Physiological Adaptations of Microorganisms to High Oxygen in Two Oligotrophic Lakes

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GENERAL INTRODUCTION

This study aimed to evaluate the effects of, and possible adaptations to, the high in situ concentration of oxygen in Lake Hoare, Antarctica. Much information has accumulated about the antarctic dry valley lakes, several of which appear unique in the world by a perpetual high dissolved oxygen in their upper waters. Six features which make these lakes, particularly Lake Hoare, (77°38'S,162°58'E) unusual are:

1) A 5.5 m-thick perennial lake ice;
2) Low light penetration through the ice (i.e.<13μEin m^{-2} s^{-1})
3) Lack of detectable turbulence or mixing;
4) Physical stratification (e.g., temperature);
5) Chemical stratification (e.g., salinity); and
6) Biological stratification (e.g., plankton and benthic microbial mats).

Details of one or more of these unusual features of this oligotrophic lake have been discussed recently (34, 35, 46).

Due to the lack of adequate baseline data on the consequences of high oxygen on natural microbial

communities, I first undertook an investigation on the planktonic heterotrophic community of Mountain Lake, Giles County, Virginia (Chapter 1). Mountain Lake is the only oligotrophic perennially oxygen saturated lake in the southern Appalachians, and therefore, is the best, nearby model for the study of the response of a community adapted to oxygen saturation. This study also developed techniques to provide comparative data for the 1981-82, 1982-83 antarctic field seasons.

Chapter 2 of this research addresses the community response of Lake Hoare to high oxygen. This study was limited to one lake to allow a more thorough assessment of effects. Lake Hoare was chosen specifically because of a relative lack of salinity which would have affected the oxygen concentration, the extensive record of dissolved oxygen seasonal data, and the presence of a nearby shelter allowing field team access to the lake for regular sampling.

Chapter 3 of this research concerns the selection of Lake Hoare isolates adapted to the high oxygen and the study of factors which might explain their survival. Isolates were obtained from the depth of maximum oxygen concentration. A higher concentration of oxygen was used for isolation (ca 4.6x Lake Hoare in situ=VHO) during the 1982-83 field season to obtain a few well adapted

isolates. Growth of the organisms under VHO was regarded as preferable to selection of isolates under ADO following brief exposure to VHO, because full metabolic activity would be involved. Physiological factors examined which potentially related to high oxygen survival and/or growth were: 1) effect of nutrient concentration, 2) measurable catalase activity, 3) superoxide dismutase specific activity and type, 4) carotenoid type and comparative growth study of a carotenoid negative mutant and parent strain.

The physiological factors chosen are reviewed in some detail in the following section to supplement information elsewhere in this dissertation. Chapters 1 and 2 have been published (29, 30).

Oligotrophy. Lake Hoare is oligotrophic (35). Less than 1% of the total bacteria stained with DAPI (4,6,-Diamidino-2-phenylindole) are culturable into visible colony forming units (=CFU) (30). CFU were maximized with dilute complex media. Percentage activity in oligotrophic environments (active uptake of tritiated amino acids) has been estimated in one report to be as high as 56% of direct microscopic counts (22). CFU is a measure of those cells, not necessarily individuals, which can grow on the selected media and produce visible colonies on a surface (agar or membrane filter). To

produce visible colonies, nutrients must be supplied in much higher than in situ concentrations in oligotrophic environments. The CFU medium contained 220 mg l^{-1} total nutrients. Total organic carbon in Lake Hoare has been determined as 16.5-18.0 mg l^{-1} (18). Furthermore, the proportion of that carbon which is utilizable for catabolism is undoubtedly much lower than in the trypticase soy-peptone-yeast extract medium which was used for production of CFU.

Oligotrophic isolates studied by Kuznetsov et. al. (21) were catalase-negative. He concluded that oligotrophy decreased oxygen toxicity. That is, organisms capable of growing in low nutrient environments respire slowly and generate lower levels of toxic byproducts (i.e., hydrogen peroxide, superoxide). The same organisms growing in eutrophic environments generate these byproducts at much higher, and usually, lethal levels.

Superoxide dismutase. Harnessing molecular oxygen as an exogenous electron acceptor imparts an energetic advantage to aerobic and facultative organisms. Cytochrome oxidases have evolved which are efficient in this four electron transfer process, yielding water in aerobic respiration. Several enzymes and hydrogen and electron carriers (e.g. flavins, NADH oxidase, xanthine oxidase, quinones) may reduce oxygen by one or two

electrons, yielding superoxide and hydrogen peroxide (23, 31, 44). The interaction of the above two products with a catalytic reduced transition metal (e.g., Fe++, Mn+) can generate the hydroxyl radical (1). All of the aforementioned products, with the exception of water, can react with a variety of cell components, resulting in adverse effects. Thus, enzymes have evolved which destroy hydrogen peroxide (catalase, peroxidase) and the superoxide radical (superoxide dismutase, (SOD)). The importance of superoxide dismutase is illustrated by the circumstantial evidence that it is found in the majority of aerobes (25), with few exceptions (23, 32). SOD induction with oxygen (or its generated byproducts), resulting in increased oxygen tolerance, is additional proof of the importance of this enzyme. Table 1 gives a literature summary of SOD inducibility in procaryotes.

Carotenoids. In photosynthesizing organisms carotenoids can act as accessory pigments transferring additional light energy to chlorophyll. Carotenoids also may stabilize cell membranes structurally, increasing their resistance to osmotic shock (17). When a light-excited photosensitizer (e.g., chlorophylls, quinones, cytochromes) and oxygen combine, an energized form of oxygen called singlet oxygen can result. Singlet oxygen can produce several detrimental reactions within

cells, one of which is lipid peroxidation (2, 3). Lipid peroxidation is an autocatalytic process which may destroy the integrity of a membrane. Carotenoids react with the three intermediates of this process to halt initiation and propagation. The three intermediates are: 1) lipid peroxide radicals (19); 2) the high energy forms of the sensitized molecules (9); and 3) singlet oxygen. The quenching of singlet oxygen by carotenoids is the most thoroughly documented. The efficiency of this reaction is proportional to the number of conjugated double bonds in the isoprenoid chain, and the minimum number for effective quenching appears to be nine (11).

Due to the short half-lives of the intermediates as well as their cross-reactions, it is hard to distinguish one mechanism from the rest. Table 2 summarizes carotenoid protection literature. Additional supportive evidence of the "anti-lipid peroxidation model" is the mode of action of herbicides inhibiting carotenoid biosynthesis with resultant plant death (39) and the light-and hydrogen peroxide-induction of carotenoid biosynthesis in *Fusarium aquaeductum* (43).

The diverse R-groups of carotenoids divide this large family of molecules into its constituent groups. They may consist of epoxides, aldehydes, alcohols, carboxyl, keto groups (41). The mechanisms and functions

of these R-groups within cells remain obscure. Keto groups can undergo reversible reduction to alcohols. Epoxide formation entails hydration and subsequent oxidation or insertion of atomic oxygen. Epoxide formation has been hypothesized as an oxygen pumping mechanism in photosynthesis (8,10). How these reactions could assist in oxygen toxicity protection is unknown.

Carotenoids in procaryotes may be both membrane bound or free within the cytoplasm. This is attributed to complexing with membrane glycoprotein (28) and/or location of polar R-groups at the membrane interfaces and bridging the membrane with the nonpolar isoprenoid chain (7, 42).

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TABLE 1. Summary of procaryote superoxide dismutase induction literature

Organism	Inducer (control)	SOD type induced	Effect	Ref.
Bacteroides fragilis	2% oxygen (anaerobic)	Fe	increased resistance to 5 atm oxygen exposure	(37)
Escherichia coli	5 atm oxygen (anaerobic)	n.d.	grew after exposure to 46 atm oxygen	(14)
"	1 atm (anaerobic)	n.d.	increased resistance to 20 atm oxygen	(15)
"	1 atm (anaerobic)	Mn	protection from cell damage on exposure to 1 atm	(16)
Streptococcus faecalis	20 atm oxygen (anaerobic)	n.d.	increased resistance to 46 atm oxygen	(14)
Bdellovibrio stolpii	1 atm oxygen (1 atm)	Fe	n.d.	(45)
Streptococcus mutans	1 atm (anaerobic)	Mn	n.d.	(24)
Propionibacterium shermanii	10-85 mm Hg pO2 (anaerobic)	n.d.	n.d.	(36)
Photobacterium leiognathi	1 atm oxygen (1 atm)	CuZn Fe	n.d.	(38)
Oscillatoria limnetica	1 atm (anaerobic)	Fe	protection from photo-oxidation	(12)
Vibrio cholerae	20 atm oxygen (1 atm)	n.d.	increased resistance to exogenous superoxide	(13)

n.d.= not determined

TABLE 2. Summary of procaryote carotenoid protection literature

Organism	Carotenoid - Control	Treatment	Protection	Ref.
Micrococcus luteus	mutant	light & oxygen	malate oxidase menaquinone	(5) (4)
Micrococcus luteus	mutant	light & oxygen	succinate dehydrogenase NADH2 dehydrogenase membrane selectivity	(27)
Rhodopseudomonas spheroides	mutant	light & oxygen	bacteriochlorophyll death	(40)
Rhodospirillum rubrum	mutant	light & oxygen	bacteriochlorophyll	(6)
Corynebacterium poinsettiae	mutant	light & oxygen	death	(20)
Azospirillum brasilense	diphenylamine inhibition	oxygen (light ?)	nitrogenase	(33)

CHAPTER 1

SENSITIVITY OF AN OLIGOTROPHIC LAKE PLANKTONIC BACTERIAL COMMUNITY TO OXYGEN STRESS

ABSTRACT

Dissolved oxygen at approximately four times normal saturation (42 mg liter^{-1}) inhibited the growth and metabolism of summer planktonic bacteria in the surface water of alpine oligotrophic Mountain Lake (Giles County, Va.). Data were derived from growth of CFU on membrane filters, D-[U-^{14}C]glucose incorporation into the extractable lipid of these CFU, and respiration and assimilation of D-[U-^{14}C]glucose by lake water samples. Statistically significant ($P < 0.05$) differences were not detected in either CFU or ^{14}C incorporation in lipid when superoxide dismutase (30 U ml^{-1}) or catalase (130 U ml^{-1}) was added to the medium. Thus, exogenous oxygen by-products apparently are not responsible for the observed inhibition of growth and metabolism.

INTRODUCTION

Oxygen, which is essential to life for all aerobic organisms, is also a very reactive molecule, often becoming toxic at concentrations exceeding atmospheric levels. The mechanisms for oxygen toxicity are not readily understood, but apparently they involve the reaction of activated oxygen or its radical byproducts at a variety of molecular sites within the cell (12). Most investigations of oxygen toxicity mechanisms concern organisms (anaerobes and microaerophiles) with high sensitivities to oxygen at or below the levels in normal atmospheres. In contrast, published studies involving community responses to naturally high dissolved oxygen (HDO) concentrations are lacking, apparently because of the rarity or ephemeral nature of such environments. For example, HDO concentrations occur ephemerally in cyanobacterial blooms with photosynthetic oxygen production, resulting in dissolved oxygen concentrations of 200 to 300% saturation (2). Prolonged existence of these HDO concentrations usually would induce photooxidative death, but they are normally brief. Among the relatively few known natural aquatic environments in which prolonged or permanent HDO conditions occur are certain oligotrophic, perennially ice-covered lakes in

dry valleys of southern Victoria Land, Antarctica (9-11). To develop techniques for studying those Antarctic lakes and to obtain base-line data for comparison, we undertook this investigation of the planktonic microbial community in Mountain Lake, Giles County, Va. during the summer of 1982. Mountain Lake is a natural alpine oligotrophic lake with perennially oxygen-saturated surface waters, about which considerable information is known (1, 6, 8, 13).

MATERIALS AND METHODS

CFU were determined by filtering measured volumes of freshly collected lake water through 0.22-μm Millipore filters (47-mm diameter) at <26 cm of Hg (vacuum). Filters were placed onto Gelman absorbant pads saturated with medium. Medium consisted of autoclave-sterilized Mountain Lake water with additions of 12.5 μCi of D-[U-^{14}C]glucose per liter (274 μCi μmol^{-1}), 0.02% trypticase soy broth and 0.002% yeast extract (BBL Microbiology Systems). These concentrations of nutrients gave the maximal CFU within 2 weeks of incubation. After preparation, one set of plates was incubated in a pressure cooker with oxygen added to give a final concentration of 42 mg liter^{-1}(11 lb in^{-2}) (HDO plates). A second set of plates was incubated in the dark at ambient atmospheric pressure with normal atmospheric

dissloved oxygen (ADO plates). All plates were incubated at 12°C for 7 to 10 days.

After the CFU were counted, total lipids were extracted in scintillation vials by the method of McKinley et al. (5). The chloroform fraction was evaporated to dryness, Aquasol 2 was added, and the [^{14}C]lipid was counted by liquid scintillation.

Total assimilated and respired carbon were determined by using lake water (50 ml) dispensed into 125-ml serum bottles stoppered with butyl rubber serum stoppers with inserted glass center wells and paper wicks (Bellco Glass, Inc.). A minimum of 10 bottles per treatment was used in each experiment. HDO bottles were then pressurized with oxygen (11 lb/in^{-2}). Both HDO and ADO bottles were agitated at 12°C for 2 h, which had been predetermined to be an adequate time to achieve equilibrium of the dissolved oxygen. Controls were fixed with glutaraldehyde (2% final concentration, v/v). All HDO and ADO bottles received 20 μCi of D-[U-^{14}C]glucose per liter (274 μCi μmol^{-1}). Bottles were incubated 4-6 h at 12°C. After incubation, unfixed ADO and HDO treatments were fixed with glutaraldehyde. Phenethylamine (150 μl) was added to the paper wick in each center well, after which all bottles were acidified with 200 μl of 2 N H_2SO_4. The bottles were slowly agitated for 2 h to

enhance both release of CO_2 from the acidified water and trapping by the phenethylamine. Water from each bottle was filtered through 0.2-μm porosity Nuclepore filters and then rinsed with an equal volume of autoclaved lake water. Aquasol 2 was added to the filters for liquid scintillation counting. Center wells and paper wicks were placed into Aquasol 2, and the total assimilated and respirated carbon was counted by liquid scintillation. Fixed control values were subtracted, and all counts were corrected to disintegrations per minute by the channels ratio method.

The total lipid fraction of the ^{14}C assimilated by lake water was determined by filtering (0.2-μm Nuclepore filter, <26 cm of Hg [vacuum]) one-half the volume of each bottle and rinsing with an equal volume of autoclaved lake water. Total lipid was extracted from filters by the previously described technique (5).

RESULTS AND DISCUSSION

CFU were significantly ($P < 0.05$) reduced with HDO as compared with ADO (Fig. 1 and Table 1). CFU differences do not necessarily indicate relative differences in biomass, because an equal number of small and large colonies on HDO and ADO plates may occur regardless of differences in colony size. Relative

differences in biomass and metabolic activity can be determined by extracting the total lipid incorporated in cells on the same filters after counting CFU. Table 1 presents a comparison of CFU development and total lipid incorporation.

It is generally recognized that pressure effects begin to be of physiological significance at 100 atm (1,500 lb/in^{-2}) (4). The pressure applied (HDO conditions) did not exceed 12 lb/in^{-2} (≅83 kPa), which is less than that encountered in 28 feet (8.53 m) of fresh water. Nevertheless, 50 lb/in^{-2} of helium was applied and compared with ADO in one experiment. No significant difference ($P > 0.05$) between these treatments existed as measured by CFU development and total lipid incorporated into CFU.

The influence of HDO on lipid incorporation need not necessarily reflect an influence on the total carbon assimilated. Whereas total carbon assimilation cannot be measured accurately with CFU, owing to high levels of abiotic absorption, the total carbon assimilated by lake water can be measured by standard procedures which also allow a portion to be fractionated for the determination of total lipid incorporation. The results of three such experiments (Table 2) indicated that 42 mg of dissolved oxygen per liter was inhibitory to both carbon and lipid

assimilation ($P < 0.05$). However, the percent lipid to total carbon assimilated was not significantly different ($P < 0.05$) within each treatment (HDO versus ADO).

Respiration of D-[U-^{14}C]glucose was also inhibited in two experiments (Table 3). It cannot be determined whether respiration was more or less inhibited than carbon assimilation on the basis of these experiments.

Superoxide dismutase and catalase were added to the standard medium in one experiment to determine whether superoxide or hydrogen peroxide was being generated exogenously to cells in inhibitory concentrations. Whereas positive evidence for such exogenous generation exists for the microaerophiles *Spirillum volutans* and *Campylobacter fetus* (3, 7), the same enzyme concentrations had no effect ($P > 0.05$) on the Mountain Lake community treated with or without the enzymes, as measured by CFU development and ^{14}C incorporation into total lipid (Table 4). Endogenous generation and activity of superoxide and hydrogen peroxide remains a possibility.

On the basis of several criteria (CFU development, total lipid incorporated into CFU, and total carbon assimilated and respired), 42 mg of dissolved oxygen per liter (HDO) inhibited the growth and metabolism of heterotrophic microorganisms in this alpine oligotrophic

lake. This constitutes the first investigation of the effects of HDO on natural heterotrophic plankton communities and has laid the basis for comparative studies with the naturally perennial HDO concentrations in lakes of the dry valleys of Antarctica.

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assimilation rates in Mountain Lake, Virginia, during its thermal stratification period. Virginia. J. Sci. 24:206-211.

TABLE 1. Comparison of ADO and HDO conditions on development of CFU and D-[*U*-^{14}C]glucose incorporation into lipid

Date	Treatment	CFU liter^{-1}	% Reduction[a]	dpm	% Reduction[a]
6/28/82	ADO	4.9×10^4		10,393	
6/28/82	HDO	2.2×10^4	54	1,191	88
8/10/82	ADO	1.4×10^5		8,206	
8/10/82	HDO	2.5×10^4	82	2,120	74
9/22/82	ADO	4.2×10^5		8,873	
9/22/82	HDO	2.1×10^5	50	1,302	85

[a] Percent reduction = [1 − (HDO/ADO)] × 100.

TABLE 2. Comparison of the effects of ADO and HDO on total carbon (T) and total lipid (L) assimilated

Date	Treatment	Total carbon assimilated (dpm)	% Reduction[a]	Total lipid fraction (dpm)	% Reduction[a]	L/T (%)
7/6/82[b]	ADO	35,350		6,102		17
7/6/82	HDO	7,709	78	1,109	82	14
7/12/82[c]	ADO	3,437		735		21
7/12/82	HDO	1,041	70	232	68	22
7/22/82[c]	ADO	3,884		774		20
7/22/82	HDO	1,532	61	345	55	23

[a] See Table 1, footnote *a*, for definition.
[b] 6-h incubation.
[c] 4-h incubation.

TABLE 3. Comparison of the effects of ADO and HDO on total carbon assimilated and respired

Date	Treatment	Total carbon assimilated (dpm)	% Reduction[a]	Total carbon respired (dpm)	% Reduction[a]
9/3/82[b]	ADO	10,724		5,145	
9/3/82	HDO	6,652	38	2,612	49
9/21/82[c]	ADO	7,228		7,884	
9/21/82	HDO	3,277	55	4,480	43

[a] See Table 1, footnote *a*, for definition.
[b] 6-h incubation.
[c] 10-h incubation.

TABLE 4. Comparison of the effects of ADO and HDO with or without enzymes on CFU development and D-[U-^{14}C]glucose incorporation into total lipid

Treatment[a]	CFU liter^{-1}	% Reduction[b]	dpm	% Reduction[b]
ADO (N)	1.4×10^5		8,206	
HDO (N)	2.5×10^4	82	2,120	74
ADO (C)	1.3×10^5		7,256	
HDO (C)	3.0×10^4	77	2,456	66
ADO (S)	1.5×10^5		8,041	
HDO (S)	2.6×10^4	83	3,062	62
ADO (CS)	1.3×10^5		7,986	
HDO (CS)	2.3×10^4	82	1,940	76

a N, No enzyme added: C, plus 130 U of catalase per ml; S, plus 30 U of superoxide dismutase per ml; CS, plus 60 U of catalase and 15 U of superoxide dismutase per ml.

b See Table 1, footnote *a*, for definition.

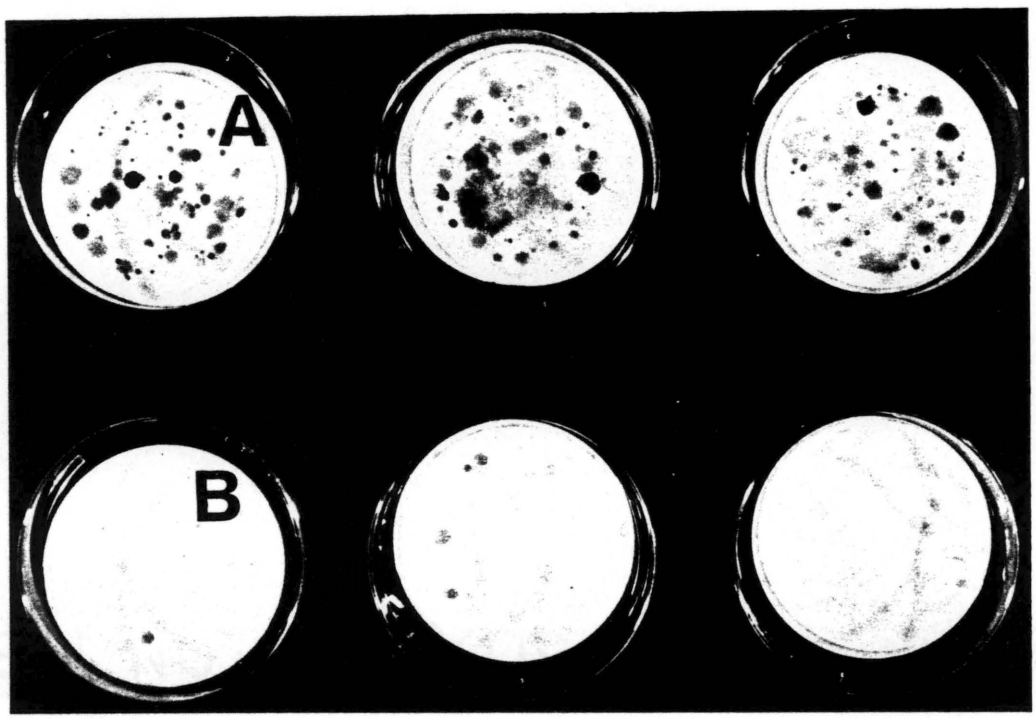

FIG. 1. Photograph of representative membrane filters (equal water volume filtered) after development of Colony forming units with atmopheric dissolved oxygen (A) or high dissolved oxygen (B).

CHAPTER 2

RESPONSE OF AN ANTARCTIC LAKE

HETEROTROPHIC COMMUNITY TO HIGH DISSOLVED OXYGEN

ABSTRACT

The upper waters of Lake Hoare, Antarctica, contain dissolved oxygen at about three times the normal saturation ($\geq$42 mg liter^{-1}). The response of the heterotrophic plankton community to this high dissolved oxygen was evaluated by the criteria of colony forming units (CFU) and D-[U-^{14}C]glucose assimilated-respired. High dissolved oxygen (HDO) was not inhibitory to D-[U-^{14}C]glucose assimilation-respiration compared with normal atmospheric dissolved oxygen in Lake Hoare water. The D-[U-^{14}C]glucose was assimilated and respired optimally at 12°C in Lake Hoare. The D-[U-^{14}C]glucose assimilated respired in the upper saturated atmospheric dissolved oxygen waters of Mountain Lake, Virginia, was inhibited by HDO in contrast to Lake Hoare ($P < 0.05$). CFU formation was inhibited in both lakes by HDO. CFU represent <1% of the fluorochrome-stained direct counts

in Lake Hoare. Lake Hoare planktobacteria are smaller than the planktobacteria in Mountain Lake. Size fractionation of particulate ATP revealed that 39% of the ATP biomass was <0.5 µm in Lake Hoare.

INTRODUCTION

Sustained high dissolved oxygen (HDO) environments are rare in nature. DO concentrations of two to three times the normal saturation values have been reported in cyanobacterial blooms (1, 6), but these concentrations are short-lived due to diurnal solar patterns or photooxidative death of the organisms. A number of perennial ice-covered lakes in Antarctica contain oxygen concentrations greater than three times the normal saturation for sustained periods (21, 30). We report here the first investigation of the metabolic response of a heterotrophic microbial community to perennial in situ HDO versus atmospheric DO (ADO) concentrations for one of these Antarctic lakes. This community response is compared with that of Mountain Lake, Va., an oligotrophic alpine lake whose waters are perennially saturated or only slightly supersaturated ephemerally with DO at certain depths (5).

Lake Hoare (77°38' S, 162°53' E), located in Taylor Valley 58 m above present sea level, is one of at least 12 such lakes located within Antarctica's largest frigid desert of southern Victoria Land (>5,000 km^2). These lakes have 3- to 6-m-thick perennial ice covers which restrict turbulence, cause sharply defined perpetual

stratification, and effectively isolate their underlying waters from the atmosphere (21). Lacking outflow streams, the lakes generally receive adequate glacial meltstream inflow each austral summer to replenish water lost through ice ablation. Despite the lack of outflow streams, these Antarctic lakes have remained fresh and oligotrophic, because nutrients and salts carried in by meltstreams have been either lost to the sediments or removed by the aerial escape of benthic microbial mats through the lake ice (18-20, 23).

Lake Hoare's surface ice measures 1.8 km^2, is 5.5 m thick, and allows transmission of only about 1.0% of surface-incident, photosynthetically available radiation to its underlying water. Despite these reduced light levels caused by rocks, gravel, soil, and pieces of microbial mat frozen in the ice, net photosynthesis by the low light-adapted eucaryotic plankton algae and benthic algal and cyanobacterial mats apparently exceeds respiratory O_2 consumption throughout most of the 32-m depth. This results in DO levels of often greater than three times the normal saturation ($\geq$42 mg $liter^{-1}$)(21).

Although oxygen is required by many organisms for respiration, it also may be toxic. Indeed, a wide continuum exists. Most research concerning oxygen toxicity has involved the very sensitive anaerobes (7,

15), microaerophiles (4, 16, 17), and the facultative aerobe *Escherichia coli* (8). Interestingly, except for our investigations of the oligotrophic Mountain Lake, Virginia. (14), no investigations of the community responses to HDO conditions have been conducted, and no studies have ever been made on perpetual HDO environments such as Lake Hoare. Consequently, we undertook this investigation of the influence of HDO versus ADO on the assimilation and respiration of D-[U-^{14}C]glucose and colony forming units (CFU) on both the planktonic and benthic heterotrophic bacterial communities of Lake Hoare. Our long-range goal is to understand the mechanisms of physiological adaptations to oxygen stress in these unique lakes.

MATERIALS AND METHODS

Water samples for 4,6-diamidino-2-phenylindole (DAPI) direct counts and CFU enumeration were taken in autoclaved biological oxygen demand bottles with a Wildco sewage sampler with a messenger-tripped valve and fixed immediately with formalin to a 2% (v/v) final concentration. Samples for chlorophyll *a* and ATP were taken with a Wildco horizontal Alpha bottle. Benthic microbial mat was collected by SCUBA (self-contained underwater breathing apparatus). All samples were

processed at the Ecklund Biological Laboratory at McMurdo Station, Antarctica, within 6 h of collection.

Water samples for DO were field fixed by the addition of alkaline manganous sulfate and later analyzed by the modified Winkler technique (3). HDO atmosphere was maintained by the application of pressurized oxygen in a modified pressure cooker for CFU experiments or to the head space in butyl rubber-sealed serum bottles for carbon assimilation-respiration experiments as previously described (14). Containers were not purged to avoid removal of normal atmospheric gases. The pressure applied (11 lb/in^{-2} [75.8 kPa]) would be equivalent to that sustained in 28 ft (ca. 8.5 m) of water and has no physiological effect (2, 13, 14).

DAPI-stained epifluorescence microscopy was performed on formalin-fixed samples, as described by Porter and Feig (25), with 0.2-μm porosity Nuclepore filters. An Aus Jena epifluorescence microscope was used to count all samples.

ATP concentrations were measured on water samples concentrated by filtration with 0.2-μm Nuclepore filters at <20-cmHg (2.66 kPa) vacuum, unless otherwise stated. ATP was extracted by boiling in citrate-phosphate buffer for 5 min (29). Photoemission was counted on an SAI integrating photometer (model 3000), and internal ATP

standards were used to correct all values. Our lowest measurable concentration was 1.0 ng ml^{-1}.

Phytoplankton samples were concentrated by vacuum filtration (<20 cmHg) onto Reeve-Angel 984H glass fiber filters. Chlorophyll *a* was extracted with dimethyl sulfoxide-acetone (27,29) and measured in a Turner fluorometer (model 430), correcting for phaeophytin (12).

Media for CFU determination consisted of autoclaved Lake Hoare water with addition of 0.02% trypticase soy and 0.002% filter-sterilized yeast extract (BBL Microbiology Systems). These concentrations of nutrients gave maximal CFU within 2 weeks of incubation at 12°C. Membrane filters (0.22μm porosity; Millipore Corp.) were used to concentrate bacterioplankton at <20-cmHg vacuum. Filters were placed onto Gelman absorbant pads saturated with media. CFU were counted after incubation at x10 magnification with a dissecting microscope.

D-[U-^{14}C]glucose assimilated and respired under ADO and HDO conditions were measured as previously described (14). Values were not corrected to disintegration per minute as relative differences were sufficient and quench was negligible in all samples. Also, we chose to use D-[U-^{14}C]glucose because of the following: (i) Previous studies revealed that it was as readily assimilated as a

number of other low-molecular-weight organic compounds (22). (ii) Glucose was available as a high-specific-activity carbon compound and is a central metabolite in a variety of microbial carbon pathways. (iii) The use of the same labeled compound as for our previous work (14) represents the only way a comparison between two ecosystems can be made.

RESULTS

Figure 1 shows the depth profile of DO concentrations in Lake Hoare. Seasonal variation through the austral summer field season was small, with maximal values averaging three to four times saturation, or $\lesssim$42 mg liter^{-1} at 12-m piezometric depth (PD). This HDO concentration was toxic to the heterotrophic planktonic community in Mountain Lake, Virginia, as evidenced by inhibition of [^{14}C]glucose assimilated and respired, CFU, and incorporation of ^{14}C into the total lipid fraction of those CFU (14).

In contrast to Mountain Lake, [^{14}C]glucose assimilated and respired in Lake Hoare water from 12-m PD exhibited no HDO inhibition (Table 1). Indeed, some samples demonstrated a stimulation of glucose metabolism with HDO. Inhibition of CFU formation (lower CFU counts) also occurred in the Lake Hoare samples under HDO (Table

2). CFU from microbial mat underlying shallow HDO waters were much more inhibited.

In one experiment, benthic mat underlying HDO waters was homogenized and kept in suspension during [^{14}C]glucose incubation. Although the percentage of glucose respired increased, there was severe inhibition by HDO in terms of ^{14}C respiration and assimilation (Table 3). However, identically homogenized samples which were allowed to flocculate quickly and settle showed no inhibition by HDO.

[^{14}C]glucose assimilation and respiration were maximal at 12°C (Fig. 2), although the mean annual temperature for Lake Hoare is 1°C. This coincides with the photosynthetic rate [^{14}C]HCO_3^--temperature response reported for Lake Hoare (19).

Size fractionation of ATP biomass at 12-m PD in Lake Hoare revealed that 46% of the filterable ATP was between 0.2 and 1 μm. A considerable portion (≥39%) of the ATP biomass in the <0.5-μm size range contains the ultramicroplankton (<0.3 μm), as defined by Sieburth (28). The majority of these organisms were achlorophyllous (determined by lack of chlorophyll autofluorescence). Attached forms were virtually absent as suspended particles are in such low numbers. DAPI-stained epifluorescence photomicrographs (Fig. 3)

confirmed the greater abundance of ultramicroplankton in the oligotrophic Antarctic lake relative to the oligotrophic Mountain Lake, Virginia.

Figure 4 shows DAPI direct counts from epifluorescence microscopy and chlorophyll *a* concentrations in Lake Hoare. Maximal values at 9-m correlated well with the higher numbers of heterotrophic plankton. We note with interest that the planktonic bacterial numbers (DAPI direct counts) and cell sizes in Lake Hoare are remarkably similar to the oceanic euplanktobacteria, whereas the benthic bacterial community of Hoare and the planktonic bacterial communities of Mountain Lake, Virginia (14), more closely resemble the larger-celled, substrate-attached marine epibacteria (28).

DISCUSSION

The HDO condition in Lake Hoare probably is derived primarily from oxygenic photosynthesis over many years. Values occur in excess, because the permanent ice cover prevents DO from achieving equilibrium with the atmosphere. Respiratory consumption of this DO may be limited by the quantity and suitability of the substrates available for chemoheterotrophy and chemoautotrophy in the upper waters and by the perpetual low temperatures (0

to 1°C) (20, 21, 30).

Small size imparts a high surface area/volume ratio characteristic of oligotrophs (24). The small size of the heterotrophic plankton in Lake Hoare may be unusual for a lake of this size. The oligotrophic conditions and a lack of turbulence probably have contributed to the development of this ultramicroplanktonic community. Despite the advantage of small size, these microorganisms might well be stressed by direct exposure to constant in situ HDO. However, we detected no evidence of HDO inhibition of assimilation or respiration of [^{14}C]glucose in these Antarctic lake planktonic microorganisms, in contrast to those of Mountain Lake under identical conditions. The Lake Hoare community was inhibited, however, by HDO as evidenced by CFU formation, which comprised $\leq$1% of the total DAPI counts. The difference in HDO effect between the metabolic indicators (i.e., respiration-assimilation) and CFU formation may have resulted from the concentration of nutrients in the CFU medium (200 mg liter^{-1}). Although low, it was far in excess of the total carbon in Lake Hoare at 12-m PD (16.5 to 18.0 mg liter^{-1}) (unpublished data). We present total carbon instead of "dissolved" organic carbon, because a significant proportion of the dissolved carbon is most certainly particulate due to separation through a

Reeve-Angel 984H filter (ca. 1.2-μm pore size). Furthermore, we do not know what fraction of the total organic carbon is biodegradable compared with trypticase soy agar and yeast extract used in the CFU medium. Also, since only organisms able to produce viable colonies on media are evaluated by this technique, they may represent HDO interference on a small subpopulation of microorganisms (CFU, $\leq$1%). Colony formation entails the multiplication of bacteria on a surface usually at elevated nutrient concentrations to shorten the time required. This represents a major departure from the oligotrophic plankton community in which in situ generation times have been estimated at 20 to 200 h (10) and in which attachment to surfaces may not take place. "Use" of oxygen in metabolism (respiration and other processes) is thought to generate toxic oxygen by-products such as peroxide and superoxide (26). It is possible under HDO incubation and CFU conditions that the organisms are self-hyperoxidized.

Oxygen toxicity has been linked to oligotrophy. Kuznetsov et. al. (11) found that many oligotrophic isolates were catalase negative. All isolates we have selected with HDO are catalase positive (unpublished data). Furthermore, all of our isolates from 12-m PD in Lake Hoare are facultative oligotrophs as defined by

Kuznetsov et. al. (11). The physiological mechanisms of this HDO resistance currently are being investigated on HDO-selected isolates in our laboratory. In contrast to the planktonic community, the benthic heterotrophic community of Lake Hoare does not appear to be physiologically adapted to HDO as evidenced by the toxicity on direct exposure in CFU and mat stir ^{14}C experiments. Large amounts of exocellular polymer were extracted from the benthic mat which may give the mat its highly cohesive nature. Exocellular polymers which cause flocculation, attachment, and diffusion limitation are common (9). The maximum number of bacteria at 9-m may result from photosynthetic extracellular products and the heterotrophic potential of such products. High concentrations of DO apparently stimulate photorespiration with extracellular release (e.g., glycolate) (18). We also have evidence that glycolate is readily assimilated in these waters (22).

In conclusion, naturally occurring bacterioplankton exist in great abundance in Lake Hoare. This community apparently is not inhibited by HDO as measured by D-[U-^{14}C]glucose assimilated or respired in contrast to Mountain Lake bacterioplankton. HDO toxicity, as measured by CFU formation, affects both lake communities. This may be an artifact of the procedure in which levels

of nutrient far in excess of in situ concentrations are used to promote rapid growth and therefore high consumption of oxygen for respiration and other reactions possibly producing toxic byproducts. Biochemical study of selected isolates from Lake Hoare represents a novel opportunity for insight into the physiological adaptations to HDO and provides a means for understanding lake typology.

ACKNOWLEDGEMENTS

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dry valley lakes. Phycologia 22:355-365.

TABLE 1. D-[*U*-^{14}C]glucose carbon assimilated and carbon respired in Lake Hoare, Antarctica, and Mountain Lake, Va.

Determination	HDO/ADO (cpm)				Probability[a]
	Lake Hoare		Mountain Lake		
	Mean[b]	Range	Mean[b]	Range	
Carbon assimilated	1.18	0.65–2.97	0.45	0.13–0.76	0.0001
Carbon respired	1.11	0.77–1.64	0.69	0.51–0.95	0.0449

[a] Probability determined by Duncan's multiple range test, using computer-conducted analysis (SAS Institute, Inc.).

[b] Mean of eight experiments.

TABLE 2. CFU in Mountain Lake, Va., and Lake Hoare, Antarctica, formed at ADO versus HDO

Location	Date	CFU (ADO)[a]	% CFU[e] (HDO/ADO)
Mountain Lake, Va., surface	6/28/82	4.9×10^4	46
	8/10/82	1.4×10^5	18
	9/22/82	4.2×10^5	50
	4/29/83	5.2×10^4	49
	6/21/83	8.0×10^4	54
	7/29/83	7.6×10^5	72
Lake Hoare, Antarctica, 6-m PD[b]	10/29/81	2.5×10^3	34
	11/02/81	5.9×10^3	<10[c]
	11/27/81	7.0×10^3	12
	12/15/81	6.1×10^3	<10
	10/29/82	1.7×10^3	30
	12/27/82	4.3×10^3	11
Lake Hoare, Antarctica, mat 12-m PD[d]	11/13/81	9.1×10^9 g (dry wt)$^{-1}$	0.32
	12/15/81	2.7×10^9 g (dry wt)$^{-1}$	0.07

a CFU l^{-1} formed under ADO incubation.
b Plankton sample.
c HDO CFU, <30 per plate.
d Benthic mat sample, underlying HDO water.
e HDO-CFU/ADO-CFU x 100.

TABLE 3. Total [^{14}C]glucose assimilated and percent respired in microbial mat, Lake Hoare, Antarctica

Treatment	Total carbon assimilated (cpm)			% Carbon respired	
ADO	164,197	$P < 0.06$	$P < 0.01$[a]	25	$P < 0.001$
HDO	143,580			28	
SHDO[b]	129,886			53	

a Probability determined by Duncan's multiple range test, using computer-conducted analysis (SAS Institute, Inc.)

b Mat homogenate kept in suspension by stirring during 4 h of incubation.

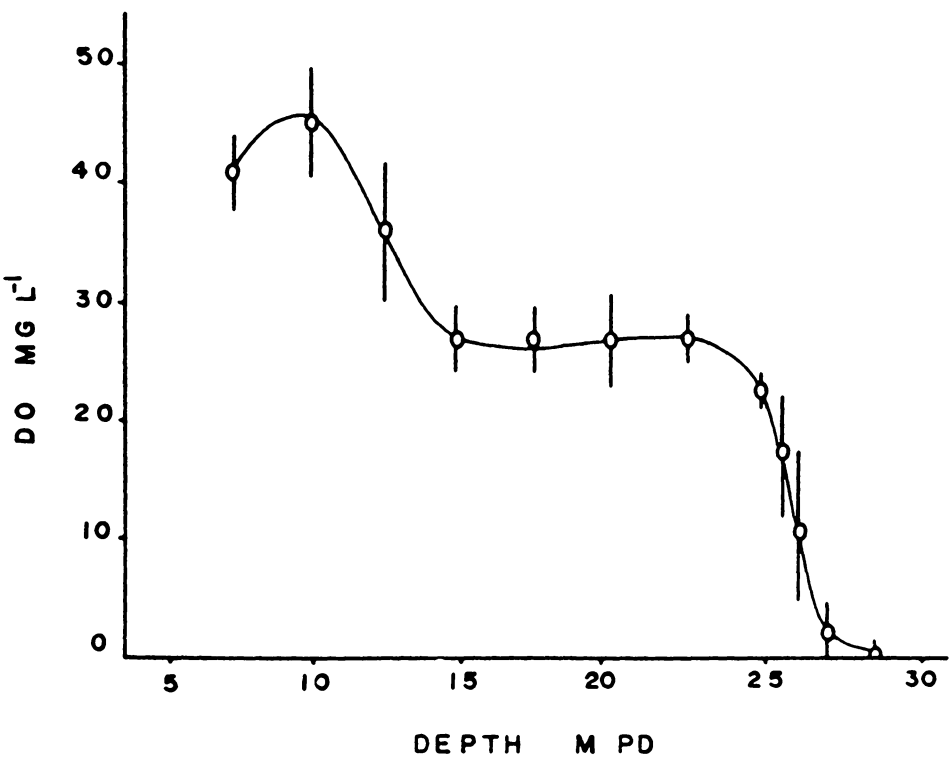

FIG. 1. DO versus depth, Lake Hoare, Antarctica. Bars represent 95% confidence intervals on samples taken over one austral field season.

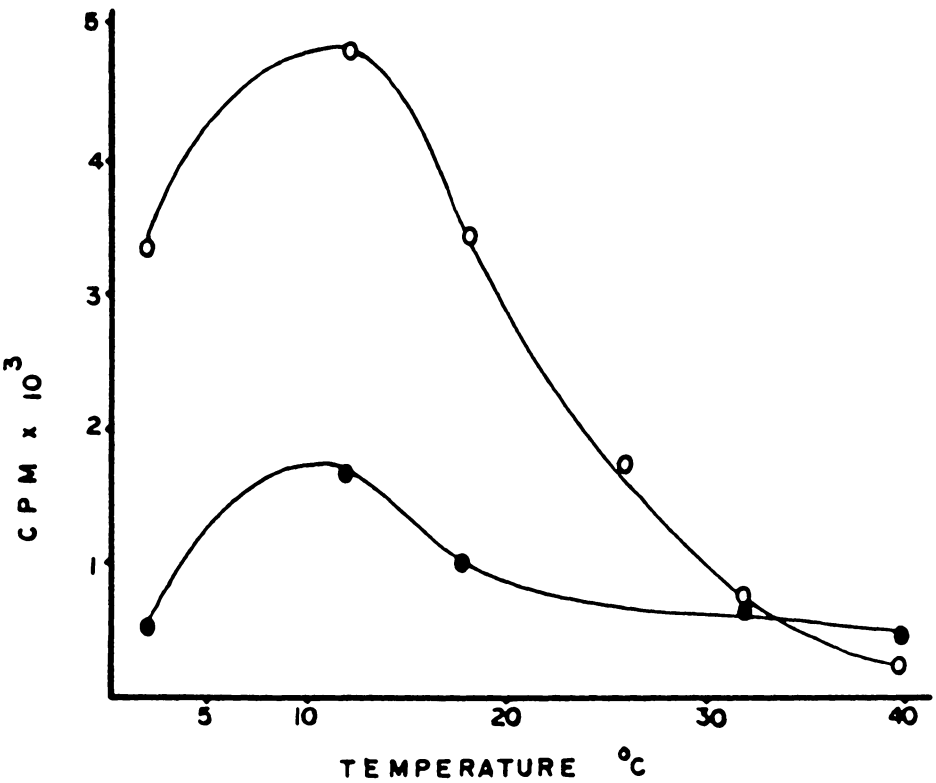

FIG. 2. Carbon assimilated (O) and carbon respired (●) in Lake Hoare, Antarctica, of D-[U-^{14}C]glucose in counts per minute versus temperature. Samples were from 6-m PD.

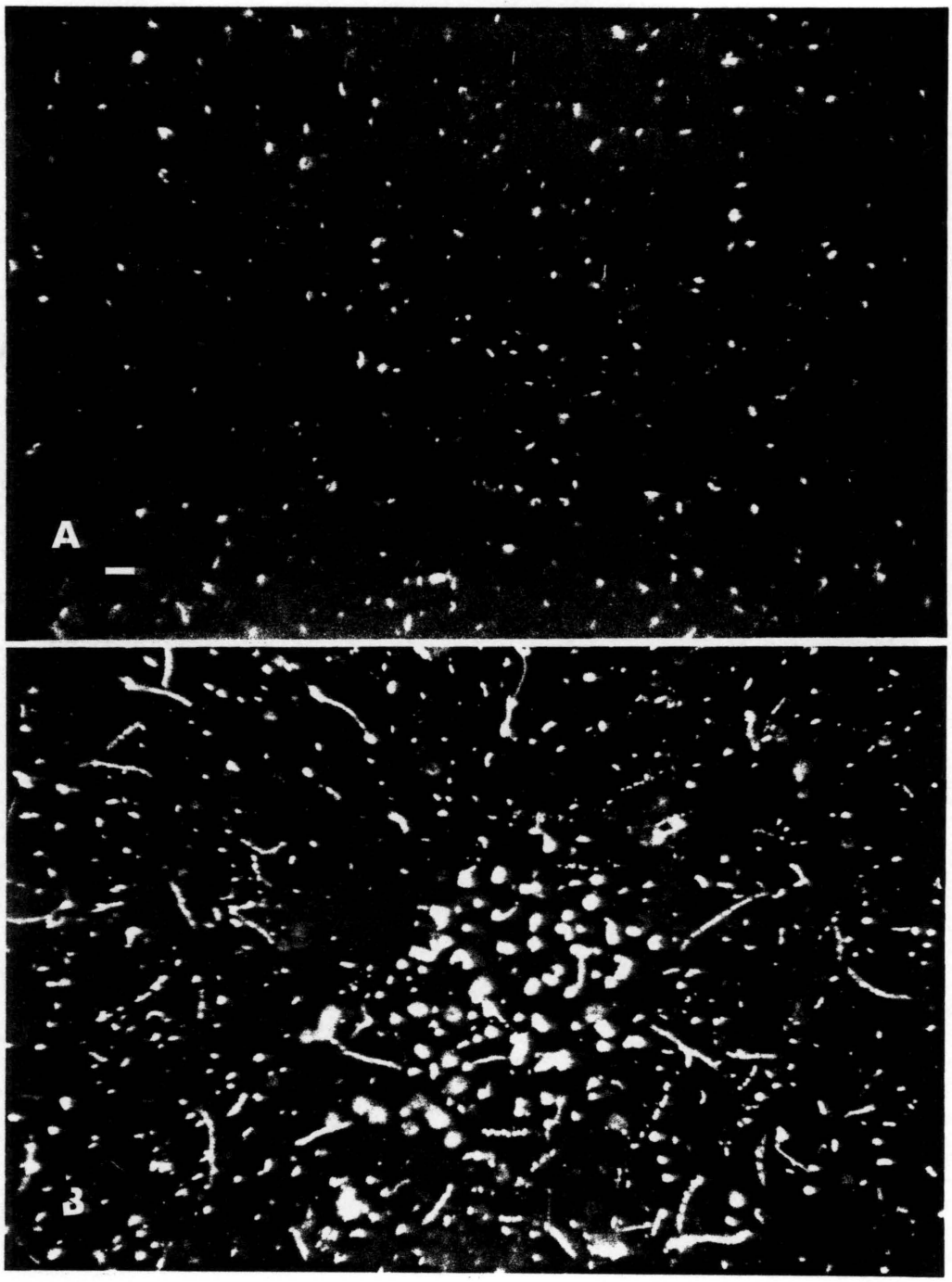

FIG. 3. DAPI epifluorescent photomicrographs. (A) Lake Hoare,Antarctica; (B) Mountain Lake, Va. Bar, 5 µm.

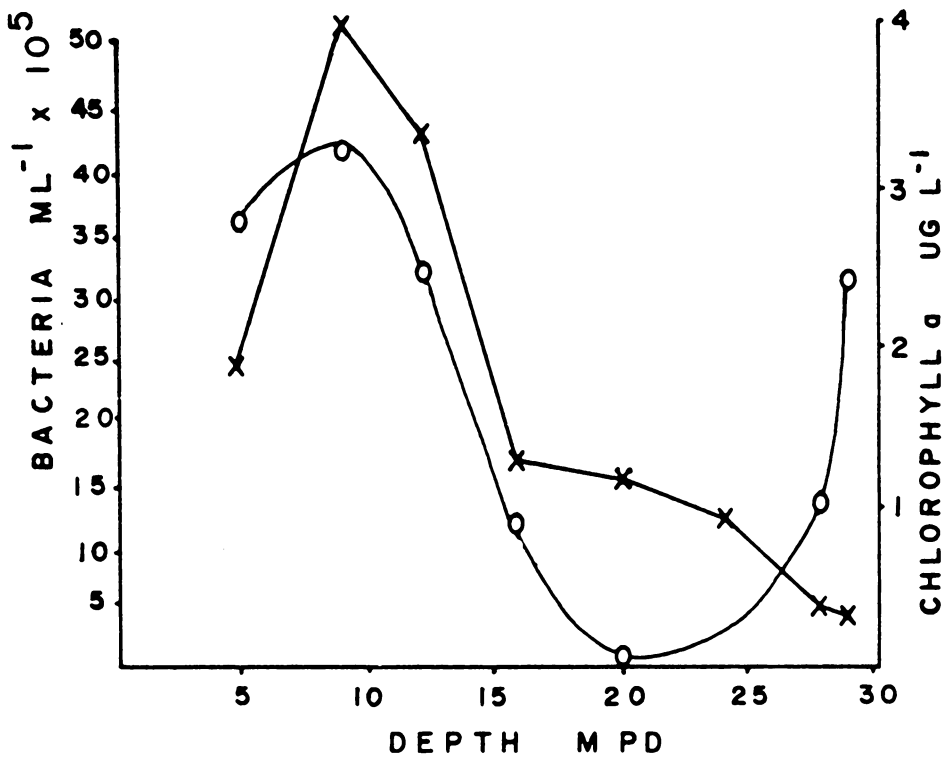

FIG. 4 . Bacteria DAPI direct count (O) and chlorophyll *a* (X) in Lake Hoare, Antarctica, versus depth.

CHAPTER 3

SOME FACTORS AFFECTING HIGH OXYGEN SURVIVAL IN MICROBIAL ISOLATES FROM LAKE HOARE, ANTARCTICA

ABSTRACT

Lake Hoare, Antarctica has an in situ dissolved oxygen concentration 3-4x normal atmospheric saturation (= high dissolved oxygen, HDO). Five microbial isolates were selected from Lake Hoare by growth under very high oxygen (=VHO, 4.6x in situ HDO, 55 lb in^{-2} of added oxygen). One isolate was selected with normal atmospheric incubation (ADO) from shallow benthic mat underlying HDO waters. We examined these isolates for selected physiological characteristics which might aid their survival in this HDO environment. VHO incubation was extremely inhibitory. While HDO incubation produced <36% of ADO incubated CFU, VHO produced <1%. The isolates were motile Gram negative rods, catalase and oxidase positive. However, they differed in their growth response to temperature and nutrient concentration. One VHO isolate was a yeast. Three bacterial isolates from this group were tested for the effect of nutrient concentration on HDO repression of maximal cell density. HDO reduced the maximal cell density in all isolates at higher nutrient

concentrations, however, all three isolates exhibited reduced repression as nutrients were lowered from 1000 to 10 mg liter^{-1} in comparison to ADO grown controls. One isolate actually produced a cell density 3x that of the ADO control. Four out of the five bacterial isolates demonstrated higher specific activities of superoxide dismutase (SOD) when grown with HDO. The inducible and constitutive SOD appear to be the manganese type and exhibited the same electrophoretic mobilities in the respective isolates. All the VHO isolates contained carotenoids. The pigmentation of the bacterial isolates differed due to the types and relative proportions of the constituitive carotenoids as evidenced by thin-layer chromatography and absorbance spectra of the purified carotenoids. A carotenoid negative mutant of one isolate grown under HDO exhibited a lengthened lag phase, decreased growth rate, maximal cell density and thereafter increased lysis compared to the same ADO grown strain and the carotenoid containing parent strain. The mutant and the parent strain produced catalase and the same specific activities of SOD.

INTRODUCTION

Oxygen is toxic to all living things. Organisms show a wide range of tolerances to oxygen from the very strict anaerobe to the obligate aerobe. Even obligate aerobes will succumb to oxygen above normal atmospheric concentrations (13). In earlier studies (31) we found that high dissolved oxygen (=HDO, 42 mg liter^{-1}) was toxic to the heterotrophic planktonic community of Mountain Lake, Virginia. This is an oligotrophic lake with waters always at or above normal atmospheric dissolved oxygen (ADO) (11).

Lake Hoare and several lakes in the dry valleys of Antarctica are extraordinary in that their upper waters perennially contain 3-4x normal dissolved oxygen saturation (defined as HDO). Nevertheless, this in situ HDO proved toxic to the planktonic heterotrophic community by the inhibition of colony forming units (CFU). Respiration-assimilation of ^{14}C-glucose approaching in situ levels was not inhibited but was stimulated in some experiments (32).

Oxygen is toxic to cells in many ways. Reactions within the cell or its environment may create oxygen byproducts which are more reactive and hazardous than

molecular oxygen such as hydrogen peroxide, superoxide radical and the hydroxyl radical (12, 26).

This multiplicity of reaction mechanisms has led to the evolution of a complex variety of adaptations. Our knowledge of oxygen defense strategies is far from complete and remains an essential goal for understanding aerobic life. Thus, study of bacteria isolated from the HDO waters of these lakes may reveal mechanisms which are new or maximally developed for oxygen toxicity protection.

We selected five planktonic microbial isolates with very high oxygen (=VHO, 19x ADO, 4.6x Lake Hoare in situ HDO) from the waters of one antarctic lake (Lake Hoare) and one with ADO incubation from the shallow benthic microbial mat underlying HDO water from that same lake. The aim of this investigation was to indentify one or more factors which may relate to the survival of these organisms in the Lake Hoare HDO environment.

All of our isolates, including the five selected with VHO, showed reduced growth rates on our standard media under HDO conditions compared to ADO growth. Our community oxygen response data indicated that increased nutrients associated with CFU production may exacerbate oxygen toxicity. This finding reinforces the theory of Kuznetsov et. al. (21) which related oxygen toxicity

protection to slow growth and toxic oxygen byproduct production associated with oligotrophy. We measured the ADO and HDO growth rates of three isolates to determine if lowering nutrient concentrations towards in situ levels would reduce the toxicity of oxygen.

Induction of superoxide dismutase (SOD) correlates with increased tolerance to oxygen in a variety of bacteria (14, 15, 34). We examined five bacterial isolates grown under HDO conditions to determine if higher specific activities of SOD were produced as compared to ADO grown cells. We also tentatively identified the type of SOD.

All five of our isolates selected with VHO contain carotenoids. Due to the low light penetration in Lake Hoare (< 13 μ Ein m^{-2} s^{-1} below the ice), photosensitized production of singlet oxygen would be minimized. The majority of the carotenoid protection research has concentrated on singlet oxygen derived from photosensitized reactions (4, 5, 29). However, carotenoids may react with other radicals as well (9). We developed a carotenoid-negative mutant of one VHO selected isolate to assess the survival advantage of carotenoids in dark HDO versus ADO growth rate experiments. We determined the relative mobility in several solvent systems with thin-layer chromatography

determined at regular intervals using a Perkin Elmer 55B spectrophotometer. The spectrophotometer had been modified to accept the serum bottles allowing a light path of 5.0 cm. Growth yield was interpreted as maximal optical density. Cultures were monitored microscopically for flocculation which would void the linear relationship between cell numbers and absorbance.

Superoxide dismutase. Cells were grown in standard growth medium to late log phase under ADO and HDO conditions. They were harvested by centrifugation (10,000 x g, 15 min), washed twice and resuspended in 50 mM potassium phosphate, 1 mM EDTA, pH 7.8. Cells were broken by sonication (Fisher sonicator, 3/4 inch tip, 60% power, 6 min). Cell extracts were clarified by centrifugation (20,000 x g, 25 min). Protein content of the supernatant fluid was determined with the Bio-Rad protein assay (Bio-Rad, Laboratories). Bovine serum albumin was used as the protein standard. Superoxide dismutase (SOD) activities were assayed according to the procedure of McCord and Fridovich (23). Inhibition of SOD by sodium azide was determined by adding the reagent to the standard assay mixture. Hydrogen peroxide inactivation of the enzyme sample was obtained by incubation of the sample at room temperature with 5 mM hydrogen peroxide + 1 mM sodium cyanide (to inhibit catalase). Subsamples

of 12°C (=VHO). In a pressure control experiment 50 lb in^{-2} of helium caused no effect on CFU formation from Mountain Lake water (31). Plates were incubated for 2 weeks at 12°C. Colonies selected with VHO and ADO were purified by streaking several times onto media containing 2 g trypticase soy, 1 g peptone and 200 mg $liter^{-1}$ filter sterilized yeast extract in lake water. Unless otherwise stated this is the standard growth and maintenance medium. Isolates on agar slants were transported to Virginia Tech packed in ice but protected from freezing.

Nutrient concentration, ADO : HDO growth yields. The standard growth medium was diluted into tap water yielding final concentrations of 1000, 500, 100, 50 and 10 mg $liter^{-1}$. Medium was prefiltered to remove suspended particulates (Whatman GF/F, Whatman Paper Ltd) and 60 ml aliquots were dispensed into 125 ml serum bottles. ADO was achieved using loose foil caps. HDO was maintained using Bellco butyl rubber serum stoppers as previously described (31). Bottles were equilibrated at the incubation temperature allowing dissolved oxygen equilibration (12-24 h). Inocula grown in standard growth media to late log phase were washed twice in 10 mg $liter^{-1}$ standard media. Inoculated serum bottles were incubated at 12°C on a reciprocal shaker (110 oscillations min^{-1}). Optical density at 550 nm was

determined at regular intervals using a Perkin Elmer 55B spectrophotometer. The spectrophotometer had been modified to accept the serum bottles allowing a light path of 5.0 cm. Growth yield was interpreted as maximal optical density. Cultures were monitored microscopically for flocculation which would void the linear relationship between cell numbers and absorbance.

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were removed at 5 min intervals for up to 1 h to determine SOD activity. Disc gel electrophoresis of clarified cell extracts was performed in 7.5% acrylimide gels (10) with a 5 mM Tris-39 mM glycine buffer (pH 8.3) at a constant current of 2.5 mA per gel. Superoxide dismutase activity was localized on the gels by the method of Beauchamp and Fridovich (6).

Carotenoid negative mutants. Mid-log phase cultures of isolates (10 ml) were centrifuged and the cells resuspended in 5 ml of the standard growth media. Ethylmethane sulfonate (Eastman Kodak Co.) was added (50 µl), and the cell suspension was vortexed and incubated in a tube roller at 12°C. After incubation, cells were washed twice in 20 mM phosphate buffer, serially diluted and spread plated onto the standard growth media. After two weeks incubation at 12°C, colonies showing color change or loss of color were picked.

HDO growth rates of ± carotenoid strains. A VHO selected isolate (*PH2A*) and a carotenoid-negative mutant (*PH2AM*) were inoculated into standard growth media. Five replicate tubes of each organism were grown under 11 lb in^{-2} (76 kPa) of added oxygen. Tubes were closed with butyl rubber stoppers designed for anaerobe culture (Bellco) to maintain pressure. ADO tubes were loosely-fitted with screw caps. The media in the HDO

replicates was allowed to equilibrate 24 h with oxygen before inoculation. Tube headspace was recharged with oxygen at 48 h intervals to maintain dissolved oxygen levels >38 mg liter^{-1} throughout the incubation period. Tubes were incubated in a tube roller (30 rpm) at 12°C. Dissolved oxygen was measured by the modified Winkler procedure (1). Growth rates were determined with a Klett colorimeter (Klett Mfg. Co., Inc.) by monitoring change in optical density at 640-700 nm. This is beyond the range of carotenoid absorbance.

Carotenoid extraction. Cells were grown on standard growth medium in the dark under HDO (11 psi, 76 kPa) in centrifuge bottles placed in modified pressure cookers and ADO replicates were both incubated on a reciprocal shaker table (110 oscillations min^{-1}). Cells were harvested by centrifugation (10,000 x g, 15 min), washed once and frozen in 20 mM phosphate buffer with 1mM EDTA. After thawing, the cells were lysed with lysozyme (0.5 mg ml^{-1}) and the carotenoids were extracted under N_2 with methanol at 4°C overnight. Cell debris was removed by centrifugation (20,000 x g, 15 min). The general precautions for handling carotenoids in all operations outlined by Britton and Goodwin (8) were followed, including working in a glove box containing a nitrogen atmosphere.

Carotenoid separation. An equal volume of chloroform was added to the methanol extract. Water containing NaCl (10% w/v) was added to prevent emulsion formation, until two phases were formed. The chloroform phase containing the carotenoids was removed, washed with water to remove methanol and salt, dried with Na_2SO_4, and the extract concentrated under N_2. This crude carotenoid preparation was stored under N_2 at -15°C. More polar carotenoids (including the xanthophylls) were separated by partioning the pigment mixtures between petroleum ether and 95% methanol as directed by Britton and Goodwin (8). The relative proportions in each phase were evaluated by their visable absorbance maxima in chloroform.

Thin-layer chromatography and absorbance spectra of carotenoids. The relative mobilities of constituent carotenoids in each of the five bacterial isolates was determined by thin-layer chromatography using Kieselgel 60 plates (EM reagents) in the following solvent systems: petroleum ether (b.p. 35-60°C)/chloroform/acetone (3:1:1 v/v), acetone/chloroform (50:50 v/v), methanol/chloroform (7:93 v/v). Maximally separated bands were eluted into chloroform and their absorbance spectra (220-600 nm) determined using a Perkin Elmer 552 or Bausch and Lomb 2000 spectrophotometer. All solvents were Photrex grade (J.T. Baker Chemical Co.).

DNA base composition. The thermal melting point of the DNA from two isolates was determined by the method of Johnson (18). The mole percent guanine plus cytosine (mol % G+C) was calculated by the equation of Mandel et. al. (27). The reference strain was *Escherichia coli b*.

RESULTS

Characteristics of the Lake Hoare isolates. Table 1 summarizes the method of selection and the physiological and morphological characteristics of the Lake Hoare isolates used in this investigation. The use of VHO was very inhibitory to CFU. Whereas <35% of planktonic ADO CFU survived HDO conditions (32), <1% of ADO CFU formed colonies with VHO incubation. All of the bacterial isolates in this study appeared similar on the basis of morphology, motility, Gram negative reaction, possession of oxidase and pigmentation. The benthic mat isolate (*MH1C*) had a larger cell diameter and demonstrated greater tolerance to increased temperature and nutrients as compared to the planktonic bacterial isolates. Isolates *PH2A* and *PH2B* demonstrated very little growth at 20°C. All these isolates from Lake Hoare had catalase, including those which we will term facultative oligotrophs (i.e. grew well at 10 mg liter^{-1}, poorly at 10 g liter^{-1} total substrate). The mat isolate (*MH1C*) did

not exhibit measurable turbidity with low concentrations of our standard growth medium; however, another medium might result in measurable growth at low concentrations as discussed by Martin and MacLeod (28). DNA isolated from two bacterial isolates, one planktonic (*PH2A*) and one benthic (*MH1C*), were subjected to DNA melting point analysis (Tm *PH2ASO* = 94.1°C, *MH1C* = 93.6°C). Both contained values in the range (G+C=55-70%) of carotenoid containing Gram negative rods suggested by McMeekin and Shewan to comprise the genus *Empedobacter* (25). Flagellar patterns were not established in these isolates; however, all bacterial isolates possessed swimming, not gliding motility.

Nutrient concentration, ADO : HDO growth yields. Only 3 of 32 isolates from lakes Hoare, Vanda, Fryxell and Bonney from the 1981-82, 82-83 antarctic field seasons failed to grow on the standard growth medium after initial isolation on our CFU medium despite the 10-fold nutrient concentration increase (0.22-3.0 g $liter^{-1}$). Nevertheless, 10 g $liter^{-1}$ (identical ratio peptone : trypticase soy : yeast extract as standard growth media) inhibited the growth of the four planktonic bacterial isolates (Table 1). A study of three of the bacterial isolates revealed that as nutrient concentrations are decreased, the HDO repression of

maximal cell density is decreased (Fig. 1). This response is illustrated as the ratio of optical density maxima, HDO/ADO. With regards to the two planktonic bacteria, *HB* and *PH2A*, there was no HDO inhibition at 10 mg liter^{-1}. In fact, a higher cell density was established with HDO in *PH2A*. Although the mat isolate (*MH1C*) showed reduced inhibition of cell yields by HDO at reduced nutrient levels, we could not measure its growth (increase in optical density) below 50 mg liter^{-1}.

Superoxide dismutase. The results of the SOD induction experiments (replicated a minimum of 5 times) are illustrated in Table 2. All the bacterial isolates, except *HB*, exhibited higher specific activities of SOD when grown under HDO as opposed to ADO conditions ($P<0.05$). Table 2 summarizes the superoxide dismutase sodium azide- and hydrogen peroxide-inhibition studies and the electrophoretic mobility patterns of SOD activity in 7.5% acrylamide gels. All bacterial isolates exhibited similar low amounts of SOD inhibition on exposure to 5 mM sodium azide and long half-lives of SOD activity in 5 mM hydrogen peroxide (Table 2). These results are consistent with the manganese containing SOD. There was no discernible difference in these inhibition patterns when comparing ADO- to HDO- induced SOD. *MH1C* resembled *HB* (0.80-0.81) and *PH2A* resembled *PH2ASO* (0.89-0.91) in

their relative mobility of their major bands of SOD activities. The relative mobility of the SOD band from *PH2B* contrasted with the other bacterial isolates (0.54-0.55). Only *PH2ASO* exhibited a secondary SOD activity band (relative mobility 0.78). No real differences in SOD relative mobility patterns were noted in respective isolates when grown under HDO or ADO conditions.

Carotenoids. All of the VHO selected isolates contained carotenoids. Each isolate produced pigmented colonies with discernible differences in their hue. Maximal pigment concentration was produced by all isolates in early stationary growth phase. Solvent extraction of the carotenoids in lysed cell preparations was in the following decreasing order of efficiency: methanol> acetone> chloroform. Fracturing the cells was a prerequisite to total one step extraction. Sonication resulted in a marked spectral change of the crude chloroform soluble extract (7-12 fold increase in UV absorbance) compared to anaerobic lysozyme cell fractionation. Crude chloroform soluble extracts of *HB*, *PH2ASO*, *PH2B* and *PH2A* produced absorbance spectra classified as "type 2" by McMeekin (24). *MH1C* exhibited a "type 1" spectrum. The majority (>50%) of the carotenoids in *MH1C* appear to be polar (i.e. xanthophylls) based on

their partitioning behavior in aqueous methanol, petroleum ether mixtures as described by Britton and Goodwin (8). The carotenoids of *HB, PH2ASO, PH2B* and *PH2A* were predominantly non-polar (i.e. carotenes). The polarity differences in constituent carotenoids contrasting *MH1C* and the other bacterial isolates is illustrated by the Rf values in Table 3. Methanol/chloroform and acetone/chloroform solvent systems produced poor carotenoid separation in the VHO isolates as they ran with the solvent front. The visual differences in pigmentation of the isolates were due to differences in carotenoid type (illustrated by Rf values and absorbance spectra) and the relative proportion of each (which could be seen as intensity of TLC separated carotenoids).

Results of ADO, HDO growth experiments on *PH2A* and the carotenoid negative mutant *PH2AM* are illustrated in Fig. 2. The carotenoid negative mutant (*PH2AM*) demonstrated reduced growth rate and maximal cell density under ADO compared to the parent strain. HDO inhibition was aggravated in the carotenoid negative mutant as illustrated in Fig. 2. It exhibited a lengthened lag phase, a decreased growth rate, reduced maximal cell density and relative increase in cell lysis upon entering stationary growth. This was confirmed by microscopic

observation as lysis and not flocculation. The colony, microscopic morphology, Gram reaction and growth inhibition at 20°C of *PH2AM* were identical to the parent strain. The specific activities of SOD in log phase cells (90 h) of the parent and mutant strain were indistinguishable in this experiment (36 vs 38). *PH2A* and *PH2AM* were catalase positive.

DISCUSSION

HDO reduced the maximal cell density at higher nutrient concentrations in the three isolates examined. This inhibition could be from toxic oxygen byproducts arising endogenously, or from exogenous toxic byproducts derived from interactions between oxygen and media constituents as demonstrated by Hoffman et. al. (17). We have tried to minimize photochemical generation of superoxide radical, hydrogen peroxide and singlet oxygen in media by dark storage and incubation. Even the most concentrated medium used in this experiment, 1000 mg $liter^{-1}$, is dilute compared to standard clinical bacterial media minimizing toxic oxygen byproducts. Nutrient reduction resulted in decreased HDO repression of maximum culture optical density. Indeed, in the planktonic isolates examined, repression of cell yield was alleviated (*HB*), or higher cell densities were

produced (*PH2A*) with HDO at <50 mg liter^{-1} of nutrients. These results support the theory of Kuznetsov et. al. (21) linking oligotrophy and oxygen toxicity. How HDO conditions contribute to acceleration of growth, evidenced in *PH2A*, is not clear. It is difficult to conceive of oxygen being a limiting factor in a low temperature oxygen-saturated environment even if these bacteria possess low oxygen affinity cytochrome oxidases. However, work by Anderson and von Meyenburg suggests that the respiration rate of *Escherichia coli* may be the growth limiting factor in static cultures (3). Increased respiration under HDO could result in increased growth rates (undetected by our technique at low nutrient concentrations) and decreased radical production by side-reactions of the electron transport system such as the production of superoxide and hydrogen peroxide by reduced quinones and flavoproteins (7, 16). The oligotrophic nature of Lake Hoare may be the most significant factor in high oxygen tolerance of the bacteria in the lake waters.

Our results do not corroborate the concept that toxicity of oxygen in these oligotrophs results from a lack of catalase activity. It may be that catalase is essential in the HDO Lake Hoare environment. Dubinina, according to Kuznetsov et. al. (21), reported an

acceleration in cell lysis of catalase-negative oligotrophic isolates when grown in rich media. This was attributed to increased hydrogen peroxide accumulation in the medium which could be alleviated by addition of catalase. In our previously reported Mountain Lake study, catalase addition to the CFU medium did not retard HDO inhibition of CFU (31).

It is interesting to note the accelerated lysis in the carotenoid negative mutant (*PH2AM*) (Fig. 3). This seems to be independant of SOD activity or catalase production. Cell lysis, resulting from membrane disruption by autocatalytic lipid peroxidation, has been established. Carotenoids have been reported to quench intermediates of this process preventing initiation and propagation of this reaction (2, 20). The carotenoids in these isolates appear membrane bound as evidenced by the difficulty in whole cell extractions as reported by Meckel and Kester (30). This fits readily into the membrane-bridging hypothesis suggested by Burton and Ingold (9) and Taylor (36). Bound carotenoids may increase membrane stability by physical reinforcement and by quenching oxygen radicals, preventing lipid peroxidation. Proteolytic pretreatment before extraction as outlined by Thirkell and Hunter (37) was not tried, so the mechanism of binding remains undetermined. Although the majority of

the carotenoid protection literature concerns photosensitized reactions, dark generation and activity of peroxides would affect cells by the same mechanisms. This would be the case in the low light environment of Lake Hoare as well as in the aforementioned HDO growth rate experiment which was performed in the dark. The possession of carotenoids may be advantageous in low light environments where high oxygen may catalyze lipid peroxidation. In this connection, it is interesting that the number of pigmented CFU planktobacteria (*Micrococcus*, *Flavobacterium*) increased during the austral summer in Lake Bonney (another Taylor Valley antarctic lake with HDO waters) (22).

Superoxide dismutase specific activities reported for these organisms are higher than those reported for *Escherichia coli* (15), albeit lower than those reported for certain aerobes such as *Bdellivibrio* (38) and *Azotobacter* (19). Four out of the five bacterial isolates examined demonstrate an increase in superoxide dismutase on growth in HDO. This induced SOD appears to be of the manganese type and does not differ in respective isolates from the ADO constituitive SOD on the basis of its relative electrophoretic mobility. If seasonally increased organic nutrients from higher productivity in the austral summer increases the toxicity of oxygen, then

perhaps an inducible enzyme system would be advantageous to survival.

The high G+C molar ratio of the two isolates examined is interesting to note as McMeekin and Shewan used the UV resistance of high G+C molar ratio strains of *Flavobacterium* to separate them from low G+C UV-sensitive strains (25). High G+C ratio types of bacteria may have a survival advantage in high light environments (35). Also, such a high G+C ratio might confer a survival advantage when free radicals are generated by means other than UV light.

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TABLE 1. Characteristics of Lake Hoare isolates used in this study

Characteristic	*PH2AS0*	*HB*	*MH1C*	*PH2A*	*PH2B*	*PH2A(1227)*
Oxygen selection[a]	VHO	VHO	ADO	VHO	VHO	VHO
Source:[b]	P	P	M	P	P	P
Gram reaction/ Morphology	- rod	- rod	- rod	- rod	- rod	yeast
Dimensions (µm)[c]	2.0x0.2	1.1x0.3	2.0x0.6	1.4x0.3	1.1x0.3	
Motility[d]	+	+	+	+	+	-
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	n.d.
Growth at: 20°C[e]	+	+	+	(-)[f]	(-)	+
30°C	-	-	+	-	-	+
Growth on 10 g/l[g]	(+)[h]	(+)	+	(+)	(+)	+
TSPEPYE 10 mg/l	+	+	-	+	+	-
Moles % G+C	n.d.	n.d	64	66	n.d.	n.d.
Pigmentation[i]	orange-red	pink	orange	orange-red	orange-red	pink

a Oxygen pressure used in initial selection: VHO=55 psi, ADO=atmospheric

b Source of isolate: P=plankton, M=benthic mat

c Average cell dimensions in standard broth media log phase cells

d Determined by hanging drop in broth standard media

e Growth by turbidity in standard media

f (-)=slightly detectable growth

g Standard media ratios of trypticase soy, peptone, yeast extract

h (+)=Growth reduced compared to 3 g/l standard media

i Standard agar media 2 week incubation 12°C

TABLE 2. Specific activities, inactivation studies and relative electrophoretic mobilities of superoxide dismutases from Lake Hoare bacterial isolates

Isolate	Growth Condition	Specific Activity	% Inhibition (mM N3)		Half-life 5 mM H202	Relative Mobility
			1	5	(min)	
MHIC	ADO	22.7	<5	8	>2 h	0.81
"	HDO	30.1+	"	13	"	0.80
PH2A	ADO	25.7	"	8	"	0.91
"	HDO	38.2+	"	22	"	0.91
PH2B	ADO	30.9	"	20	"	0.54
"	HDO	42.1+	"	21	"	0.55
HB	ADO	12.9	"	20	72	0.80
"	HDO	10.0	"	7	112	0.81
PH2ASO	ADO	19.8	"	14	>2 h	0.90, 0.79*
"	HDO	37.7+	"	16	"	0.89, 0.77*

*= minor SOD band

+= specific activity > ($P<0.05$)

TABLE 3. Relative mobilities of carotenoids from Lake Hoare bacterial isolates on thin layer chromatography

Bacterial isolate					Color[a]	Rf[b] value	Absorbance Maxima[c]						
HB	*PH2ASO*	*PH2B*	*PH2A*	*MH1C*									
				*	yellow	0.06	468	272	265	260	238		
				*	"	0.10	478	273	265	260	238		
				*	"	0.27	480	273	265	260	238		
				*	"	0.35	492	272	266	260	238		
				*	"	0.65	487	273	266	260	238		
				*	"	0.78	469	272	264	260	238		
*	*	*	*		yellow	0.77	488	462	432	274			
*	*	*	*		orange	0.87	516	484	458	368	296		
*		*	*		pink	0.93	538	504	473	392	373	322	308
	*				pink	0.93	518	500	476	276			
			*	*	orange	0.94	468	272	265	258	238		
	*	*	*	*	yellow	0.96	460	271	264	259	239		
			*		orange	0.97	515	486	454	259	239		

[a] Standard broth media, 12°C incubation, stationary phase

[b] Kieselgel 60, petroleum ether/chloroform/acetone, 3:1:1

[c] Absorbance maxima in chloroform

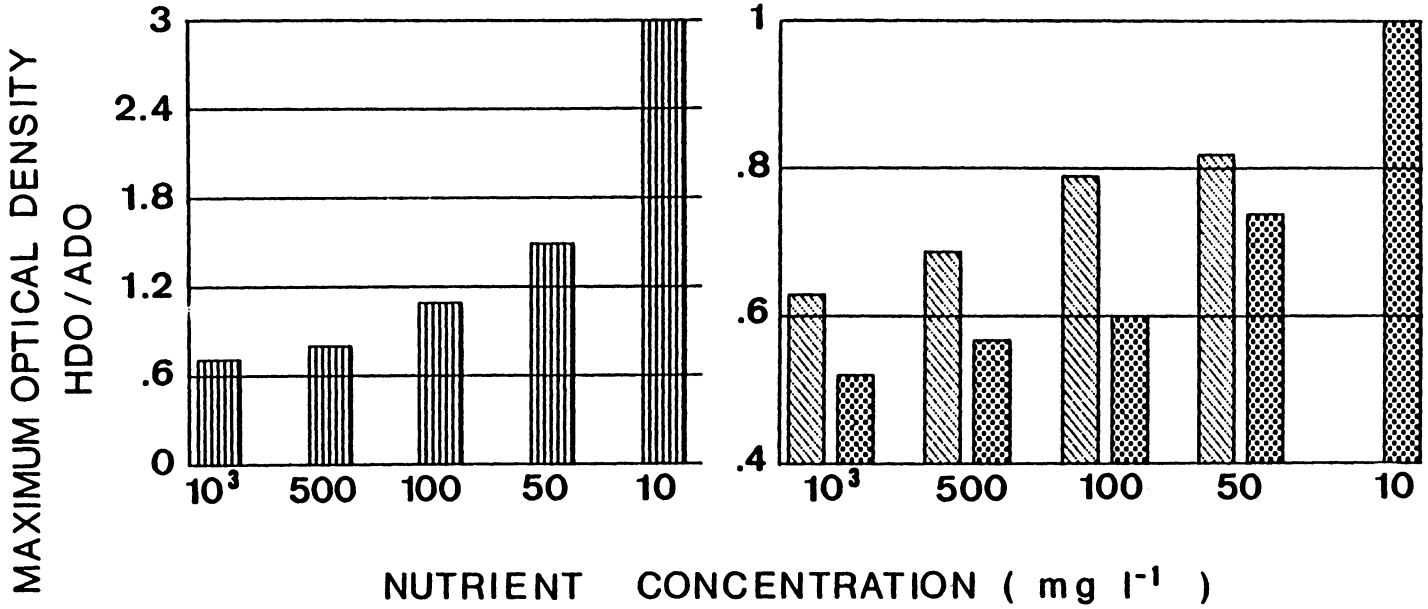

FIG. 1. Ratio of HDO:ADO maximal cell densities (optical density) of isolates *PH2A* , *HB* and *MH1C* grown in 1000, 500, 100, 50, 10 mg liter^{-1} dilutions of standard growth media.

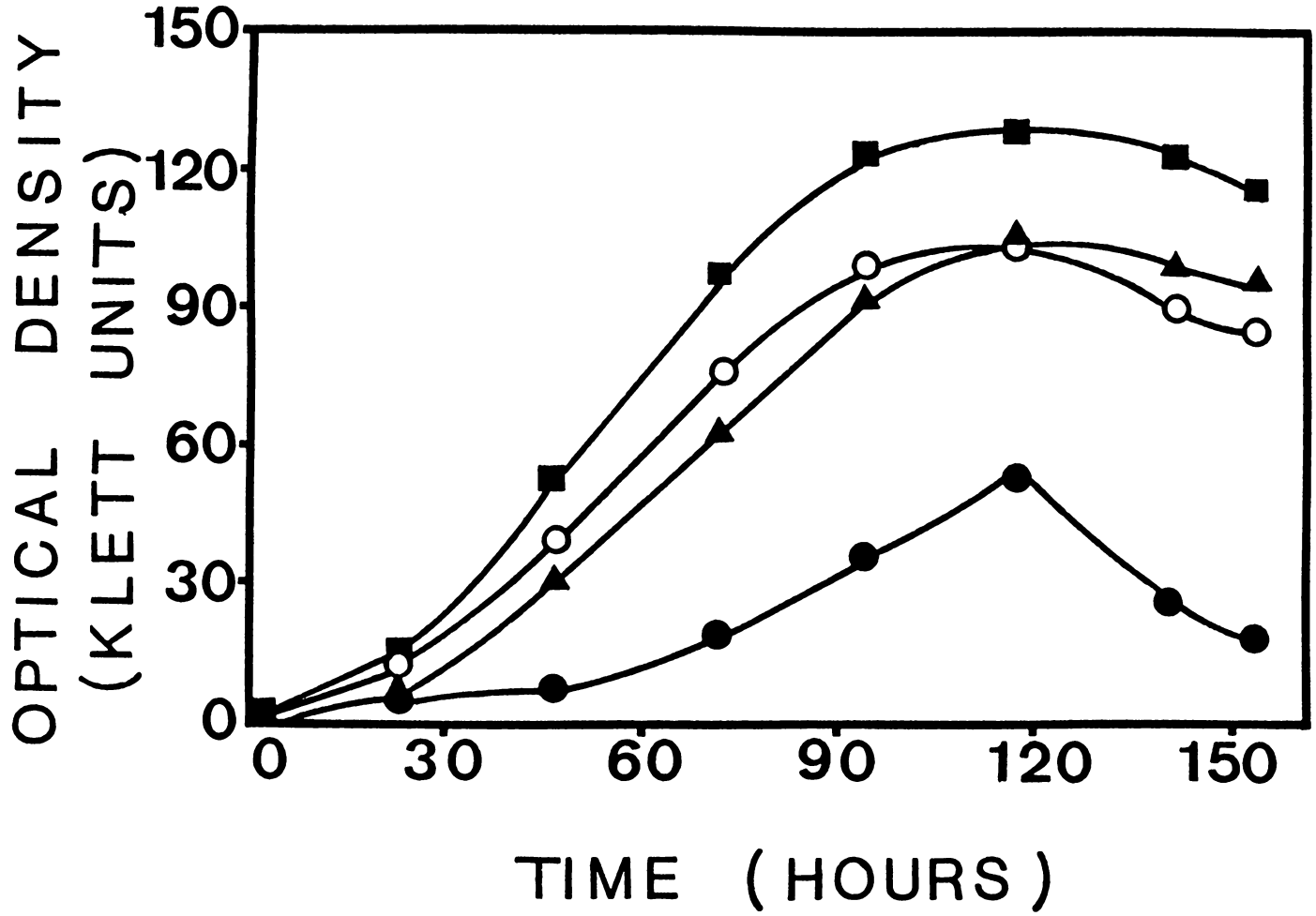

FIG. 2. Growth curves (change in optical density) of ADO grown *PH2A* ■ and *PH2AM* ▲. HDO grown *PH2A* ○ and *PH2AM* ●

GENERAL CONCLUSIONS AND DISCUSSION

Oxygen at only four times the normal atmospheric saturation value was toxic to the heterotrophic planktonic community of Mountain Lake, Virginia. This toxicity was demonstrated by relative inhibition of ^{14}C-glucose assimilation and respiration and colony formation (Chapter 1). Inhibition of colony formation was independent of exogenous oxygen byproducts as evidenced by the neutral effect of media additives (i. e. superoxide dismutase, catalase) (Chapter 1). Planktobacteria in Mountain Lake were of large cell size and frequently attached to particulates.

Unlike the planktobacteria in Mountain Lake, those in Lake Hoare, Antarctica, were free-living and not attached to substrate debris. They also had smaller dimensions like the ultraplankton from other oligotrophic systems such as the open oceans (Chapter 2). Their small size, which imparts a large surface area to mass ratio is probably advantageous in oligotrophic environments. Small size would decrease sinking velocity and maintenance energy and would increase surface area for nutrient transport relative to cell mass. However, the Lake Hoare planktobacteria have little high oxygen protection by physical diffusion distance as do the attached benthic bacteria or even the larger epibacteria, which are

attached to particles in the plankton. High oxygen repression of heterotrophic activity in mat (from underlying HDO waters) was increased when mat was dispersed, exposing more cells to oxygen diffusion (Chapter 2).

Only a small fraction of the potentially living bacteria from Lake Hoare could be cultured. To study the physiological adaptations, microbial isolates from the lake that could achieve sufficient biomass under controlled laboratory conditions had to be used. These isolates do not necessarily represent the dominant bacteria in the lake; however, they were a living part of the lake's high oxygen surviving community. The isolation of the 5 isolates under very high oxygen (=VHO, 4.6x in situ levels) is further evidence of their adaptation to high oxygen concentrations. Study of these isolates has contributed to the knowledge of oxygen defense mechanisms in microorganisms.

The VHO planktobacterial isolates appeared similar by several criteria (Chapter 3). However, the mat isolate differed in its constituent carotenoids, tolerance of higher nutrient concentrations and broader temperature range for growth.

In Lake Hoare, Antarctica, the in situ concentration of high dissolved oxygen did not inhibit the assimilation

and respiration of ^{14}C-glucose (Chapter 2). Higher nutrient concentrations inhibited colony formation. This unaturally high nutrient concentration effect suggests a clue to the nature of obligate oligotrophy. That is, nutrient levels above in situ, although required for colony formation, accompany increases in metabolism and formation of toxic oxygen metabolites beyond the normal defense capabilities of the cells. Subsequent growth experiments in 3 bacterial isolates from Lake Hoare supports this hypothesis (Chapter 3). In each case the inhibition of maximal cell growth was reduced at lower nutrient concentrations. It would be interesting to examine this trend to in situ nutrient concentrations which might be <1 mg liter^{-1}. However, measuring growth at these low nutrient concentrations could be complicated by adsorption of cells on the container surface as well as detection of turbidity. At nutrient concentrations below 10 mg liter^{-1} there was a tendency towards adhesion of bacteria to the glass container. Thus, some method to resuspend all adsorbed cells and the use of direct microscopic counts might be necessary.

Due to the investigations of Kuznetsov et. al. (Chapter 3, ref. 21) we tested our isolates for the presence of catalase. All isolates produced catalase. Although this contrasts with the aforementioned study,

the difference may be attributed to the high oxygen concentration of this oligotrophic environment necessitating catalase protection. We also used an isolation medium with a higher nutrient concentration than that used by Kuznetsov et. al.

In 4 out of 5 isolates tested, superoxide dismutase was inducible. Modulation of enzyme activity in response to need is economical to the cell. Economy is of the upmost importance in the nutrient limited oligotrophic Lake Hoare environment. The horizontal distribution of oxygen appears to be constant, judging from low fluctuations during the austral field season. The distribution is uniform under the ice at shallow depths. The area of the oxycline and hypolimnion are limited in Lake Hoare to a few deep pockets. This, coupled with low turbulence, probably makes for a continual homogenous exposure of microorganisms to high oxygen. The heterotrophic planktobacteria in this antarctic lake are dependent primarily on photosynthetic production. This varies dramatically in this polar region due to seasonal variation of light input. Increased productivity in the austral summer would increase nutrient input (as well as contribution from glacial melt streams) and aggravation of oxygen toxicity. Modulation of superoxide dismutase might be advantageous to survival in Lake Hoare as

dormant bacteria in the winter would not need the protection of active forms in the austral summer.

Light input in these lakes is minimal due to the thick ice covers. Nevertheless, adequate light energy does penetrate to drive photosynthesis. Under these high oxygen conditions the light energy for photosensitized generation of singlet oxygen might be reduced. The production of toxic oxygen byproducts can also proceed in the dark. Membranes exposed to high oxygen diffusion might be particularly vulnerable. A membrane defense mechanism to thwart lipid peroxidation would, of course, be advantageous. Carotenoids in heterotrophic organisms are known to fit this need. The importance of carotenoids is supported by the high oxygen dark growth studies of the carotenoid containing isolate and the carotenoid negative mutant. Oxygen affected the growth as well as the rate at which cells lysed in the nonpigmented mutant (Chapter 3).

These investigations have shown that some heterotrophic microorganisms have a variety of mechanisms for protection against toxic byproducts of oxygen. These mechanisms include: inherent oligotrophy or slow growth on low substrate, minimizing toxic byproduct accumulation; the enzymes catalase and superoxide dismutase; and membrane-bound carotenoids. Bacterial isolates from Lake

Hoare, Antarctica, selected with very high oxygen, appear to employ all of these protective mechanisms.

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Physiological Adaptations of Microorganisms to High Oxygen in Two Oligotrophic Lakes

by Alfred Thomas Mikell Jr.

ABSTRACT

Dissolved oxygen at four times normal saturation inhibited growth and metabolism of summer planktobacteria in surface waters of alpine oligotrophic Mountain Lake (Giles County, Virginia). Data included viable colony counts, D-[U-^{14}C]glucose incorporation into extractable lipid of colonies, and respiration-assimilation of D-[U-^{14}C]glucose by lake water samples. Significant ($P < 0.05$) differences were not detected in either colony counts or ^{14}C-lipid when superoxide dismutase (30 U ml^{-1}) or catalase (130 U ml^{-1}) were added to the medium.

The upper waters of Lake Hoare, Antarctica, contain dissolved oxygen at $\geq$42 mg liter^{-1} (=HDO). HDO did inhibit D-[U-^{14}C]glucose assimilation-respiration compared with normal atmospheric dissolved oxygen (=ADO) in Lake Hoare water. D-[U-^{14}C]glucose was assimilated and respired optimally at 12°C in Lake Hoare. Colony formation was inhibited in both lakes. Colonies represented <1% of the fluorochrome-stained direct counts in Lake Hoare. Lake Hoare planktobacteria were smaller than the

planktobacteria in Mountain Lake. ATP size fractionation revealed that 39% of ATP biomass was <0.5 μm in Lake Hoare.

Five microbial isolates were selected from Lake Hoare by growth under very high oxygen (=VHO, 4.6x in situ HDO, 55 lb in^{-2} of added oxygen). One isolate was selected under ADO from shallow benthic mat underlying HDO waters. Isolates were examined for physiological characteristics which might enhance their survival in the HDO environment. While HDO incubation produced <36% of ADO incubated CFU, VHO was more selective producing <1%. Bacterial isolates were motile Gram negative rods, catalase and oxidase positive, differing in their growth response to temperature and nutrient concentration. One VHO isolate was a yeast. HDO reduced the maximal cell density in three isolates tested at higher nutrient concentrations, however, all three exhibited less repression as nutrients were lowered from 1000-10 mg $liter^{-1}$ in comparison to ADO grown controls. One isolate actually produced a cell density 3x that of the ADO control. Four of five bacterial isolates demonstrated HDO inducible superoxide dismutase (SOD). The inducible and constitutive SOD were the manganese type and had the same electrophoretic mobilities in respective isolates. All VHO isolates contained carotenoids. Pigmentation of the

bacterial isolates differed due to the types and relative proportions of the constituent carotenoids. A carotenoid-negative mutant of one isolate grown under HDO exhibited a lengthened lag phase, decreased growth rate, maximal cell density and thereafter increased lysis compared to the same ADO grown strain and the carotenoid containing parent strain. The mutant and parent strain produced catalase and indistinguishable specific activities of SOD.

Microorganisms in the high oxygen Lake Hoare waters may be protected from oxygen toxicity by the lake's oligotrophic nature as well as a combination of cellular defenses.