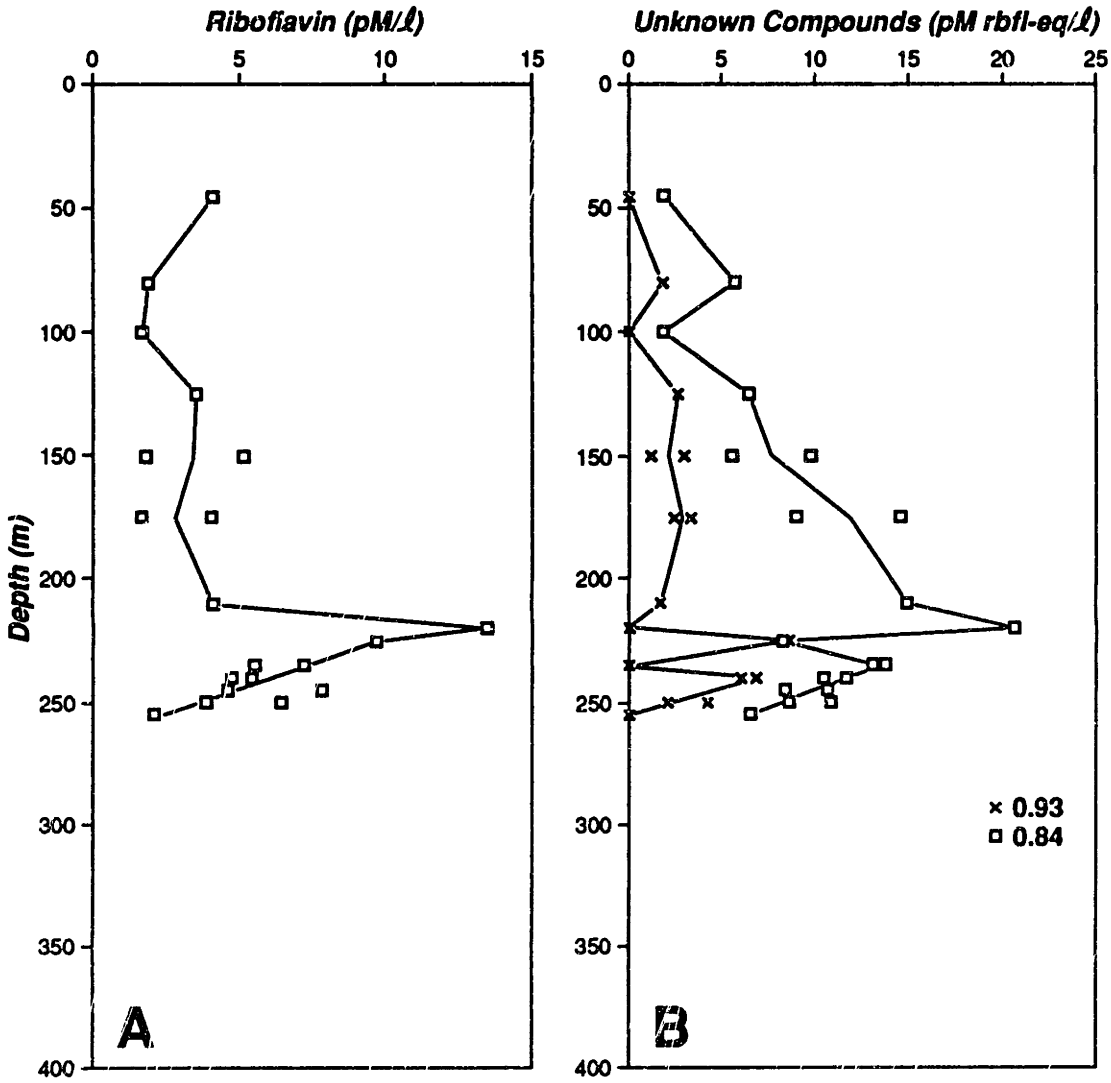
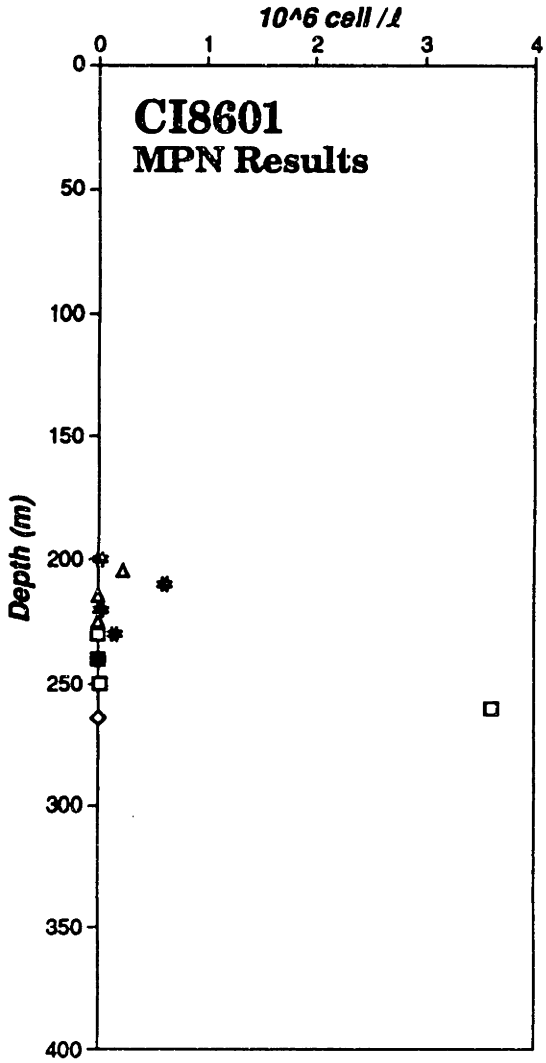


Figure 5.07. Depth profile of results of most probable number (MPN) analysis of denitrifying bacteria in the Cariaco Trench. Different symbols indicate data from separate casts.


10^6 cell /ℓ
0
1
2
3
4
CI8601
MPN Results
Depth (m)
0
50
100
150
200
250
300
350
400

in the denitrification zone. The depth at which maximum values occurred was near the sulfide interface in both studies.

The population of bacteria quantified in the MPN method represents less than 10% of the total bacterial population as measured by acridine orange direct cell counts (AODC) (Ward 1987). In the depth range of 200 to 260 m, total densities range from 3.3×10^4 to 4.0×10^5 cells per ml, with maximum values occurring at 240 and 250 m depth. While some differences are expected due to counting of particles other than live bacteria, this result also illustrates the importance of other bacterial groups within this depth zone. For example, a local maximum in methane oxidation rates coincided with the maximum in AODC (Ward et al. 1987).

Maxima in bacterial biomass as measured by MPN and AODC occurred at depths where much lower cell densities were usually found and therefore may represent "hot spots" distinct from ambient conditions. "Hot spots" of riboflavin concentration (this study; Mopper and Kieber 1986; Vastano 1988), nitric oxide concentration and uptake rates (Zafiriou 1986), and methane oxidation rates (Ward 1986) were also observed during the study period. Unfortunately, these observations were made on samples taken from different Niskin bottles. The nominal depths at which maxima in the various parameters occurred were also different, but the possibility exists that internal waves may displace a particular parcel of water by a distance of up to 10 m. To test this hypothesis, a least squares regression between potential temperature and salinity was run for all CTD data taken between 200 and 260 m from the station in the western basin. The coefficient of correlation was 0.9736 and the standard deviation was 0.0062. Samples

were plotted on the regression line to determine if they showed the same T-S properties. Temperature data for some samples taken on the latter half of the cruise were not available due to equipment failure, so these were plotted based solely on observed salinities and temperatures were interpolated. Data are shown in Table 5.03. It can be seen in Fig. 5.08B that nominal sample depths correctly position the samples in the temperature-salinity field and we can therefore assume that the maxima really did occur at different depths. The difference in salinities of the samples (Table 5.03) is greater than the standard deviation of 0.006, from which we conclude that depth differences were not simply due to displacement of a discrete layer by internal wave motion.

In summary, depth distributions of riboflavin and two unknown flavins in the Cariaco Trench showed maxima in the region of the oxic/anoxic boundary. Maxima in most probable numbers of denitrifying bacteria, total bacterial cell counts (Ward 1986), nitric oxide concentrations, nitric oxide uptake rates (Zafiriou 1986), and methane oxidation rates (Ward 1986) were also observed in this region, but measurements were not made on the same sample of water. It would appear from these observations that parcels of water containing high bacterial activities and/or numbers are randomly distributed in the water column between 210 and 260 m. From our observations they appear to be small, localized and discontinuous in both time and space. Therefore, despite intensive sampling in this zone, we were unable to adequately characterize the number, size or relative importance of these "hot spots".

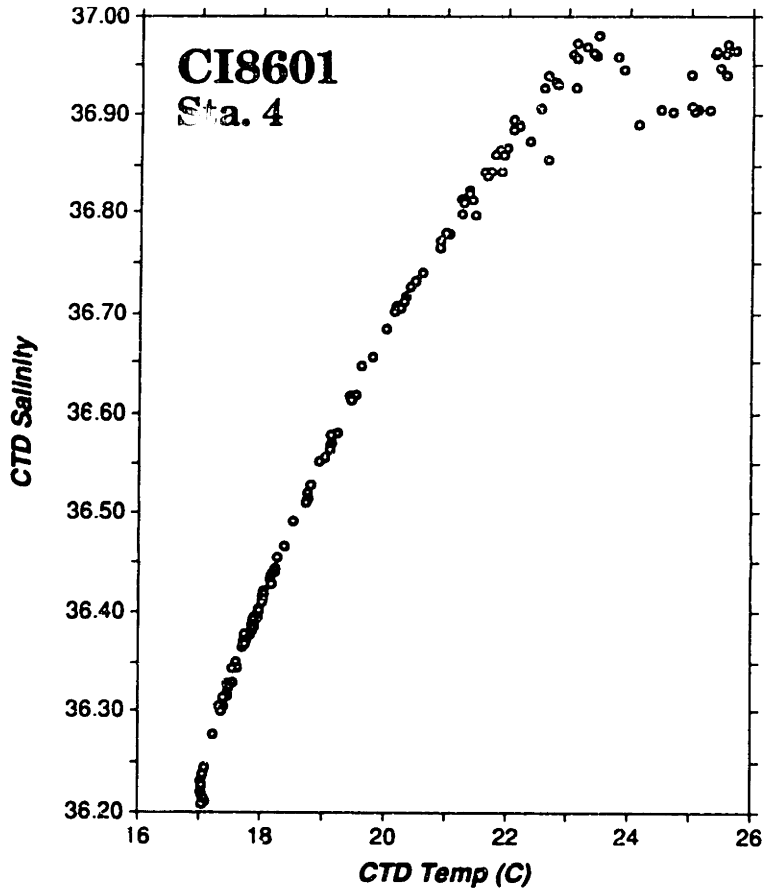
Table 5.03. Summary of maxima in chemical and biological parameters during Cariaco Trench expedition, CI8601. Numbers in left hand column identify points in Fig. 5.09B.

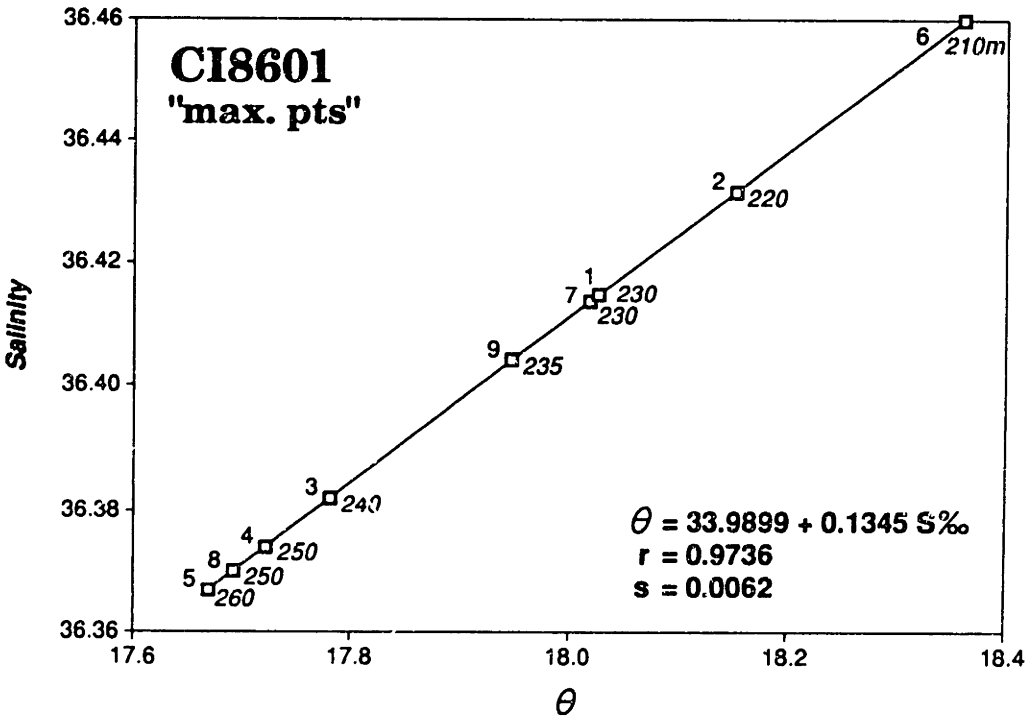
	DEPTH (m)	THETA	SALINITY (ppt)	DATE	CAST	DATA	SOURCE	NITRATE+NITRITE (µM)
1	230	18.025	36.415	MAR 5	14	RBFL	KM	-
2	220	18.151	36.432	MAR 6	17	RBFL	PGC	6.7
3	240	17.780	36.382	MAR 8	552	AODC	BBW	0.1
4	250	17.72	36.374	MAR 8	552	AODC	BBW	0.3
5	260	17.668	36.367	MAR 8	25	MPN	PGC	0.3
6	210	18.360	36.460	MAR 16	601	MPN	PGC	-
7	230	18.018	36.414	MAR 16	601	MPN	PGC	-
8	250	17.691	36.370	MAR 4	523	NO	OCZ	0.3
9	235	17.943	36.404	MAR 15	581	NO	OCZ	2.9

SOURCES:

KM = Mopper 1987
PGC = This study
BBW = Ward 1987
OCZ = Zafiriou 1987

Figure 5.08. A. Temperature-salinity diagram for all data collected by CTD during cruise CI8601 to the Cariaco Trench in March 1986. B. Temperature and salinity properties of samples showing maxima in various parameters collected between 210 and 260 m in the water column of the Cariaco Trench. The regression line for T-S relationship between these points is: $T = 33.9899 + 0.1345\ S$, $r = 0.9736$, $s = 0.0062$. Depth at which each sample was collected in also indicated. The T-S properties correctly position these samples with respect to depth.


CI8601
Sta. 4
CTD Salinity
CTD Temp (C)
37.00
36.90
36.80
36.70
36.60
36.50
36.40
36.30
36.20
16
18
20
22
24
26

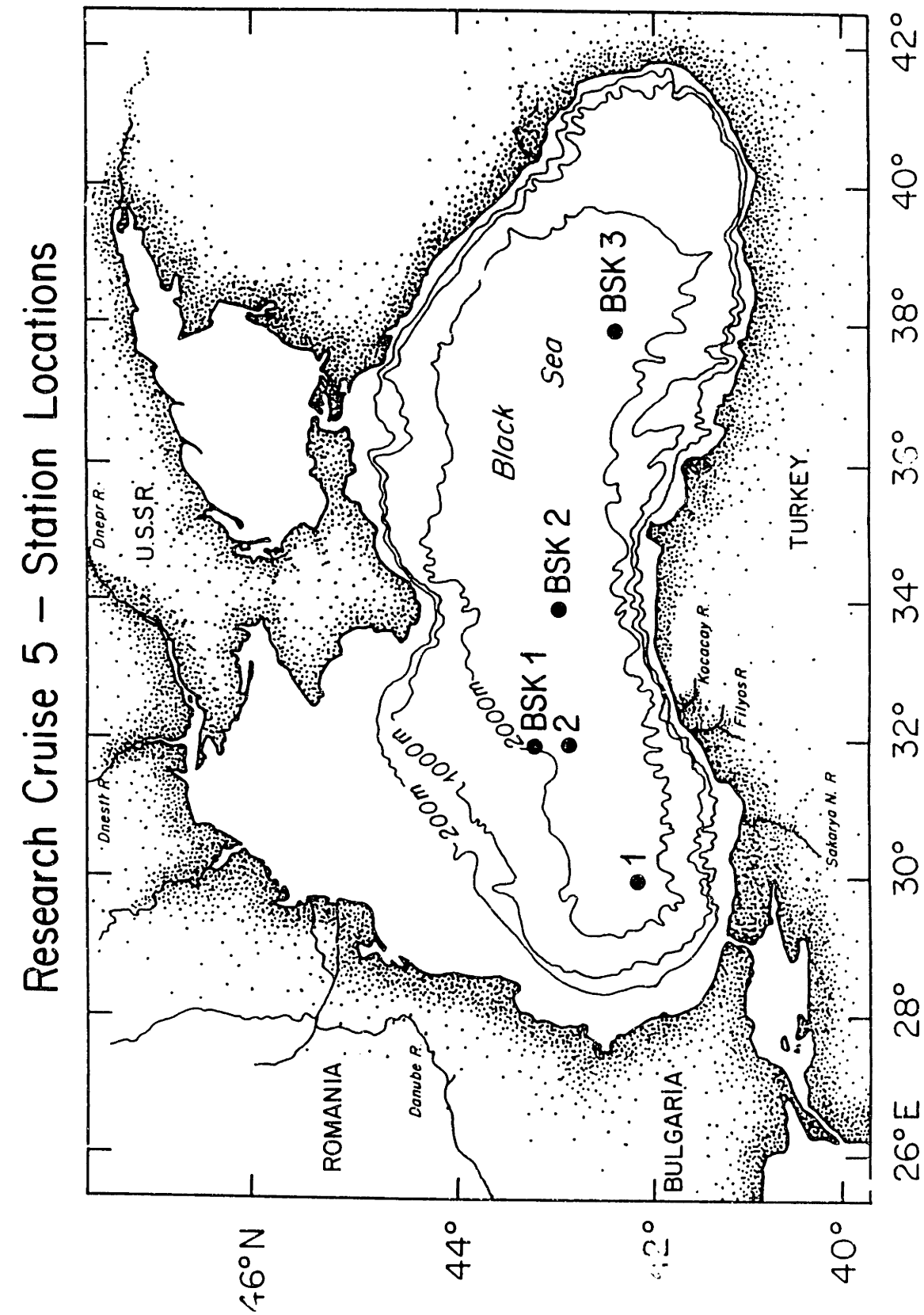

CI8601
"max. pts"
Salinity
θ
θ = 33.9899 + 0.1345 S‰
r = 0.9736
s = 0.0062
1 230
2 220
3 240
4 250
5 260
6 210m
7 230
8 250
9 235
36.46
36.44
36.42
36.40
36.38
36.36
17.6
17.8
18.0
18.2
18.4

B. THE BLACK SEA

The Black Sea is the world's largest anoxic basin, located between the countries of Turkey and the U.S.S.R. (Fig. 5.09). The maximum depth is 2200 m, and a large area in the central part of the basin is deeper than 2000 m (Ross et al. 1974). The major river input is from the Danube (Tolmazin 1985a), and exchange with the waters of the Mediterranean is through the Bosporus Straits, via the Sea of Marmora and the Dardanelles (Tolmazin 1985b). All data were collected during R/V Knorr cruise KN134-12, Leg 5 of the 1988 Black Sea Expedition, during July 1988. The sample site was located near the center of the basin at 43°N, 34°E (Fig. 5.09).

Vertical distributions of the hydrographic and nutrient parameters during the study period are discussed in detail in Chapter 4 (Coble et al. 1989). A thermocline at about 20 m and a halocline at 60 - 70 m divided the water column into three zones - a well-mixed surface layer, a stratified intermediate zone, and an isohaline, isothermal deep zone. Except for small vertical displacements of less than 10 m in the positions of the thermocline, halocline and nutriclines, there was little variation in the distribution of hydrographic parameters from day to day during the study period. In general, vertical profiles of the chemical parameters were similar to those found in the Cariaco Trench, with the important exception that the depth of the oxygen and sulfide interfaces was much shallower in the Black Sea, and there was a suboxic zone where neither oxygen nor sulfide occurred in measurable concentrations (Murray et al. 1989).

Figure 5.09. Map of the Black Sea showing the study site located near the center of the basin.


Research Cruise 5 – Station Locations
U.S.S.R.
ROMANIA
BULGARIA
TURKEY
Black Sea
Dnepr R.
Dnestr R.
Danube R.
Kocacay R.
Filyos R.
Sakarya N. R.
BSK 1
BSK 2
BSK 3
1
2
2000m
1000m
200m
46°N
44°
42°
40°
26°E
28°
30°
32°
34°
36°
38°
40°
42°

During Leg 5 of the expedition, this anoxic zone was located between 100 and 120 m at the study site.

Flavin concentrations were measured from the surface to 270 m at two pump stations. The raw data for all flavins are shown in Table B.03. Riboflavin concentrations (Fig. 5.10A) showed a maximum of 8 pM/l in the euphotic zone, decreased to 2 pM/l at intermediate depths, and showed a secondary maximum of 10 pM/l just below the depth of the sulfide interface at 130 - 140m. FMN concentrations (Fig. 5.10B) were less than 10 pM/l in the oxic zone and undetectable in the anoxic zone. FAD concentrations (Fig. 5.10C) ranged between 10 and 50 pM/l in the oxic zone and were also undetectable in the anoxic zone. Quantitation of FMN and FAD was more difficult than for riboflavin, even though concentrations were the same or higher. This was due to lower fluorescence yield and to peak shapes, which were low and broad.

Lumichrome and lumiflavin are photodegradation products of the other flavins and their distributions reflect this source. Riboflavin, FAD, and FMN are photo-oxidized in a matter of seconds in surface waters to produce lcr and lfl in a ratio of 24:1 (Dunlap and Susic 1986; Mopper and Zika 1986). Lumichrome concentrations (Fig. 5.11A) showed a maximum of 80 pM/l at the surface, then gradually decreased with depth to zero below 140 m. Lumiflavin concentrations (Fig. 5.11B) were comparable to those of riboflavin. Concentrations were low and variable in the upper 120 m of the water column and were undetectable below the sulfide interface. The depth profile for the unknown compound Unk 1 which was tentatively identified as a flavin and was found to be produced by marine bacteria in Chapters 2 and 3 is shown in Fig. 5.11C. The variability in concentration in the upper

Figure 5.10. Depth profiles of riboflavin (rbfl), FMN, and FAD in the Black Sea during cruise KN134-12. Different symbols indicate data collected from two casts separated by a time interval of three days. Horizontal bars connect duplicate values obtained on the same sample.

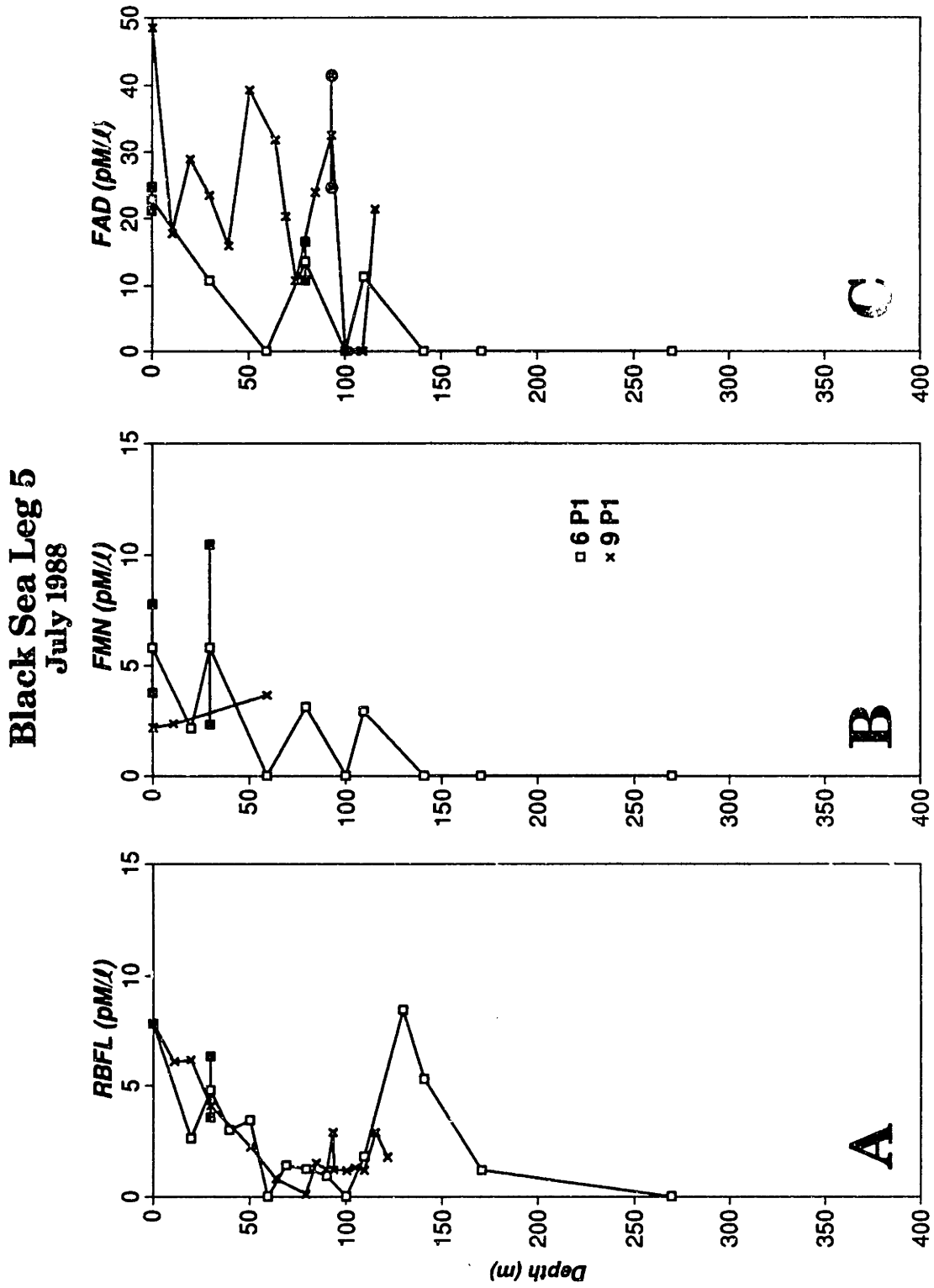
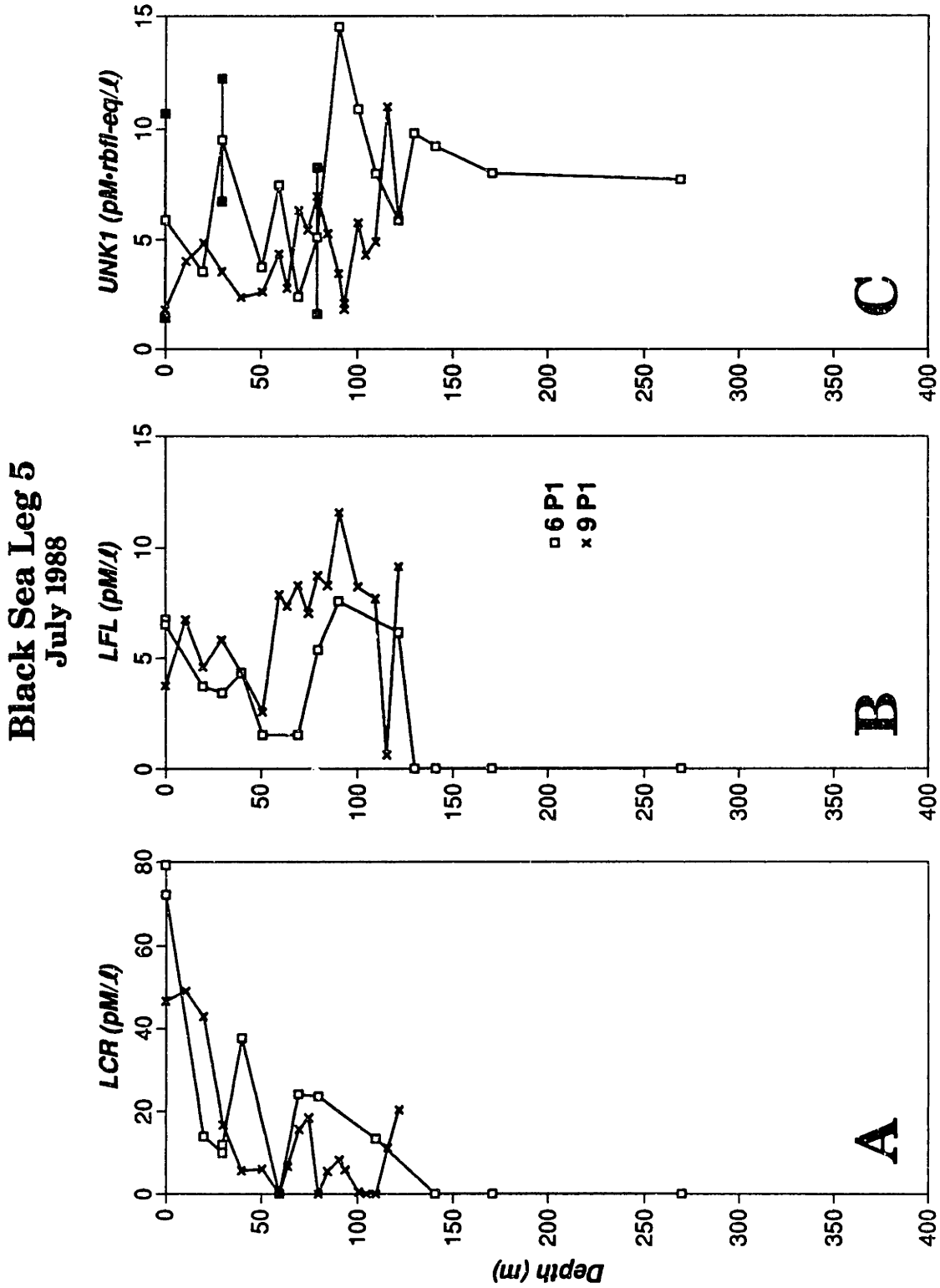

Black Sea Leg 5
July 1988
RBFL (pM/l)
FMN (pM/l)
FAD (pM/l)
Depth (m)
6 P1
9 P1
A
B
C

Figure 5.11. Depth profiles of lumichrome (lcr), lumiflavin (lfl), and unknown 1 (Unk 1) in the Black Sea during cruise KN134-12. Different symbols indicate data collected from two casts separated by a time interval of three days. Horizontal bars connect duplicate values obtained on the same sample.


Black Sea Leg 5
July 1988
LCR (pM/l)
LFL (pM/l)
UNK1 (pM-rbfl-eq/l)
Depth (m)
6 P1
9 P1
A
B
C

100 m is within the range shown by duplicate sample runs and is therefore not significant. However, the fact that this compound was found in the anoxic water is significant and suggests that, unlike lcr and lfl, Unk 1 is produced by processes other than photo-oxidation.

Profiles of three unknown flavins are shown in Fig. 5.12. Unknowns are designated by their retention times relative to riboflavin. Concentrations of compound 1.15 (Fig. 5.12A) showed two maxima, one in the euphotic zone and another at the depth of the sulfide interface. Compound 0.82 (Fig. 5.12B) represented the largest peak in most chromatograms, with concentrations (in riboflavin equivalents) of greater than 100 pM/l in the region of the halocline at 75 m. Concentrations of compound 2.2 (Fig. 5.12C) were low in the upper oxic waters and increased sharply just below the sulfide interface. These three compounds illustrate three distinct patterns of depth distributions which may be related to the role of these compounds in the water column. The distribution of compound 1.15 is similar to that of riboflavin and chlorophyll (Fig. 4.04), and may be indicative of biological production. Compound 0.82 peaks between 50 and 100 m in the cold intermediate waters which originate in the northwest shelf region of the Black Sea (Tolmazin 1985) and therefore may have a terrestrial source. Compound 2.2 is found primarily in the anoxic waters and thus may have as its source one of the processes which are unique to sulfide-bearing waters.

Estimates of living biomass made using the electron transport system (ETS) assay are presented in Table B.04. The highest ETS activities measured were at the surface, with a secondary maximum between 100 and 120 m (Fig. 5.13). Activities in the subsurface

Figure 5.12. Depth profiles of three unknown components present in the C18 fraction of seawater having retention times relative to that of riboflavin of 1.15 (A), 0.82 (B), and 2.2 (C). Data were collected in the Black Sea during cruise KN134-12. Different symbols indicate data collected from two casts separated by a time interval of three days. Horizontal bars connect duplicate values obtained on the same sample.

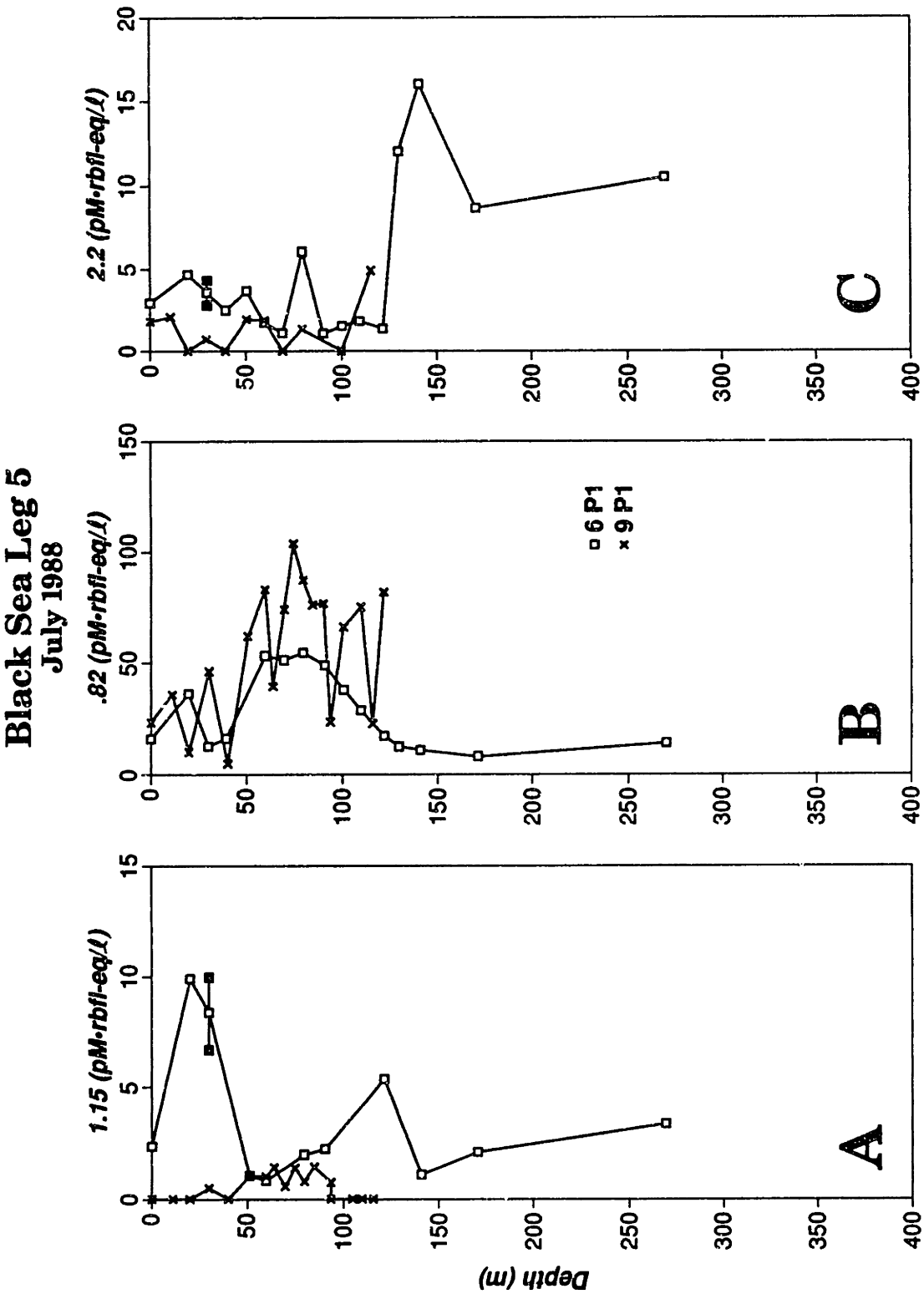
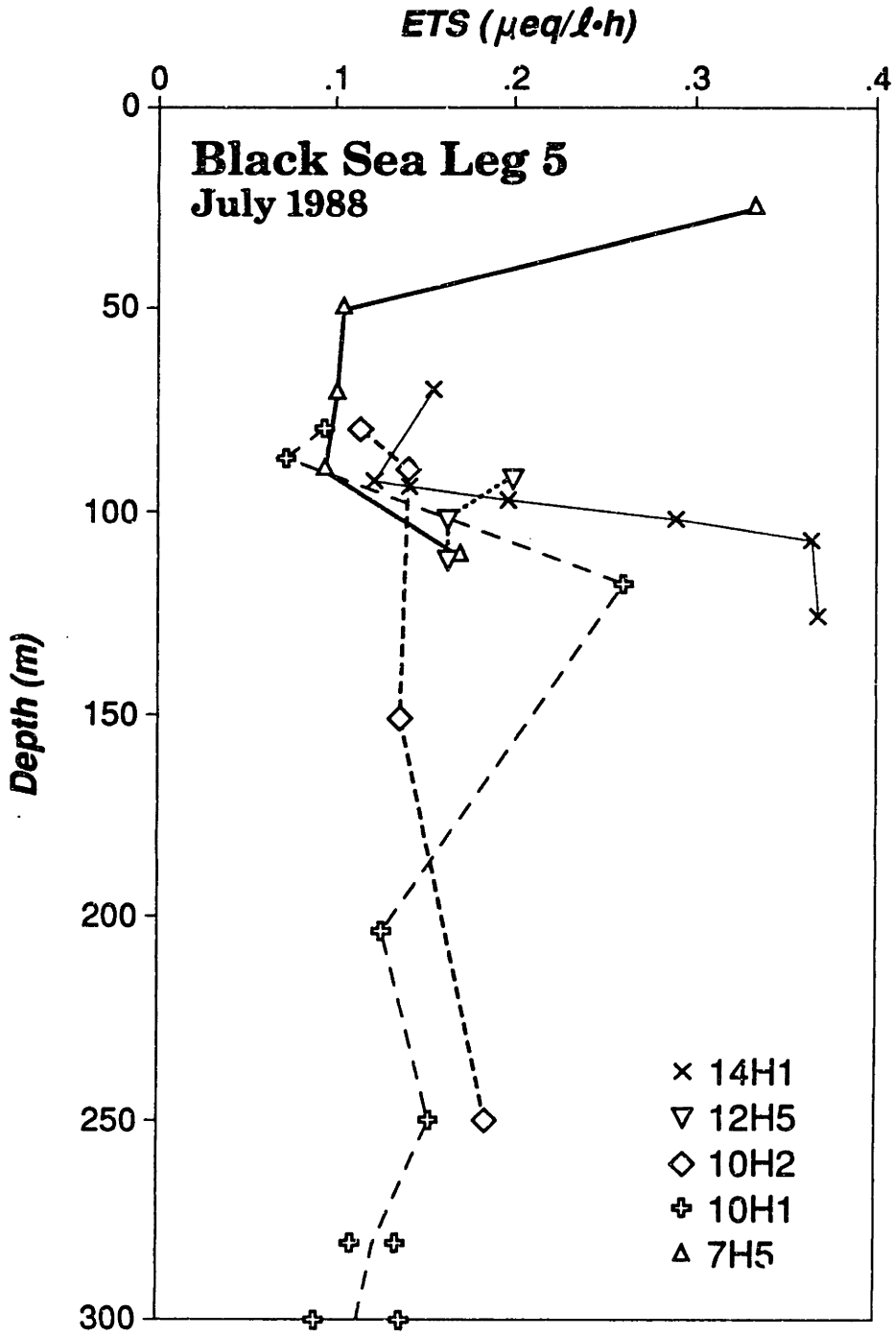

Black Sea Leg 5
July 1988
1.15 (pM•rbfl-eq/l)
.82 (pM•rbfl-eq/l)
2.2 (pM•rbfl-eq/l)
Depth (m)
6 P1
9 P1
A
B
C

Figure 5.13. Depth profile of microbial biomass as determined by electron transport system (ETS) activity assay. Data were collected in the Black Sea during cruise KN134-12.


ETS (μeq/ℓ•h)
0
.1
.2
.3
.4
Black Sea Leg 5
July 1988
Depth (m)
0
50
100
150
200
250
300
× 14H1
▽ 12H5
◇ 10H2
✤ 10H1
△ 7H5

maximum were two times higher than background levels and approximately equal to euphotic zone levels. The depth of the subsurface ETS activity maximum corresponded to the depth of the particle maximum as determined by a maximum in beam attenuation coefficient (Coble et al. 1989; Chapter 4).

Although ETS activities and flavin concentrations were not measured on the same casts, we can assume that highest ETS activities were associated with the particle maximum which was located between 100 - 120 m at 6P1. Riboflavin (Fig. 5.10A) and unknown compounds 1.15 and 2.2 (Fig. 5.12 A,C) all showed maxima in this region of the water column.

Bacterial abundance as determined by total direct acridine orange cell counts (AODC) ranged from 5×10^4 to 1×10^6 (Reeburgh et al. 1988). Values were highest in the euphotic zone and lowest between 75 and 100 m. Values between 100 and 130 m were equal to those at 50-70 m, or about twice as high as those at 100 m. This agrees well with our measurements of ETS activities, however, a sharp maximum in AODC like the one observed in the Cariaco Trench was not observed here.

In summary, flavin concentrations in the Black Sea are generally less than 15 pM/l, except for FAD and lumichrome which attained maximal values of 50 and 80 pM/l, respectively. Riboflavin and two unknown flavins showed maxima at the depth of the sulfide interface where ETS activities were also elevated.

DISCUSSION

A. CONCENTRATION OF FLAVINS IN THE OCEAN

Flavins are present in both the Cariaco Trench and the Black Sea in concentrations ranging from 1 to 100 pM/l. Riboflavin, FMN, and FAD concentrations are highest in the surface waters, undoubtedly as the result of biological input.

The riboflavin profiles also show a secondary maximum below the euphotic zone in the region of the oxic/anoxic interface coincident with elevated bacterial numbers and metabolic rates. The riboflavin maximum was more pronounced in the Cariaco Trench than in the Black Sea, however this may be due to a lower sampling frequency in this zone of the Black Sea. In both study sites, the riboflavin maximum occurred in the denitrification zone. However there were indications from other parameters measured concurrently, such as methane oxidation rates and bacteriochlorophyll e concentrations, that bacteria of other metabolic types were also present. Although no conclusions can be drawn as to the which bacteria may have been responsible for the observed maximum, the data strongly indicate that the source is microbiological.

Lumichrome and lumiflavin concentrations are also highest at the surface, probably as a result of photochemical degradation of the other flavins. There have been no previous reports that these two flavins are biosynthesized, and our own results indicate that they are found in bacterial culture filtrates only after exposure to light (Chapter 2). Results from this study are similar to previous observations of flavins in other regions of the ocean (Vastano 1988).

At the Cariaco Trench site, several parameters showed maxima in the region of rapidly decreasing nitrate and oxygen (210 - 260 m), including riboflavin concentration, MPN of denitrifiers, nitric oxide (Zafiriou 1986), total bacterial cell counts, and methane oxidation rates (Ward 1986). Our initial hypothesis was that maxima in all these parameters (except methane oxidation) would co-occur in a layer at a depth of constant oxygen or nitrate concentration. This was based on the assumption that these "hot spots" represented locally high concentrations of denitrifying bacteria whose distribution was controlled by the vertical distribution of chemical parameters, which we asumed would be fairly constant. Indeed, the depth of the chemoclines (i.e., for oxygen, nitrate, or sulfide) changed little throughout the study period, whereas depth of our presumed indicators of elevated microbial activities varied widely. Concentrations of chemical parameters such as nitrate plus nitrite were not good indicators of "hot spot" distribution, as they were sometimes high and sometimes low (Table 5.03). We have ruled out the possibility that the observed variation in depth of "hot spots" was simply due to displacement of a discrete layer by internal wave motion. The maxima actually occurred at different depths and in different layers of a stratified water column.

Unfortunately, due to the fact that MPN, AODC, methane oxidation, riboflavin concentration, and nitric oxide concentration and uptake rates were not measured in the same bottles, we cannot prove or disprove the initial hypothesis. However, regardless of whether or not there is a correlation between the various parameters, there is strong evidence that individual distributions are extremely patchy in

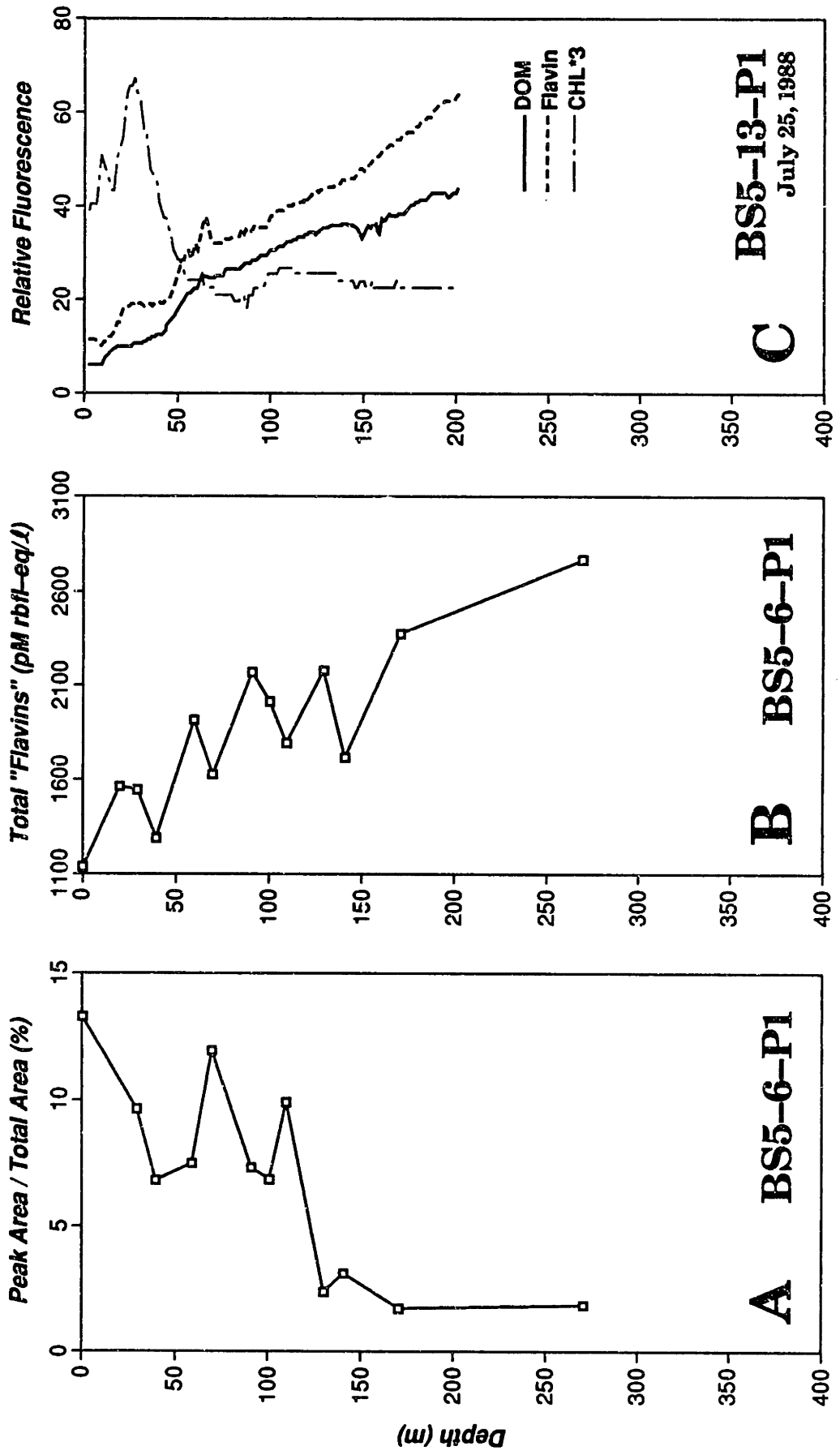
both time and space. The "hot spots" are either very small and rare, or are only active for short periods of time.

A similar analysis for the Black Sea study site is not possible at this time, since data collected by other investigators have not been fully analyzed and collated.

Perhaps the most interesting comparison of the two study sites is the finding that DFC samples from both study sites exhibit the same distribution of components, as evidenced by the similarity in patterns of peaks seen in the HPLC chromatograms (Table 5.01). Peaks having the same relative retention times were observed with such predictability as to suggest that they have some significance even though they represented components of unknown purity and identity. Estimated concentrations of material in these individual unknown peaks were generally less than 15 pM rbfl-eq/l. Despite quite a bit of noise in the data, general trends in the depth profiles suggest that these components have different sources and sinks in the water column and that they may be potentially useful biomarkers.

The material quantified in this study represents only a fraction of the total dissolved fluorescence. We have investigated only the material which is retained on a C18 absorbent cartridge. We know that additional material is retained on Florisil, but have made no estimates of how this compares to the C18 fraction, nor of the amount of material which is not retained by either type of Sep-Pak. Combined peak areas of identifiable flavins in samples from the Black Sea (rbfl, lcr, lfl, FAD, FMN) represent only a few percent of the total area in each chromatogram, ranging from 13% at the surface to 2% at 270 m (Fig. 5.14A). The largest percentage of the area is the

Figure 5.14. Depth distributions of the material present in the C18 fraction of dissolved organic matter (DOM) isolated from seawater in the Black Sea. A. Percent of identifiable flavins (rbfl, FAD, FMN, lcr, lfl) to total DOM. B. Total DOM. Comparisons are based on fluorescence uncorrected for response to various compounds.


A
BS5-6-P1
Peak Area / Total Area (%)
0
5
10
15
B
BS5-6-P1
Total "Flavins" (pM rbfl-eq/l)
1100
1600
2100
2600
3100
C
BS5-13-P1
July 25, 1988
Relative Fluorescence
0
20
40
60
80
DOM
Flavin
CHL*3
Depth (m)
0
50
100
150
200
250
300
350
400

background "hump" of unresolved compounds. This material probably relates in some way to marine humics, which are complex materials derived from soils or formed by complexation of organic compounds in seawater (Kalle 1966). Our method of extracting DOM from seawater is too different from those used in previous studies of humics to permit direct comparisons (Harvey et al. 1983), however if we are indeed able to isolate and analyze these humic materials using our method it may be possible in the future to devise faster and more accurate methods for studying this complex and poorly understood fraction of DOM.

A gross attempt was made to compare estimates of dissolved DOM based on the two methods employed in this study: dissolved fluorescence and HPLC analysis of individual dissolved fluorescent compounds (DFC). An estimate of the total DFC concentration was made by integrating the entire area of chromatograms for samples from station 6-P1 (Fig. 5.14B). Since the detector responded to emission at wavelengths greater than 418 nm, this signal is more like that of DOM fluorescence than flavin fluorescence. However, this is a minor point since the comparison is qualitative and since both fluorescence channels showed a similar depth profile (Fig. 5.14C). Dissolved fluorescence and total DFC in the C18 fraction both show a gradual increase with depth. More work is needed before we can say how much of the dissolved fluorescence signal the total DFC concentration represents, but these results indicate that the two parameters are closely related.

It would be informative to compare our findings with depth profiles of dissolved organic carbon (DOC) in the Black Sea, however there are serious discrepancies in published DOC data. Deuser's

(1971) DOC profile is similar to our dissolved fluorescence and total DFC profiles. He reported a gradual increase in DOC from a value of 125 μM at 200 m to 500 μM just above the bottom. However, data collected only a few months before our study (Karl and Knauer 1988) show concentrations in this same depth range of 60 - 70 μM, with little depth variability. The reason for the discrepancies in DOC results is unclear, but could relate to methodology.

In any event, the predicted relationship between DOC and DFC or dissolved fluorescence is still not known. Dissolved fluorescence and DFC should correlate since both were detected using fluorescence, however only a portion of the total DOC is fluorescent. A significant correlation between DOC and fluorescence has been found for coastal waters (Smart et al. 1976; Laane and Koole 1982) but not for oligotrophic ocean waters (Karabashev and Agatova 1984). In Chapter 4 we present four potential explanations for the distribution of dissolved fluorescence substances in the ocean: release in conjunction with nutrient regeneration, release associated with increased solubilization of mineral phases in anoxic waters, diffusion out of sediments, and degradation due to photo-oxidation. These processes would not be expected to effect DOC and fluorescence in the same way. For example, photo-oxidation may drastically decrease fluorescence while having no effect on the DOC concentration. Diffusion out of sediments would be expected to result in increased concentrations of both DOC and dissolved fluorescence. The process of nutrient regeneration should decrease DOC while increasing fluorescence. This problem needs further study to determine what

fraction of DOC is fluorescent and how that relationship may vary temporally or spatially.

B. THE ROLE OF FLAVINS IN THE OCEAN

Our results indicate that riboflavin does not exist below the euphotic zone simply because it is not photo-oxidized at these depths, but rather it can be produced by indigenous populations of bacteria. Since many species of phytoplankton and bacteria have been shown to put these compounds into their growth media and therefore probably also into the ocean, the puzzle would seem to be not where the riboflavin is coming from, but where does it go? Only a few species of bacteria and phytoplankton have been shown to have a specific growth requirement for riboflavin (Droop 1962; Burkholder 1963; Provasoli 1963; Provasoli and Carlucci 1974). A number of ciliates do require riboflavin (Lilly 1967), however those grazers and predators which are unable to synthesize this vitamin (B2) should be able to obtain their nutritional requirements from the ingested material, and are not likely to need additional dissolved riboflavin.

The importance of flavins to the photochemical degradation of organic matter in the surface waters is totally out of proportion to its contribution to the carbon pool. Estimates of DOC concentrations in the Black Sea are within the range of values reported for the ocean in general, or 50-300 μM C/l between the surface and 2000 m (Sugimura and Suzuki 1986). Even at maximal concentrations of 20 pM/l riboflavin, 50 pM/l FMN and 1000 pM/l FAD, flavins would represent only 0.01-0.10 % of the total DOC. Despite these seemingly minor concentration levels, Mopper and Zika (1986) have shown that

riboflavin at natural seawater concentrations can account for 13 - 70% of the total photosensitizer activity in seawater. They have also shown that it is a significant producer of hydrogen peroxide, which, in turn, can initiate additional degradation reactions. It has been suggested that flavins may be an important pathway by which high molecular weight refractory DOM is recycled in the oceans (Mopper et al. 1987).

Since many denitrifying bacteria are facultative anaerobes, they can grow as well or better in oxygenated waters as in anoxic waters. We could therefore expect them to be present in the surface waters as well as below the euphotic zone. In fact, they were found at nearly all depths sampled in the Peru Upwelling region, from 30 to 1500 m (Coble, unpublished). There has been a recent report that the deep waters in the oceans are more photochemically active than surface waters (Mopper et al. 1987), that is, they support greater rates of photo-oxidation. This finding indicates that there is perhaps a subsurface source of flavins and pterins, and our work identifies one potential source of these compounds. Marine bacteria could represent a source of flavins and other photosensitizers throughout the entire water column.

The significance of this part of our study lies not only in reporting concentrations and vertical distributions of individual fluorescent compounds such as flavins, but also in the report that we are now able to separate "dissolved fluorescence" in the oceans into numerous components. The identities of these components awaits further research. Some of the peaks reported here may not be single compounds, but rather a mixture of two or more compounds. This is one

possible explanation for their erratic distributions in the Black Sea. Improvements in separation technology and improved detectors will eventually result in improved resolution of individual components. Combined with the knowledge of compound identities, we will then be able to assess their oceanic significance, such as their usefulness as biomarkers or their role in the DOM cycle.

C. CONTRIBUTION OF BACTERIA TO FLAVIN AND FLUORESCENCE DISTRIBUTIONS IN THE BLACK SEA

The major objective of this project was to test the hypothesis that an indigenous population of marine bacteria could be a source of fluorescent compounds of sufficient concentration to be detected using in situ fluorometry. Two pieces of evidence have been obtained in support of the hypothesis.

1. A maximum in riboflavin was observed at the depth of the oxic/anoxic interface at both study sites in association with elevated bacterial numbers. In the Cariaco Trench the distribution of high densities of bacteria was very patchy, and different physiological types, including denitrifiers, may have been responsible for the maximum. In the Black Sea, the riboflavin maximum was associated with a layer of the photosynthetic bacterium Chlorobium which persisted at the top of the sulfide zone. The zone of active denitrification was located higher in the water column.

2. Small maxima in flavin fluorescence (Ex/Em = 450/525 nm) were observed in the denitrification zone at the Black Sea site.

(Fluorescence data were not collected at the Cariaco Trench site.) The fluorescence maxima accounted for only about 10% of the total flavin fluorescence signal, but were observed at the same depth in several profiles taken over the course of two days.

We had not expected that flavin fluorescence would be so high in the deep waters. There are no previous reports of flavin fluorescence in the ocean, however the fluorescence properties of DOM had led us to predict that most of the dissolved fluorescence signal would be in the DOM channel (Em/Em = 350/450), and that flavin fluorescence, if measurable, would be closely related to the concentration of dissolved flavins. Our results indicate that the contribution of actual identifiable flavins (riboflavin, FMN, and FAD) to the DFC concentration and to in situ flavin fluorescence is minor, but that this small fraction of the total signal may contain the biological information we set out to find.

The evidence relating in situ flavin fluorescence to the presence bacteria is far from compelling and more work is needed to verify these results. By the same token, however, the original hypothesis cannot be rejected based on the data presented here. Additional detailed profiles are needed in low oxygen waters as well as in oxygenated water columns to establish the relationship between bacteria, dissolved fluorescence, DFC concentrations and DOC in the ocean.

(Fluorescence data were not collected at the Cariaco Trench site.) The fluorescence maxima accounted for only about 10% of the total flavin fluorescence signal, but were observed at the same depth in several profiles taken over the course of two days.

We had not expected that flavin fluorescence would be so high in the deep waters. There are no previous reports of flavin fluorescence in the ocean, however the fluorescence properties of DOM had led us to predict that most of the dissolved fluorescence signal would be in the DOM channel (Em/Em = 350/450), and that flavin fluorescence, if measurable, would be closely related to the concentration of dissolved flavins. Our results indicate that the contribution of actual identifiable flavins (riboflavin, FMN, and FAD) to the DFC concentration and to in situ flavin fluorescence is minor, but that this small fraction of the total signal may contain the biological information we set out to find.

The evidence relating in situ flavin fluorescence to the presence bacteria is far from compelling and more work is needed to verify these results. By the same token, however, the original hypothesis cannot be rejected based on the data presented here. Additional detailed profiles are needed in low oxygen waters as well as in oxygenated water columns to establish the relationship between bacteria, dissolved fluorescence, DFC concentrations and DOC in the ocean.

V. CONCLUSIONS

1. Flavins are present in the waters of the Cariaco Trench and the Black Sea at the level of pM/l. A maximum in riboflavin was found to occur in the denitrification zone in both areas. Results support the hypothesis of *in situ* production of flavins from a bacterial source.

2. Although most of the peaks obtained using IP-HPLC could not be identified, the same pattern of components could be seen in chromatograms for samples from both locations. This suggests that ihis apporach will be useful in characterization of individual components of the dissolved fluorescence in the oceans.

3. Estimates of dissolved fluorescence based on HPLC analysis show general agreement with those obtained using continuous fluorescence profiling. Both data sets show a gradual increase with depth in the Black Sea.

4. The distribution of presumed indicators of high microbial activity ("hot spots") showed an unexpected and previously undocumented degree of heterogeneity.

REFERENCES

Blasco, D., T.T. Packard, and P.C. Garfield. 1982. Size dependence of growth rate, respiratory electron transport system activity, and chemical composition in marine diatoms in the laboratory. J. Phycol. 18: 58-63.

Broenkow, W.W., and J.D. Cline. 1969. Colorimetric determination of dissolved oxygen at low concentrations. Limnol. Oceanogr. 14: 450-454.

Burkholder, P.R. 1963. Some nutritional relationships among microbes of sea sediments and waters, pp. 133-150. In: Symposium on Marine Microbiology. C.H. Oppenheimer, ed. C.C. Thomas. Springfield.

Carpenter, J.H. 1965. The Chesapeake Bay Institute technique for the Winkler dissolved oxygen method. Limnol. Oceanogr. 10: 141-143.

S.A. Casso, Q.S. Hanley and O.C. Zafiriou. 1986. Preliminary data report, R/V Columbus Iselin Cruise CI8601.

Clarke, K.R., and N.J.P. Owens. 1983. A simple and versatile micro-computer program for the determination of 'most probable number'. J. Microbiol. Meth. 1: 133-7.

Cline, J.D. 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. 14: 454-458.

Coble, P.G., R.B. Gagosian, L.A. Codispoti, G.E. Friederich, and J.P. Christensen. 1989. Dissolved fluorescence in the Black Sea. Deep-Sea Res. (submitted).

Demain, A.L. 1972. Riboflavin oversynthesis. Ann. Rev. Microbiol. 26: 369-388.

Droop, M. 1962. Organic micronutrients, pp. 141-159. In: Physiology and Biochemistry of Algae. R.A. Lewin, ed. Academic Press. N.Y.

Dunlap, W.C., and M. Susic. 1985. Determination of pteridines and flavins in seawater by reverse-phase, high-performance liquid chromatography with fluorometric detection. Mar. Chem. 17: 185-198.

Dunlap, W.C., and M. Susic. 1986. Photochemical decomposition rates of pteridines and flavins in seawater exposed to surface solar radiation. Mar. Chem. 19: 99-107.

Gaill, F. and A. Momzikoff. 1975. The presence of riboflavin and two pterins in ascidians (Tunicata) and their excretion into seawater. Mar. Biol. 29: 315-319.

Gentien, P. 1981. Fluorescent metabolites in coral reefs off Townsville, Queensland. Aust. J. Mar. Freshwater Res. 32: 975-980.

Hanley, Q., and O.C. Zafiriou. 1986. Nitrate data. In: Preliminary data report, R/V Columbus Iselin Cruise CI8601. S.A. Casso, Q.S. Hanley and O.C. Zafiriou, eds.

Hungate, R.E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. 14: 1-49.

Momzikoff, A. 1969a. Etude de quelques substances fluorescentes presentes dans deux echantillons de plancton marin. Cahiers Biol. Mar. 10: 429-37.

Momzikoff, A. 1969b. Recherches sur les composes fluorescents de l'eau du mer. Identification de l'isoxanthopterine, de la riboflavine et du lumichrome. Cahiers Biol. Mar. 10: 221-30.

Momzikoff, A. 1973. Mise en evidence d'une excretion de pterines par une population naturelle de copepodes planctoniques marins. Cahiers Biol. Mar. 14: 323-328.

Momzikoff, A., and J.-M. Legrand. 1973. Etude de quelques substances fluorescentes presentes dans un lot de plancton marin naturel compose de copepodes. Identification de l'erythropterine, de la drosopterine, de l'isodrosopterine et de la neodrosopterine. Cahiers Biol. Mar. 14: 249-259.

Momzikoff, A., R. Santus and M. Giraud. 1983. A study of the photosensitizing properties of seawater. Mar. Chem. 12: 1-14.

Mopper, K. and D. J. Kieber. 1986a. Redox sensitive organic compounds in the chemocline of the Cariaco Trench. Extended Abstr. Conf. on Chem. and Phys. Oceanogr. Black Sea, June 2-4, 1986. Goteborg, Sweden.

Mopper, K. and D.J. Kieber. 1986b. Flavin data. In: Preliminary data report, R/V Columbus Iselin Cruise CI8601. S.A. Casso, Q.S. Hanley and O.C. Zafiriou, eds.

Mopper, K., and R.G. Zika. 1986. Natural photosensitizers in sea water: riboflavin and its breakdown products, pp. 174 - 190 In: Photochemistry of Environmental Aquatic Systems. R.G. Zika and W.J. Cooper, eds. American Chemical Society. Washington, D.C.

Mopper, K., R. Sikorsky, D. Kieber and J. McDaniel. 1988. Photochemical incorporation and fragmentation of DOM in relation to oceanic carbon cycling. (abstract) EOS 68(50): 1752.

Morris, I., H.E. Glover, W.A. Kaplan, D.P. Kelly, and A.L. Weightman. 1985. Microbial activity in the Cariaco Trench. Microbios 42: 133-144.

Murray, J.M., H.W. Jannasch, S. Honjo, R.F. Anderson, W.S. Reeburgh, Z. Top, G.E. Friederich, L.A. Codispoti, and E. Izdar. 1989. Unexpected changes in the oxic/anoxic interface in the Black Sea. Nature 338: 411-413.

Packard, T.T., P.C. Garfield, and R. Martinez. 1983. Respiration and respiratory enzyme activity in aerobic and anaerobic cultures of the marine denitrifying bacterium, Pseudomonas perfectomarinus. Deep-Sea Res. 30: 227-243.

Packard, T.T. 1985. Measurement of electron transport activity of microplankton. Advances in Aquatic Microbiology 3: 207-261.

Provasoli, L. 1963. Organic regulation of phytoplankton fertility, pp. 165-219. In: The Sea. Vol. 2. M.H. Hill, ed. Interscience. N.Y.

Provasoli, L. and A.F. Carlucci. 1974. Vitamins and growth regulators, pp. 741-787. In: Algal physiology and biochemistry. W.D.P. Stewart, ed. Univ. Cal. Press. Berkeley.

Reeburgh, W.S., B.B. Ward, S.C. Whalen, K.A. Sandbeck, K.A. Kilpatrick, and L.J. Kerkhof. 1988. Tracer studies of methane oxidation in Black Sea water column and sediments. EOS 69: 114.

Richards, F.A. 1975. The Cariaco Basin (Trench). Oceanogr. Mar. Biol. Ann. Rev. 13: 11-67.

Scranton. M.I., F.L. Sayles, M.P. Bacon, and P.G. Brewer. 1987. Temporal changes in the hydrography and chemistry of the Cariaco Trench. Deep-Sea Res. 34: 945-963.

Sugimura, Y., and Y. Suzuki. 1988. A high-temperature catalytic oxidation method for the determination of non-volatile dissolved organic carbon in seawater by direct injection of a liquid sample. Mar. Chem. 24: 105-131.

Tolmazin, D. 1985a. Changing coastal oceanography of the Black Sea. I: Northwestern shelf. Prog. Oceanogr. 15: 217-276.

Tolmazin, D. 1985b. Changing coastal oceanography of the Black Sea. II: Mediterranean effluent. Prog. Oceanogr. 15: 277-316.

Vastano, S.E. 1988. Processes affecting the distribution of flavins in the ocean. MS Thesis. Univ. Miami. 67p.

Ward, B.B. 1986. Methane oxidation data, In: Preliminary data report, R/V Columbus Iselin Cruise CI8601, S.A. Casso, Q.S. Hanley and O.C. Zafiriou, eds.

Ward, B.B., K.A. Kilpatrick, P.C. Novelli, and M.I. Scranton. 1987. Methane oxidation and methane fluxes in the ocean surface layer and deep anoxic waters. Nature 327: 226-229.

Zafiriou, O.C. 1986. Nitric oxide concentration and uptake rate data. In: Preliminary data report, R/V Columbus Iselin Cruise CI8601. S.A. Casso, Q.S. Hanley and O.C. Zafiriou.

CHAPTER 6

MARINE BACTERIA AS A POTENTIAL SOURCE OF FLAVINS IN THE OCEAN

INTRODUCTION

Flavin excretion is a normal growth process in many bacteria. Certain organisms produce such prodigious amounts that they are grown in large quantities to provide a commercial source of riboflavin. Demain (1972) defined flavin "overproducers" as those organisms which were capable of producing total flavin concentrations in excess of 10 mg/l in culture. "Normal" bacteria produce much less than that amount.

The gradual accumulation of extracellular flavins in growth media is much greater than for other essential compounds. Ratios of excreted (extracellular) to intracellular metabolites range from 0.01 to 0.05 for amino acids, nucleic acid bases, and porphyrins in normal bacteria, as compared to 0.8 to 8.0 for flavins. Absolute amounts of flavins produced are low due to a much slower rate of synthesis. Cell quotas for flavins remain constant during exponential growth, but as production exceeds the cell quota, flavins are released into the growth media. This was termed "overproduction" by Wilson and Pardee (1962) who made a thorough study of the phenomenon. They concluded that the reason for flavin overproduction was the fact that flavin synthesis does not exhibit feedback inhibition, but rather is controlled by repression, which means that flavin biosynthesis is controlled only by the concentration of one or more of the enzymes required for its production. This would seem to insure that flavins are always present in the cell in excess of the amount needed for the protein-bound pool.

Marine bacteria have been shown to produce extracellular flavins (Burkholder 1963), but production rates have not been investigated. We have shown that isolates of marine bacteria produce riboflavin, FMN, and FAD during normal growth (Chapter 2) and that these flavins are present in measurable concentrations in the oceans (Chapter 5). In this chapter, we will present results of laboratory studies in which we measured flavin production rates for two species of marine bacteria. We will develop a simple model using this experimental data as well as field observations to determine the potential contribution of bacteria of this type to the observed ambient concentrations of flavins in the water column of the Cariaco Trench.

METHODS

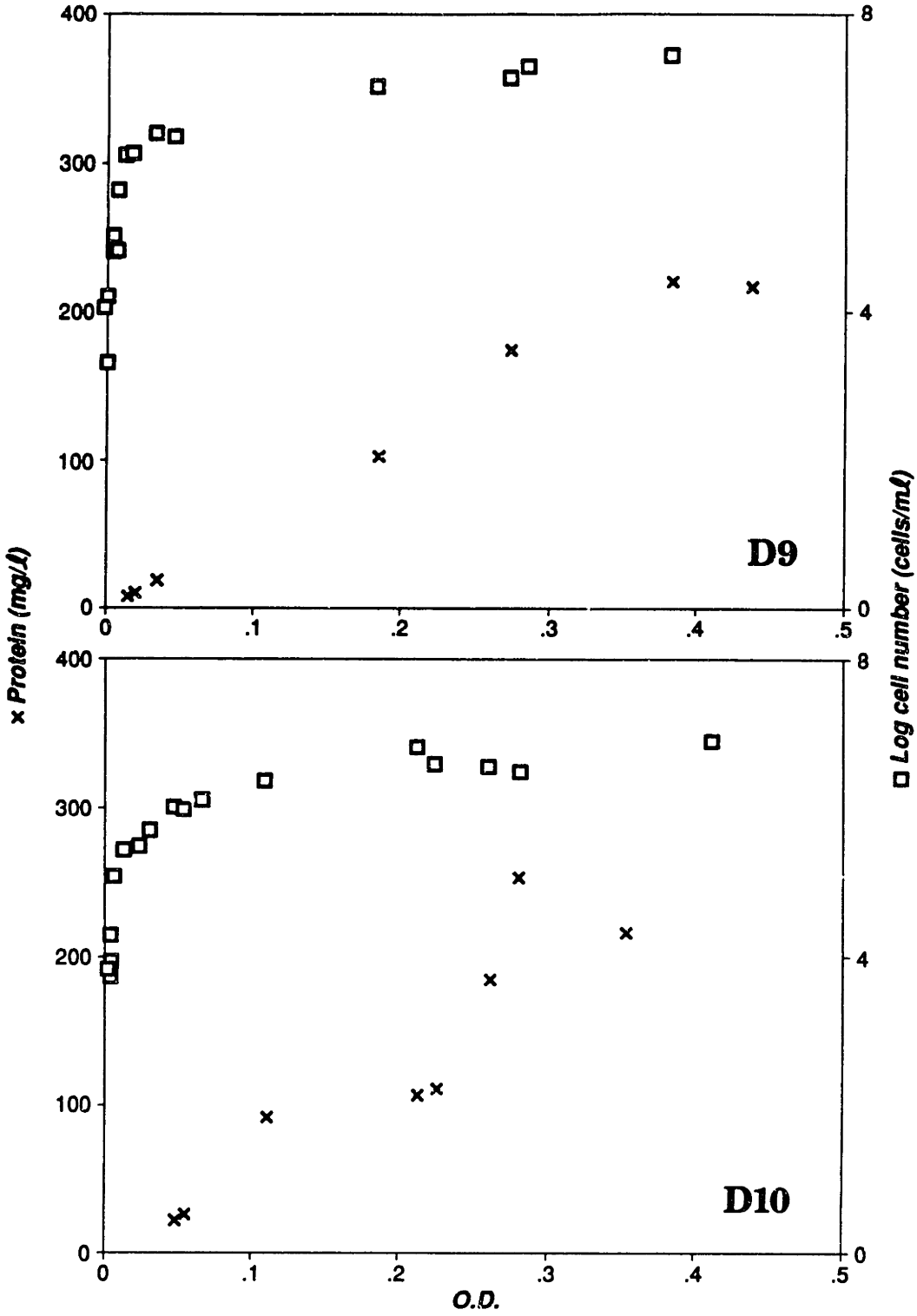
Pure clones of bacteria isolated from the low oxygen waters of the Cariaco Trench in March 1986 were used. (See Methods, Chapter 2 for details of isolation, characterization and media.) Cultures were grown aerobically in 1500 ml Asn-2S media in 2800 ml Fernbach flasks, kept in the dark. Flasks were stoppered with gauze-wrapped cotton through which a sampling siphon had been inserted. Cultures were not stirred, except for ten minutes just prior to and approximately five minutes during the period required for sampling for analysis of biomass and dissolved fluorescent compounds (DFC). A three-way luer-lock fitting was attached to the end of the siphon. Before samples were drawn, a sterile syringe was locked onto this fitting and used to pull off sufficient culture volume to rinse the entire siphon apparatus with at least three volumes. The same syringe was then used to pull off sufficient volume for cell count and protein assay samples. The valve was then opened to permit the culture to flow in a stream which was used first to rinse out a clean, solvent-rinsed graduated cylinder and then to fill the cylinder to the desired volume for DFC analysis. Sample volume for DFC analysis varied between 250 and 50 ml, depending on the age of the culture. The valve was then closed and care was taken not to allow the liquid in the siphon tube to be drawn back into the flask. In this way the sterility of the culture was maintained.

Biomass was measured using three methods. Acridine orange direct cell counts (AODC) were made using a Zeiss epifluorescence microscope on formalin-preserved (2 % final conc.) samples following the

procedure of Hobbie et al. (1977). These same preserved samples were used to measure cell density by optical density at 600 nm. Protein concentration was determined on frozen samples using the Coomassie Brilliant Blue method (Bradford 1976) as adapted for use on bacterial cultures by Nelson et al. (1982). Aliquots of whole culture were incubated at 55°C for 1 hr in 0.1 N sodium hydroxide, then neutralized with 1.0 N phosphoric acid. The remainder of the procedure, including concentration of dye added, depended on amount of protein in the samples, and the assay followed the recommendations outlined by BioRad in the instruction manual accompanying the reagent. Cell protein concentrations were standardized daily versus egg albumin.

The relationships between the three biomass measurements are shown in Figure 6.01. The relationship between turbidity of the culture (O.D.) and cell counts was best described by a log-log fit for both clones. For D9 that equation was: log(cells/ml) = 8.32 + 1.44 log(OD 600), r = 0.9620, n = 17. For D10 the relationship was: log(cells/ml) = 7.53 + 1.31 log(OD 600), r = 0.9567, n = 16. Protein concentrations showed a linear correlation with OD for both clones. The linear regression equation was protein (µg/ml) = 7.73 + 536 OD 600, r = 0.9792, n = 8 for D9, and protein (µg/ml) = -9.11 + 700 OD 600, r = 0.9183, n = 8 for D10. Protein per cell ranged from 5 to 12 pg for D9 and from 23 to 82 pg for D10. There was a slight increase in protein per cell with age in the D10 culture, but not in D9. Such a result could be caused by overestimate of protein in the presence of extracellular proteins, by underestimate of cell densities due to excessive clumping, or by an actual change in cell composition during post-exponential growth phase.

Figure 6.01. Cell number (squares) and protein concentration (x) as a function of cell density as measured by optical density (O.D.) for clones D9 (A) and D10 (B).


400
300
200
100
0
8
4
0
0
.1
.2
.3
.4
.5
D9
D10
× Protein (mg/L)
□ Log cell number (cells/mL)
O.D.

RESULTS

A. GROWTH EXPERIMENTS

Experimental results are shown in Fig. 6.02 and Table 6.01 for D10. Increase in dissolved extracellular flavins paralleled increase in cell numbers for the first two days after inoculation, during which time the cells were growing exponentially. As cells entered stationary growth phase, the concentrations of flavins increased only slightly. Experimental results are shown in Fig. 6.03 and Table 6.02 for clone D9. Cells of this clone were smaller than D10 and had higher growth rates. Again, flavin concentrations paralleled cell density.

Normalized to a per cell basis (Fig. 6.04), large concentrations of extracellular FAD are produced by both clones. D9 produced lower concentrations of every flavin on a per cell basis than did D10, perhaps because of its smaller size. There seems to be a slight increase in riboflavin and FMN per cell with increasing age in batch culture for clone D10, but not for clone D9. FAD per cell increased with age for both clones.

The intracellular content of a few bacteria are shown in Table 6.03. Clostridium kluyveri is classified as a weak overproducer, according to Demain (1972) because it produces more than 10 mg flavin per liter. Its intracellular flavin content is 6.0 μM/l. "Normal" micro-organisms produce less than 10 mg/l and have an intracellular flavin content of 0.21 to 0.33 μM/g protein, or an order of magnitude less than weak overproducers. This value has been found to be fairly constant for a wide range of bacterial types, regardless of growth

Table 6.0∮. Growth study results for clone D10.

Age (days)	cll/nl	O.D.	Protein (ug/ml)	Rbfl (nM/l)	FMN (nM/l)	FAD (nM/l)	rbfl/cll (fg/cll)	FMN/cll (fg/cll)	FAD/cll (fg/cll)	FMN:rbfl	FAD:rbfl	FAD:FMN
.00	.006	.003										
.09	.007	.000										
.10		.006										
.20	.009	.004										
.47	.019	.003										
.96	.301	.025										
.96	.118	.006										
1.18	.270	.015		.090	.061	.227	.126	.108	.661	.855	5.258	6.149
1.20	.482	.032										
2.00	.970	.048	22.40	.425	1.045	3.055	.165	.515	2.474	3.125	15.000	4.800
2.00	1.230	.067										
2.14	.940	.055	25.60	.491	1.101	5.844	.197	.561	4.884	2.852	24.855	8.714
2.21		.065										
3.00	6.230	.212	106.00									
3.02	2.230	.110	91.20	.399	.987	3.974	.067	.212	1.400	3.142	20.777	6.612
3.03		.091										
4.08		.120										
6.09	3.850	.224	110.00	4.251	3.345	33.097	.416	.416	6.753	1.000	16.250	16.250
7.00	3.500	.261	185.00	2.126	2.843	17.414	.229	.389	3.909	1.700	17.100	10.059
7.19	3.740			2.657	1.902	23.907	.267	.243	5.021	.910	18.780	20.637
7.98	3.080	.281	252.00									
10.04		.353	216.00									
11.00	3.480		264.00	5.553	10.975	27.789	.601	1.509	6.273	2.512	10.445	4.158
16.00	7.890	.412		16.128	14.529	60.339	.769	.881	6.008	1.145	7.809	6.820
mean							.301	.525	4.080			
s.d.							.230	.430	2.200			

Figure 6.02. Growth curve and extracellular flavin concentration for clone D10 in batch culture.

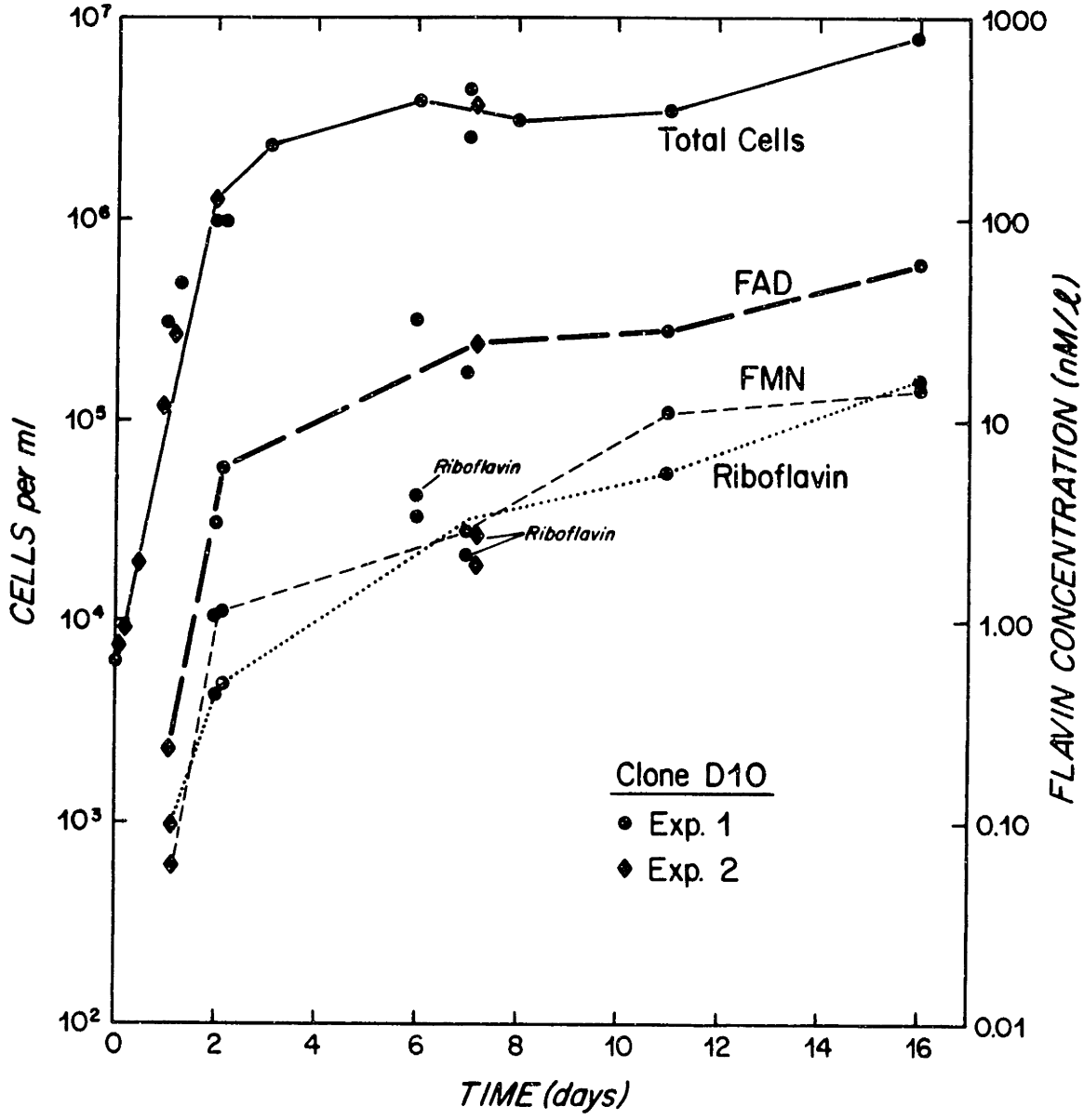

Total Cells
FAD
FMN
Riboflavin
Riboflavin
Riboflavin
Clone D10
Exp. 1
Exp. 2
CELLS per ml
FLAVIN CONCENTRATION (nM/ℓ)
TIME (days)
10⁷
10⁶
10⁵
10⁴
10³
10²
1000
100
10
1.00
0.10
0.01
0
2
4
6
8
10
12
14
16

Figure 6.03. Growth curve and extracellular flavin concentration for clone D9 in batch culture. Data from experiment #2 were shifted by 0.6 days so that growth curves would overlap.

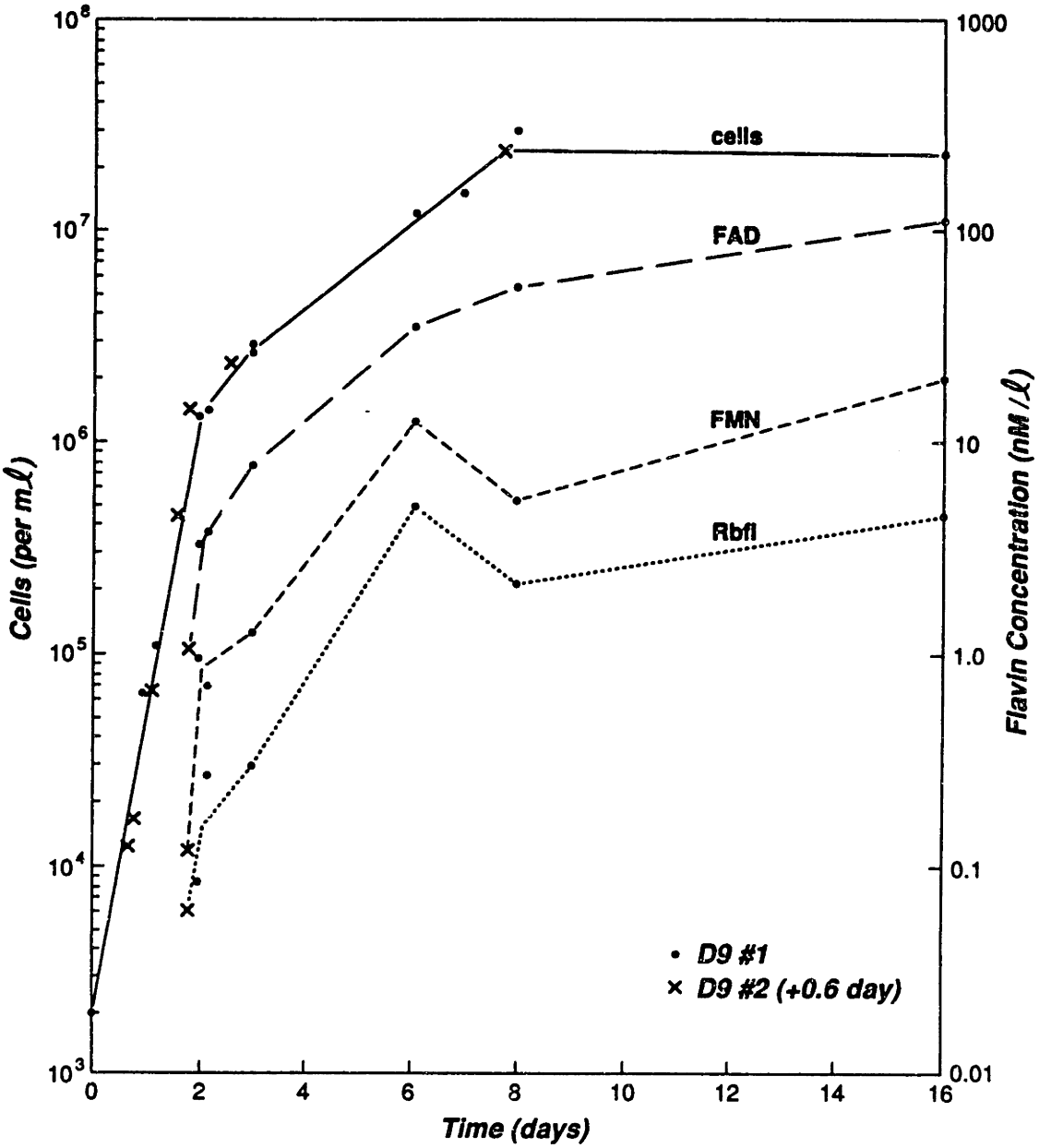
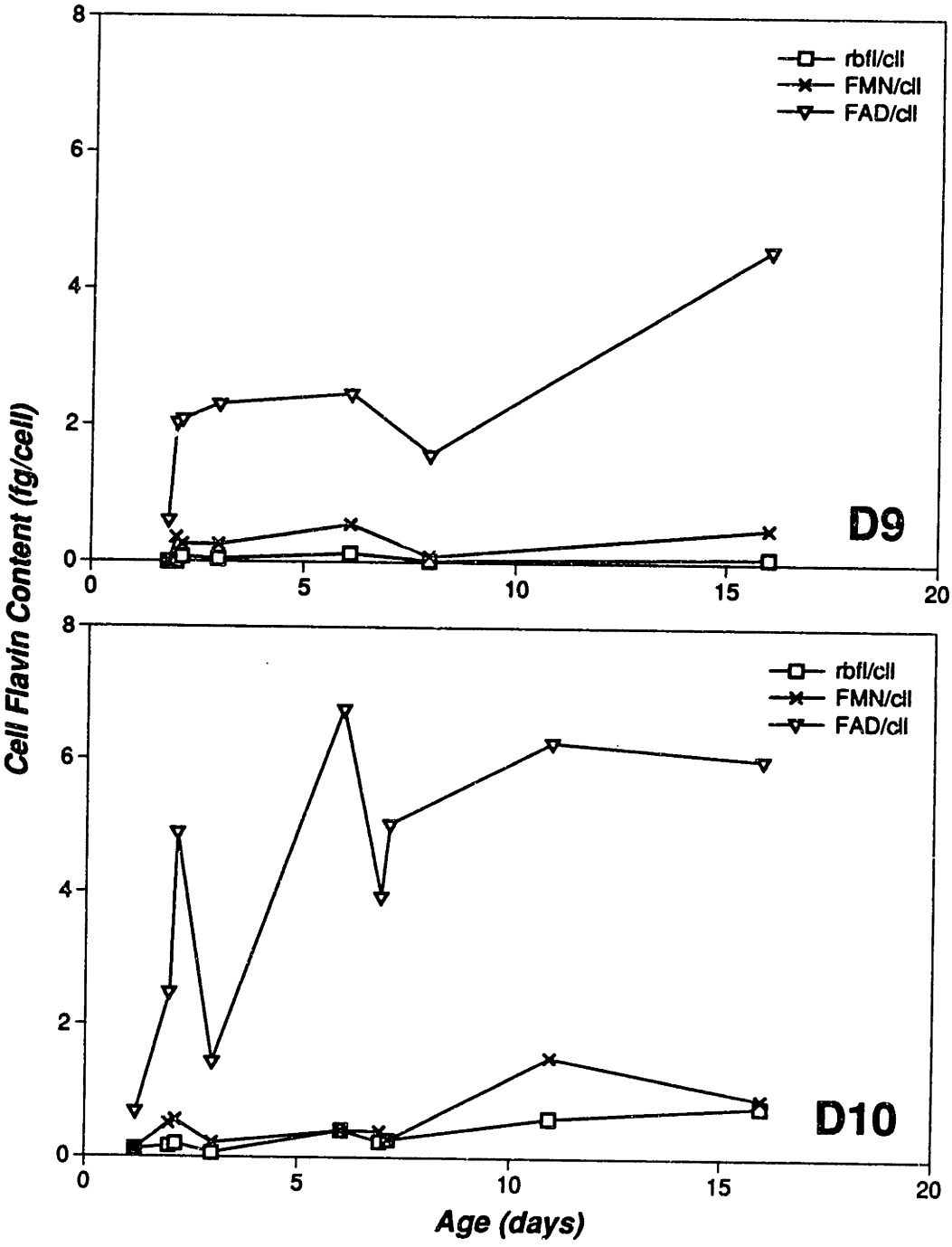

cells
FAD
FMN
Rbfl
• D9 #1
× D9 #2 (+0.6 day)
Cells (per mℓ)
Flavin Concentration (nM/ℓ)
Time (days)
10^8
10^7
10^6
10^5
10^4
10^3
1000
100
10
1.0
0.1
0.01
0
2
4
6
8
10
12
14
16

Table 6.02. Growth study results for clone D9.

Age (days)	cll/nl	O.D.	Protein (ug/ml)	Rbfl (nM/l)	FMN (nM/l)	FAD (nM/l)	rbfl/cll (fg/cll)	FMN/cll (fg/cll)	FAD/cll (fg/cll)	FMN:rbfl	FAD:rbfl	FAD:FMN
.69	.011	.000										
.00	.002	.001										
.80	.016	.001										
.96	.064	.005										
1.08	.067	.008										
1.20	.102	.004										
1.57	.442	.009										
1.79	1.420	.019		.069	.116	1.044	.018	.039	.577	2.128	31.371	14.743
2.00	1.310	.015	7.20	.082	.979	3.398	.023	.357	2.037	15.250		5.701
2.13	1.370	.020	9.60	.260	.709	3.629	.071	.247	2.081	3.463	29.125	8.409
2.60	2.270	.047										
2.80		.044										
3.00	2.650	.035	18.40	.303	1.300	7.839	.043	.235	2.324	5.452	54.008	9.906
3.60		.077										
4.65		.126										
6.11	11.500	.185	102.00	3.986	13.045	36.025	.130	.543	2.461	4.160	18.867	4.535
7.00	14.300	.274	173.00									
7.80	23.100											
7.98	28.700	.384	220.00	2.179	5.519	57.055	.029	.092	1.562	3.220	54.659	16.977
10.04		.438	216.00									
16.00	20.700	.287		4.836	21.533	120.207	.088	.498	4.562	5.659	51.885	9.168

Figure 6.04. Extracellular flavin concentration per cell as a function of age in batch culture for clones D9 (A) and D10 (B).


rbfl/cll
FMN/cll
FAD/cll
D9
D10
Cell Flavin Content (fg/cell)
Age (days)
0
2
4
6
8
0
5
10
15
20

phase (Wilson and Pardee 1962). All the species of Psuedomonas listed in Table 6.03 would thus be classified as "normal". We do not have any measurements of intracellular concentrations for our clones, however ratios of extracellular to intracellular flavins have been found to range from 0.8 to 8.0 (Wilson and Pardee 1962). Our values for extracellular flavin content ranged from 0.29 - 0.62 μM/g protein for D9 and from 0.12 - 0.37 μM/g protein for D10 Table 6.03). If we use a ratio of 1.5 for extracellular to intracellular flavins, we calculate intracellular values of 0.08 to 0.41 uM/g protein for our marine clones (Table 6.03). These concentrations are in the range for "normal" cells and are too low to qualify our clones as weak overproducers.

Previous workers have reported that the intracellular riboflavin content of cells is so low as to be attributable solely to breakdown of FAD and FMN. Values ranged from less than 3% of total flavin content in animal tissue (Bessey et al. 1949) to less than 10% for microorganisms. However, riboflavin is reported to be a major component of extacellular flavins (Demain 1972). Hence, our finding of extracellular FAD concentrations roughly 10-fold higher than riboflavin is surprising. The increase in FAD per cell with age of the culture suggests that flavins in the media are not the result of breakdown of intracellular flavin nucleotides, but rather, may be the result of release of flavin bound to proteins.

B. FLAVIN PRODUCTION RATE ESTIMATES

The purpose of estimating production rates is two-fold. We can compare rates from our clones to published values for other bacteria

Table 6.03. Intracellular and extracellular flavin content of some bacteria.

INTRACELLULAR FLAVIN CONTENT

	nMFAD[0] (gm dry wt.)	nM flavin[0] (gm dry wt.)	nM flavin[1] (gm protein)
Clostridia kluyveri	2140	2930	6000
Escherichia coli	81	245	300-700
Pseudomonas fluorescens	69-256	108-287	210-300
P. stutzeri	185	310	--
P. aeruginosa	217	360	--
Marine clones			
D9			190-410*
D10			80-250*

EXTRACELLULAR FLAVIN CONTENT

	nM flavin[2] (gm protein)
Marine clones	
D9	290-620
D10	120-370

[0]Peel, 1958
[1]Demain, 1972
[2]This study
* Calculated using ratio of 1.5 for extracellular:intracellular flavin content.

to see if they are reasonable, and we can evalute the relative importance of our clones to flavin concentrations observed in the oceans. The following equations are based on assumptions of balanced growth, which implies that production of a metabolite is controlled by a specific cell quotient. During exponential growth phase when nutrients are not limiting growth, the cell quotient is an intrinsic property of the cell. The following approach was patterned after Taylor and Jannasch (1976).

In order to estimate production rates of riboflavin, we must assume that its concentration in the media is related to its rate of production by the cells. The rate of change in concentration of the metabolite is therefore proportional to the cell density:

$$dC/dt = qN$$

where C = concentration of metabolite (moles/volume), t = time, q = the metabolism quotient (moles/cell x hr), and N = cell density. This equation is valid during "balanced" growth, when the the cell density can be expressed as an exponential:

$$qN = RN_0 \, e^{ktln2}$$

where R = metabolite production rate, k = reciprocal doubling time (hr^{-1}), and N_0 = initial cell density. The total concentration of metabolite in the media at any given time is equal to the integral from time = 0 to time = t, or after integration:

$$TC = (RN_0/kln2)(e^{ktln2} - 1)$$

where TC equals the total concentration of the dissolved compound. After 3 generation times, e^(ktln2) >> 1, and the total metabolite

concentration is proportional to growth. The equation can be simplified to:

$$\ln(TC) = (RN_0/k\ln 2) + k\ln 2\ t$$

and solved either graphically or, as we have done here, by linear regression for the intercept, $(RN_0/k\ln 2)$. N_0 and k were taken from the intercept and slope calculated by least squares regression analysis of cell count data. TC was measured by HPLC analysis of individual flavins, so R can be calculated.

Results of production rate calculations are listed in Table 6.04. Rates of total flavin production ranged from 4 to 12 aM (1 aM = 10^{-18} moles) per cell per day. FAD production rates represented 60 - 85 % of total flavin production rate and magnitudes were similar for D9 and D10. Rates of FMN production were about 10 - 25 % of total rates, and again were of similar magnitude for the two clones. Riboflavin production rates were lowest, accounting for less than 10 % of the total (excluding one value of 24 % for D10), and were lower for D9 than for D10. Our extracellular production rates show ratios of FAD:FMN:riboflavin of 7:2:1, which are more typical of previously reported intracellular than extracellular ratios.

Average rates for coenzyme synthesis are about 10 molecules per cell per sec (McIlwain 1946), which is equivalent to 1.4 aM per cell per day. The rates of flavin synthesis measured here are 3 to 8 times higher. Wilson and Pardee (1962) found total protein-normalized flavin production rates to be constant with time during exponential growth of Escherichia coli at a rate of 0.3 to 0.9 µM/ g protein/ hr. Based on average protein content per cell, our rate estimates fall in

Table 6.04. Flavin production rates for clones D10 and D9 calculated based on data shown in the first four column and equations given in text. Production rates (R) are in units of a moles per cell per day.

clone	t (day)	No (cl/ml)	k (/day)	e^(ktln2)	rbfl (ug/l)	FMN (ug/l)	FAD (ug/l)	R(rbfl) (a moles/cell/day)	R(FMN) (a moles/cell/day)	R(FAD) (a moles/cell/day)
D10	1.18	4680	4.87	54	.034	.029	.179	1.214	.814	3.061
	2.00	13500	3.28	94	.160	.500	2.400	.759	1.866	5.453
	2.14	13500	3.28	130	.185	.527	4.590	.638	1.430	7.586
D9	1.19	7590	6.25	173	.026	.056	.820	.227	.385	3.437
	2.00	2450	4.45	478	.031	.468	2.670	.217	2.578	8.957
	2.13	2450	4.45	713	.098	.339	2.850	.460	1.251	6.402

the range of 0.83 to 15.0 μM/g protein/hr. Again, our rates are somewhat higher, however it is hard to compare results directly since we did not measure intracellular flavin content.

Our results show that flavins are produced primarily during active growth and suggest that their appearance in the culture media results from some leakage out of the cell associated with normal growth processes. The slight increase in concentrations during stationary phase may not represent new production but may result from lysed or damaged cells. If the intracellular concentrations are about equal to the extracellular concentrations, as is the case for most bacteria (Wilson and Pardee 1962), we would expect only a 2-fold overall increase in concentration in the media due to release of the intracellular pool. This agrees well with the observed 2- to 3-fold increase observed in total flavin concentration between days 6 and 16 in both clones.

C. MODEL RESULTS FOR THE CARIACO TRENCH

Flavin production rates determined in the laboratory can be used to estimate potential *in situ* production rates in the Cariaco Trench between 200 and 260 m by extrapolation. We have constructed a simple model based on the following assumptions: 1) bacteria are growing exponentially, 2) there is no grazing of bacteria under these low oxygen conditions, 3) there are no losses of flavins through advection or uptake, and 4) maximum riboflavin concentrations are 14 pM (Chapter 5). A summary of pertinent observations are shown in Table 6.05. Estimates of the rate of riboflavin production from triplicate samples for each of two clones range from 0.08 to 0.46 fg/cell/day, at

Table 6.05. Data used in model and model results. R = riboflavin production rate, k = growth rate. Projected FMN and FAD concentrations are based on minimum and maximum rates of production of these compounds listed in Table 6.04.

Initial cell # (cells/l)	R (fg/cell/d)	k (/day)	Time Required (day)	Projected [FMN] (pM/l)	Projected [FAD] (pM/l)
clone D10					
6×10^3	0.232	3.3	4	20	75
1×10^6	0.457	5.5	1	22	90
clone D9					
6×10^3	0.081	4.0	3.7	54	488
1×10^6	0.173	7.0	1.0	68	236

measured growth rates of 3 to 7 divisions per day. Most probable number estimates of denitrifier cell densities measured in the Cariaco Trench range from 6 x 10^3 to 10^6 cells per liter. We can use these numbers as ranges to compute the minimum and maximum time required to produce the maximum observed riboflavin concentration. Results of this calculation (Table 6.05) indicate that it would take these cells 1 to 4 days to produce this concentration of riboflavin.

Although we did not measure FMN and FAD concentrations in the Cariaco Trench, we can use the same type of approach to calculate how much of these flavins would be present in the water column after the same time period if these bacteria were the sole source. The pertinent data are shown in Table 6.05. After four days at the slower growth rates, predicted concentrations would be 20-54 pM/l for FMN and 75-488 pM/l for FAD. After 1 day at the faster growth rates, predicted concentrations would be 22-68 pM/l for FMN and 90-236 pM/l for FAD. These values are higher than any we measured in the Black Sea samples, however unusually high concentrations of 35 pM FMN/l and 750 pM FAD/l were found in association with the chlorophyll maximum in the Sargasso Sea (Vastano 1988).

Results of the model presented here suggest that resident populations of bacteria such as the Cariaco Trench isolates cultured in our laboratory could, at ambient cell densities, produce all of the flavins in the water column in a few days time. The fact that such a short time is required may be a partial explanation for the observed patchiness in concentrations (Chapter 5). These organisms are probably not be the only sources of flavins at intermediate depths in the oceans, and there are undoubtedly loss terms which we have ignored

in this simple model. Nevertheless, these results support the hypothesis that bacteria may be a significant source of dissolved flavins in the water column.

CONCLUSIONS

Our estimates of extracellular flavin production (Table 6.05) show that the marine denitrifier clones we tested were not "overproducers", i.e., they produced less than 10 mg flavin/l in culture. Moreover, in the species we tested, flavin production was associated with active growth with little or no further increase in flavins after the beginning of stationary phase. This is similar to results for "normal" bacteria (Wilson and Pardee 1962) and unlike the case for "overproducers," in which excess flavin production is initiated at the end of the growth phase. This would appear to be further evidence that these organisms are not "overproducers".

Although our estimates of flavin production rates are based on two replicate experiments for two marine clones, and may or may not be representative of rates in the water column, we have shown that rates of production are potentially high enough to significantly influence the in situ concentrations of some of these compounds.

The simple model presented represents an attempt to determine if marine bacteria can be a significant source of flavins in the water column. Much more information regarding flavin sources and sinks are needed to make a complete model, however our initial estimates indicate that bacteria similar to our clones can produce all the ambient riboflavin in a few days time. Similar results were found for FAD and FMN.

1. Marine bacteria may be a significant in situ source of flavins in sub-euphotic zone waters. Their riboflavin production rates are high

enough to explain the magnitude of the observed signal, while the short times required to produce the entire amount of dissolved riboflavin are suggestive of the degree of spatial and temporal heterogeneity which has been observed in dissolved flavin distributions.

2. Amounts of flavins released into media and rates of flavin production are comparable to those reported for other "normal" bacteria, and much lower than expected for even weak "overproducers".

3. Flavin production rates were highest during active growth phase, and showed little increase after cessation of growth.

4. FAD was the major component of excreted flavins.

REFERENCES

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. Anal. Biochem. 72: 248-254.

Burkholder, P.R. 1963. Some nutritional relationships among microbes of sea sediments and waters, pp. 133-150. In: Symposium on Marine Microbiology. C.H. Oppenheimer, ed. C.C. Thomas. Springfield.

Demain, A.L. 1972. Riboflavin oversynthesis. Ann. Rev. Microbiol. 26: 369-388.

Hobbie, J.E., R.J. Daly, and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33: 1225-28.

Nelson, D.C., J.B. Waterbury, and H.W. Jannasch. 1982. Nitrogen fixation and nitrate utilization by marine and freshwater Beggiatoa. Arch. Microbiol. 133: 172-7.

Taylor, C.D., and H.W. Jannasch. 1976. Subsampling technique for measuring growth of bacterial cultures under high hydrostatic pressure. Appl. Enivern. Microbio. 32: 355-359.

Vastano, S.E. 1988. Processes affecting the distribution of flavins in the ocean. MS Thesis. Univ. Miami. 67p.

Wilson, A.C., and A.B. Pardee. 1962. Regulation of flavin synthesis by Escherichia coli. J. gen. Microbiol. 28: 283-303.

CHAPTER 7

SUMMARY AND SIGNIFICANCE

This thesis has attempted to bridge the gap between such diverse areas of research as the structural chemistry of bacterial pigments, the relative importance of bacteria in the oceans, the amount and specific composition of organic carbon in the ocean, and the nature and distribution of the dissolved fluorescence of seawater. Advances in the past in these areas have been hampered by inadequate methodology. A major contribution of the present work is combining the application of a recent technological developments in column chromatography (HPLC) with an interdisciplinary approach to advance our understanding of the interaction between biological, chemical and optical signals in the ocean. In light of the major advances in instrumentation and separation technology which have occurred during the period in which this thesis was completed, it seems highly likely that this approach will be even more fruitful in the decade to come.

A. SUMMARY

1. Bacterial pigments and their contribution to dissolved fluorescence and flavin concentrations in the ocean

In answer to the questions posed in the introduction section, we can summarize our findings as follows. We have shown that natural isolates of marine bacteria produce a variety of fluorescent compounds. Three of these have been identified as the flavins riboflavin, FAD, and FMN. Other fluorescent compounds are also produced which show a greater similarity to pterins than to flavins, but these have not been identified.

The pigments produced by the marine bacteria used in this study were not the same as pyoverdine, however they were similar to some of the minor fluorescent metabolites produced by Pseudomonas fluorescens. We have demonstrated the quantitative relationship between pyoverdine and other fluorescent metabolites released into the growth medium by Pseudomonas fluorescens and recommended a method utilizing high performance liquid chromatography (HPLC) as a replacement for the King's B test for pigment production in bacteria.

We have shown that rates of flavin production by these natural isolates are fast enough to explain the magnitude and variability of flavin distributions in the water column, however rates were not high enough to classify these bacteria as "riboflavin overproducers".

Maxima in riboflavin concentrations and bacteria were observed near the oxic/anoxic boundary in both the Black Sea and the Cariaco Trench, however the magnitude of the maximal values represented only a two-fold elevation over background levels. The riboflavin at the depth of the maximum would appear to be of biological origin, however due to the complex microbial ecology in these environments, we cannot determine what type of bacteria are responsible.

2. Optical signals from bacteria

Continuous profiles of fluorescence in the Black Sea revealed the presence of a deep chlorophyll maximum. This maximum was associated with a maximum in beam attenuation coefficient, a maximum in respiratory electron transport system (ETS) activity, and a maximum in bacteriochlorophyll e. The coincidence of these features as well as the fact that detrital pigments were not present strongly suggests

that the chlorophyll fluorescence maximum was due to the presence of a viable, active population of the purple non-sulfur bacterium Chlorobium.

Continuous profiles of fluorescence, using a second channel optimized for flavin fluorescence, showed small but consistent maxima in the region of the oxycline. These maxima were in the region of maximal denitrification rates but no maximum in beam attentuation coefficient was observed. The limited vertical extent of the feature and high background fluorescence signal on which it was superimposed make it very difficult to draw any definite conclusions, however the magnitude of the flavin fluorescence maximum is perhaps even greater than would be predicted given the relatively low rates of bacterial activities observed during the study period.

3. Relationship between specific compounds and dissolved fluorescence

Perhaps the most significant contribution from this research is the recognition that dissolved fluorescence can be separated into numerous individual components using HPLC techniques. We have investigated only that fraction which is retained by octadecylsilane (C18) adsorbent, however additional fluorescent material from seawater and culture filtrates is retained by magnesium silicate (Florisil) adsorbent. Although only a few individual components have been identified so far, estimates of dissolved fluorescence based on HPLC analysis show general agreement with those obtained using continuous fluorescence profiling.

B. FUTURE WORK

The direction of future research on this project may best be described as "ChemoOptical Oceanography". It involves using optical signals to investigate the organic biogeochemistry of the ocean. Fluorescence studies provide information concerning the distribution and concentration of both dissolved fluorescence, due to organic compounds, some of which are of biological origin, and particulate fluorescence due to biotic and abiotic particles. Analysis of individual components of both dissolved and particulate fluorescence signals provides detailed compositional information.

1. Fluorescence studies

Fluorescence profiling of dissolved and particulate organic matter should be investigated in other areas of the ocean, especially in oxygenated water columns and in surface waters. We need to identify other sources of fluorescence, such as other bacterial types and other organisms.

Most previous studies of dissolved fluorescence in the oceans have measured two parameters: fluorescence intensity, and the wavelengths of the excitation and emission maxima. The former parameter has been used to estimate the quantity of material present and the latter as a measure of the nature of the material. What has emerged is the finding that while fluorescence intensities vary widely in time and space, the excitation and emission maxima are remarkably similar regardless of whether samples have a marine or terrestrial origin (Zepp and Schlotzhauer 1981). Improved sensitivity can be

obtained if a laser excitation source is used, or by using a conventional spectrofluorometer equipped with double emission monochrometers to reduce scatter. The technique of synchronous fluorescence scanning, in which excitation and emission monochromators are scanned simultaneously at a fixed wavelength separation interval, has been used to resolve mixtures of organic matter from four riverine sources (Cabaniss and Shuman 1987). Fluorescence contouring, which involves repeated emission scans at numerous excitation wavelengths, results in excitation-emission matrices which can provide even more information regarding the nature of compounds present in mixtures (Lochmuller and Saavedra 1986). Seawater is a complex mixture of fluorescent compounds which will never be totally resolved by in situ fluorescence measurements, however application of advanced techniques may at least allow us to detect changes in the nature of the mixture associated with changing sources and environments.

2. Individual compound analysis

We need to improve the methodolgy used to analyze individual fluorescent compounds in seawater and increase the "catalog" of fluorescent compounds in seawater. High performance liquid chromatography (HPLC) is one of fastest growing analytical techniques in biological, medical and chemical research today. The development of packing materials with smaller particle size, greater surface areas, and additional functional groups continues to improve the resolution and increase the number of compounds which can be analyzed. Materials such as mixed mode resins and coated fused plates of porous alumina (Biotage, "Unisphere-PBD") have been developed to improve

separation of basic, polar organic compounds, such as are found in seawater. Improved detection of flavins could be achieved with the recently developed post-column photochemical reactor (ict Corp., "Beam Boost"). All these new products have become available during the last two to three years and their efficacy with separation of seawater organic compounds should be tested. Lastly, HPLC systems can be interfaced with state-of-the-art fluorometers to provide information pertaining to the purity and identity of individual components using excitation-emission matrices (EEMs) alone or in combination with time-resolved and phase-resolved techniques (McGown 1989).

Once the analytical methods have been improved, the relationship between fluorescent and total DOM in the ocean should be explored in more detail. Specifically, we need to know what percentage of total DOC is fluorescent, and if this relationship changes in time and space. High DOM and flavin fluorescence in the deep waters of the Black Sea are suggestive of accumulation of these substances over a long time period. The nature of the material in surface and deep waters of the Black Sea was also different, perhaps due to differences in age or source of the material. Previous attempts to correlate dissolved fluorescence with total DOC have succeeded in estuaries, but not in the ocean. With new methods for measuring both of these parameters, new studies may succeed where others have failed. These findings may have important implications regarding our understanding of the age, size, and cycling of DOC in the ocean.

3. Chemical Oceanography and remote sensing

The interaction of light with organic matter in the oceans should be regarded not simply as an interference in measurements of chlorophyll (Carder et al. 1989), but rather as a source of information regarding the composition, distribution, and sources of organic molecules. Although detection of the intrinsic DOM and flavin fluorescence signals on a global scale using satellites would probably require the use of active sensors (an unlikely development), such measurements are quite feasible in the water column. The real power of fluorescence measurements derives from the ability to make nearly continuous measurements in time or space using in situ instruments or by connecting instruments to a pumping system. Previous studies of the relationship between DOC and fluorescence have shown a correlation only in coastal waters, however, we can now measure both parameters with greater sensitivity, and the relationship between these two parameters in the ocean should be reinvestigated. In situ fluorometry is ideally suited for mapping small scale features and examining patchiness or heterogeneities in distributions of organic matter in the ocean. Improving our ability to measure and interpret dissolved fluorescence will increase our understanding of DOM in the oceans.

REFERENCES

Cabaniss, S.E., and M.S. Shuman. 1987. Synchronous fluorescence spectra of natural waters: tracing sources of dissolved organic matter. Mar. Chem. 21: 37-50.

Carder, K.L., R.G. Steward, G.R. Harvey, and P.B. Ortner. 1989. Marine humic and fulvic acids: Their effects on remote sensing of ocean chlorophyll. Limnol. Oceanogr. 34: 68-81.

Lochmuller, and Saavedra. 1986. Conformational changes in a soil fulvic acid measured by time-dependent fluorescence depolarizatiion. Anal. Chem. 58: 1978-1981.

McGown, L.B. 1989. Fluorescence lifetime filtering. Anal. Chem. 61: 839A-847A.

Zepp, R.G., and P.F. Schlotzhauer. 1981. Comparison of photochemical behaviour of various humic substances in water: III. Spectroscopic properties of humic substances. Chemosphere 10: 479-486.

APPENDIX A

CHROMATOGRAPHIC DATA FOR ADDITIONAL MARINE BACTERIAL ISOLATES

Figure A.01. Comparison of pterin reference compounds (top and bottom panels) with the Florisil fraction of pigments from marine isolates MD2 (second from top) and D9Sm (third from top) using SCX-HPLC. Retention times are indicated in minutes. Numbers in parentheses are designated labels for unknown compounds from all the marine isolates (MFlor series). Refer to Table 2.02 for a complete listing of unknowns and their corresponding relative retention times. Chromatographic conditions were as follows: 4.5 x 250 mm Partisil SCX column with 10 µm particle size; isocratic elution with ammonium phosphate (pH = 2.8), 7 % methanol, and 5 % acetonitrile at 1.5 ml/min.

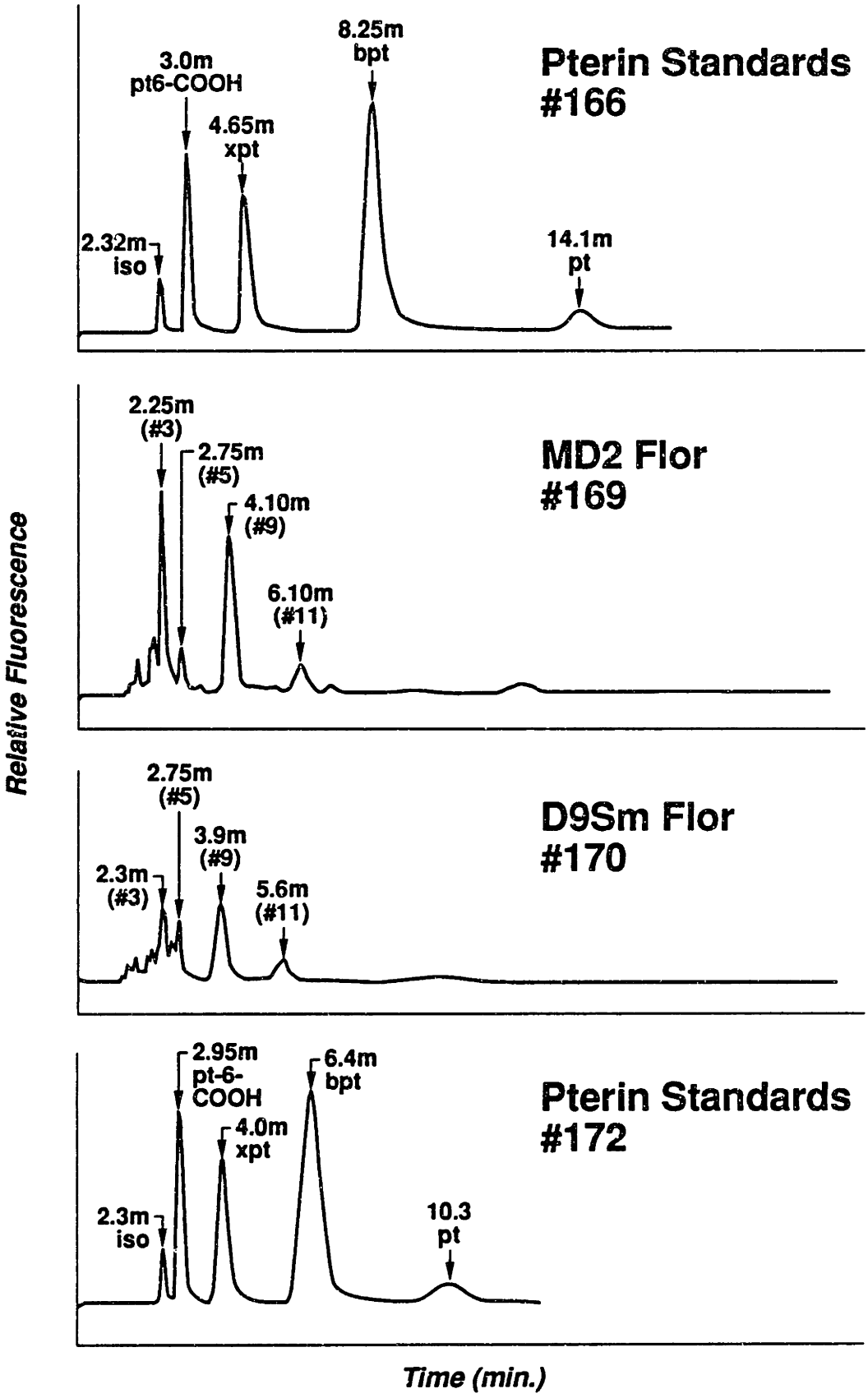
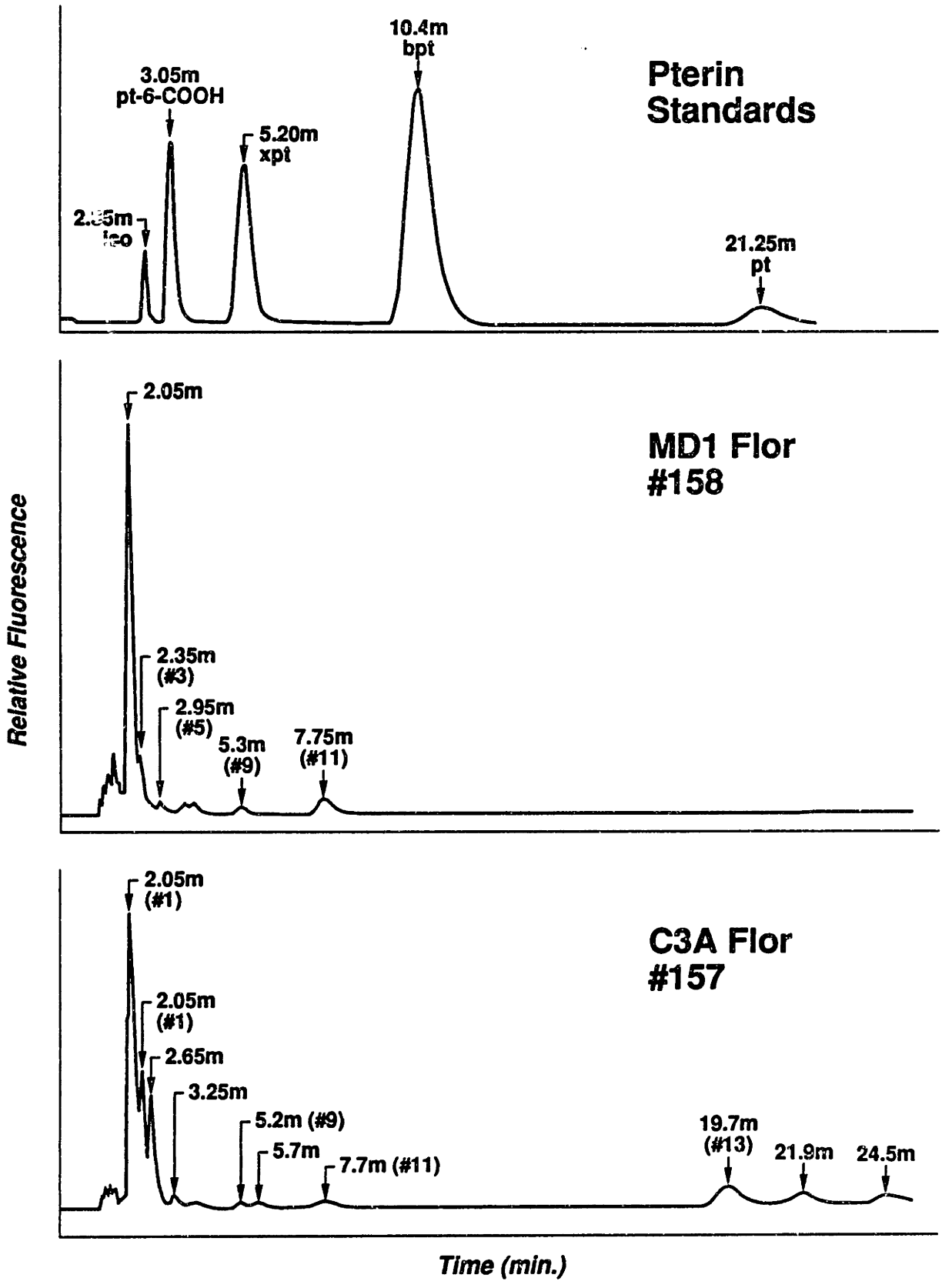

Pterin Standards
#166
3.0m
pt6-COOH
4.65m
xpt
8.25m
bpt
2.32m
iso
14.1m
pt
MD2 Flor
#169
2.25m
(#3)
2.75m
(#5)
4.10m
(#9)
6.10m
(#11)
D9Sm Flor
#170
2.75m
(#5)
3.9m
(#9)
2.3m
(#3)
5.6m
(#11)
Pterin Standards
#172
2.95m
pt-6-
COOH
4.0m
xpt
6.4m
bpt
2.3m
iso
10.3
pt
Relative Fluorescence
Time (min.)

Figure A.02.Comparison of pterin reference compounds (upper) with the Florisil fraction of pigments from marine isolates MD1 (middle) and C3A (bottom) using SCX-HPLC. Retention times are indicated in minutes. Numbers in parentheses are designated labels for unknown compounds from all the marine isolates (MFlor series). Refer to Table 2.02 for a complete listing of unknowns and their corresponding relative retention times. Chromatographic conditions were as follows: 4.5 x 250 mm Partisil SCX column with 10 µm particle size; isocratic elution with ammonium phosphate (pH = 2.8), 7 % methanol, and 5 % acetonitrile at 1.5 ml/min.


Pterin Standards
10.4m bpt
3.05m pt-6-COOH
5.20m xpt
21.25m pt
MD1 Flor #158
2.05m
2.35m (#3)
2.95m (#5)
5.3m (#9)
7.75m (#11)
C3A Flor #157
2.05m (#1)
2.05m (#1)
2.65m
3.25m
5.2m (#9)
5.7m
7.7m (#11)
19.7m (#13)
21.9m
24.5m
Relative Fluorescence
Time (min.)

APPENDIX B

TABULATED CRUISE DATA

Table B.01. Summary of flavin data collected in the Cariaco Trench during cruise CI8601, March 1986.

Cast	Depth	RBFL (pM/l)	0.84 (pM/l)	0.93 (pM/l)
10	45	4.110	1.884	.000
10	80	1.889	5.735	1.844
10	100	1.704	1.914	.000
10	125	3.572	6.435	2.598
10	150	1.838	5.532	1.199
10	150	5.157	9.779	2.947
10	175	1.670	9.016	2.444
10	175	4.057	14.621	3.342
13	210	4.172	14.977	1.712
17	220	13.487	20.700	.000
570	225	9.710	8.272	8.631
570	235	7.259	13.167	
570	235	5.553	13.762	.000
17	240	4.774	10.498	6.042
13	240	5.474	11.680	6.831
570	245	7.835	10.741	
570	245	4.628	8.434	
17	250	6.446	10.888	4.204
13	250	3.894	8.623	2.132
570	255	2.104	6.554	.000

Table B.02. Summary of most probable number (MPN) results for cruise CI8601, March 1986.

Cast	Depth meters	mpn cells/ml	Range
17	220	6.1	1.6-23
25	230	11	3-36
25	240	2.3	0.7-7
25	250	25	8-81
25	260	3616	1091-12000
578	205	230	76-796
578	215	6	2-23
578	225	7	1.7-30
601	200	15.9	5-48
601	210	615	168-2256
601	220	15.9	5-48
601	230	159	52-483
601	240	9.3	2.7-31
613	260	9.3	2.7-31

Cast	DEPTH	.82	.93	RBFL	1.15	1.30	1.40	1.50	LFL
BS5-6P1 July 18,		1988							
	0			7.82	2.35	5.08			6.72
	0	16.18				1.06	1.53	4.80	6.56
	20	36.90		2.63	9.91	6.98		4.58	3.72
	30	13.26		6.37	9.98		9.26		
	30			3.59	6.71	7.93	1.04	5.43	3.47
	40	16.46		3.00				6.76	4.28
	51			3.45	1.00		7.22		1.56
	60	53.56		.00	.82	4.84		6.08	
	70	51.72		1.40		2.27	1.91	5.91	1.58
	80	55.32		14.26		1.53			
	80			1.22	2.00	3.30	1.97		5.40
	91	49.39		.96	2.24	6.87	1.85		7.60
	101	38.48		.00		6.39	1.46	7.31	
	110	29.21		1.83		6.75		9.21	
	122	17.50			5.41	7.37			6.21
	130	12.37		8.46		3.93		4.20	.00
	141	11.00		5.29	1.10	1.63		3.38	.00
	171	8.08		1.16	2.10			3.80	.00
	270	13.97		.00	3.41			3.74	.00
BS5-9P1 July 21,		1989							
	0	23.38		7.92	.00	1.74	1.65	1.65	3.76
	11	36.43		6.09	.00	1.36	.90	.90	6.76
	20	10.36		6.19	.00	1.20	1.77	1.23	4.60
	32	46.61		4.07	.44	2.10	1.26	.00	5.82
	41	4.85			.00	.00	.00	.00	4.38
	50	62.14	2.86	2.25	1.05	3.56	.61	1.55	2.59
	60	83.39	14.57		.99	3.38	1.64	.00	7.88
	64	39.81		.80	1.40	2.60	.83	.00	7.35
	69	74.25			.55	2.73	1.39	.00	8.29
	75	104.28	18.24		1.39	4.38	1.59	.00	7.02
	81	87.66	13.82	.13	.79	3.10	1.70	.00	8.72
	85	76.48	12.04	1.54	1.42	4.00	.74	.00	8.28
	90	76.92	10.48	1.16		4.98	1.14	.00	11.59
	94	23.68		2.91	.76	3.57	1.05	3.35	
	94			1.21	.00	4.42	.77	4.05	
	100	66.94	11.23	1.19		5.31	.84	.00	8.24
	105			1.29	.00	3.31	.70	.00	
	105				.00	3.74			
	110	75.38	7.09	1.18	.00	8.59	.57	.00	7.71
	116	22.82		2.92	.00	.00	.00	.00	.64
	121	82.38		1.81		5.67	2.10	.00	9.16

Table B.03. Summary of flavin data collected in the Black Sea during cruise KN134-12, July 1988. Concentrations are in p moles/l.

Cast	DEPTH	UNK1	1.90	2.05	2.20	2.45	2.55	2.8	3.00
BS5-6P1 July 18,									
	0	10.64							
	0	1.45		1.95	2.92		5.47		3.63
	20	3.56		1.81	4.62	1.99	2.24		9.76
	30	12.23		5.49	2.83	6.03	6.38		3.31
	30	6.67		2.48	4.28	1.38			9.46
	40			2.93	2.49	3.66	4.13		3.97
	51	3.75			3.65	2.23	1.54		
	60	7.44		4.49	1.74	2.66			6.19
	70	2.38		4.07	1.07	2.76	3.39		5.36
	80	8.23			19.89	6.07			
	80	1.59			5.98		4.20		1.12
	91	14.55		4.48	1.07	3.30	1.56		4.88
	101	10.85		3.81	1.52	2.31			3.32
	110	7.94		3.54	1.80	1.71	2.53		2.81
	122	5.85		5.66	1.39	2.41			4.36
	130	9.75			12.06				
	141	9.19			16.05				
	171	7.97		8.10	8.63				
	270	7.65		8.90	10.51				
BS5-9P1 July 21,									
	0	1.81	1.07	2.09	1.79	.93	4.33	1.69	1.37
	11	4.02		2.10	2.10	.98	4.89	2.16	8.61
	20	4.87		2.69	.00	1.15	9.81	3.50	7.76
	32	3.58		1.88	.72	.81	3.53	1.78	
	41	2.36	1.50	.00	.00	.67	4.56	1.62	.93
	50	2.61	2.45	1.37	1.91	1.12	1.97	.96	1.41
	60	4.39	5.04	1.27	1.89	.67		2.45	
	64	2.81	5.40	.00		.00		.00	2.37
	69	6.31	7.56	4.00	.00	.00	3.19	1.73	4.28
	75	5.44	7.73	1.05		.00		1.38	4.27
	81	6.92	7.40	.90	1.38	.00		1.69	
	85	5.28	6.23	.00		.00		.00	.98
	90	3.45	6.64	.91		.00	3.22	.00	
	94	1.82	6.70	6.34		.00	1.84	1.04	
	94	2.14	4.05	5.67		1.24		2.95	
	100	5.78	5.61	1.24	.00	.00		.00	
	105	4.31	2.87	3.58		.00		.00	
	105			4.82		.00			
	110	4.90	3.28	.90		.00		.00	
	116	10.99	.00	3.12	4.92	.00		2.93	
	121	6.01	2.29	7.68		.00	3.59	1.78	

Table B.03. (cont'd.)

Cast	DEPTH	FMN	FAD	A	4.60	5.00	LCR	8.0	TOT FL
BS5-6P1 July 18,		1988							
	0	7.79	21.16				79.37		
	0	3.79	24.64				72.02		1133
	20	2.18					13.76		1566
	30	2.32					10.04		
	30	10.48	10.67				11.66		1546
	40						37.59		1290
	51								
	60	.00	.00				.00		1918
	70						24.12		1626
	80		16.60				23.50		
	80	3.19	10.54						
	91								2169
	101	.00	.00						2015
	110	2.97	11.33				13.48		1793
	122								
	130								2179
	141	.00	.00				.00		1714
	171	.00	.00				.00		2379
	270	.00	.00				.00		2763
BS5-9P1 July 21,									
	0	2.18	48.57	2.19	.00	.00	46.51		64
	11	2.38	17.76	.00	.00	.00	48.97		37
	20		28.86	.00	1.10	.00	42.87		45
	32		23.53	.00	3.47	2.37	16.62		37
	41		15.92	1.28	4.12	2.68	5.51		23
	50		39.32	.00	6.99	3.82	6.04		47
	60	3.66		.00	10.58	4.40	.00		
	64		31.84	.00	16.48	4.25	6.64		43
	69		20.33	.00	11.33	3.07	15.54		35
	75		10.67	.00	10.60	.00	18.47		23
	81			3.96	10.44	.00	.00		
	85		24.03	1.08	8.95	2.17	5.50		39
	90			.00	10.39	.00	8.33		
	94		24.49	.00	16.95	.00	5.89		29
	94		41.49		9.46				45
	100		.00	.00	9.66	2.38	.52		
	105				7.31				
	105		.00	.00	11.26	.00	.00		
	110		.00		7.63	2.94	.00		
	116		21.45	1.48	6.52	.00	11.04		36
	121			.00	4.04	.00	20.30		

Table B.03. (cont'd.)

	CAST					
DEPTH	14H3	14H1	12H5	10H2	10H1	7H5
25						.3336
50						.1043
70		.1539				
71						.0998
80				.1122	.0919	
87					.0712	
90				.1394		.093
92			.1972			
93		.1201				
94		.14				
97		.1951				
102		.2873	.1604			
107		.3634				
111						.1706
112			.1609			
118					.2585	
126		.367				
151				.1339		
204					.1249	
250				.1832		
251					.152	
281					.1336	
281					.108	
307					.1358	
307					.0879	
401				.2374		
408					.1597	
500	.1323			.1173		

Table B.04. Summary of electron transport system (ETS) activities in the Black Sea during cruise KN134-12, Leg 5, July 1988. Activities are in units of u eq/l*hr.