UNIVERSITY OF MANITOBA

BACTERIAL NUMBERS AND SUBSTRATE UTILIZATION AS RELATED TO PHYTOPLANKTON ACTIVITY IN WEST BLUE LAKE, MANITOBA

by

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ABSTRACT

Bacterial numbers in West Blue Lake, Manitoba, as determined by plate counting, showed fluctuations of a 14 day or longer duration which were apparently related to changes in chlorophyll a. Seasonal cycles were measured for both bacterial populations and chlorophyll a concentrations. These were found to be similar for some of the depths sampled, particularly the epilimnial depths. Chlorophyll a values in the epilimnion showed spring and mid-summer maxima, with some indication of an impending early fall peak. Bacterial plate counts from Cytophaga medium (Anderson and Ordal 1961) were out of phase relative to chlorophyll a, that is, their peaks, particularly the epilimnial midsummer peaks, succeeded chlorophyll a peaks. The most significant correlation as determined by multiple regression was the negative correlation of temperature with chlorophyll a. Times of maximum temperature change were usually coincidental with maximum carotenoid:chlorophyll a ratio changes.

Although heterotrophic potential was being measured by the direct inoculation of both Cytophaga agar (Anderson and Ordal 1961) and succinate (basal salts solution) agar, comparative measurements were made on a variety of organic acids, glutamate and glucose by means of replica plating from Cytophaga medium. The proportion of Cytophaga agar counts able to use the various media varied with respect to time and substrate. But the mean proportion of each substrate was specific resulting in a relative sequential order as follows; glutamate (highest proportion), glucose, succinate, malate, fumarate, pyruvate, acetate, citrate, tartarate, glycolate and lactate. As substrates in West Blue Lake the latter two are probably insignificant. The proportions varied

from 0% to 50% and higher. The calculated absolute numbers for the replica plating media correlated very highly with Cytophaga agar plate counts.

INTRODUCTION

For many years comparisons have been made between direct microscopic examination and agar plate counts as means of enumerating bacteria. Jannasch and Jones (1959) concluded that the former is a more valid method of estimating the total number of cells. But plate count methods were chosen to estimate bacterial numbers and activity based on the premise that they provide a truer representation of the biochemically active and potentially active heterotrophic planktonic cells.

During the present study investigations were conducted to determine the most suitable media for plate counting and for estimating fluctuations in planktonic bacterial populations in West Blue Lake, Manitoba during 1972. The relationship between the planktonic bacterial population size and phytoplankton activity (chlorophyll and possible soluble organic excretion) was studied.

Although chlorophyll a as a measure of biomass is the topic of much controversy (Parsons and Strickland 1968) it must measure important parameters of the phytoplankton population, considering its importance to algal cell physiology and photosynthesis. That is, chlorophyll a must to some extent estimate algal activity, species composition and possibly biomass.

The carotenoid:chlorophyll a ratio was related to bacterial number fluctuations both quantitatively and qualitatively. This ratio (Yentsch and Vacarro 1958, Manny 1969) and a similar ratio D_{430}/D_{665} (Margalef 1965) is believed to be an indicator of physiological conditions in the phytoplankton community and/or of species composition and its changes.

As such it could be related to the availability of a bacterial

assimilable substrate via primary production and excretion or autolysis or to the release of antibiotic substance.

Both graphical and computer multiple regression analyses and comparisons were carried out between bacterial plate counts, chlorophyll a, the carotenoid: chlorophyll a ratio and temperature.

In lieu of the familiar ^{14}C heterotrophic uptake experiments (Robinson etal 1973), the technique of replica plating (Lederberg etal 1952) was applied to the problem of determining the potential of the bacterial population to utilize single organic acid substrates.

LITERATURE REVIEW

1. Plate counts

The various techniques used in acquiring plate counts and the validity of the counts obtained have been the subject of much debate. Do plate counts underestimate viable numbers due to the inability of the inoculating technique in dispersing bacterial clumps? Are the media used relatively suitable for enumeration? Do they sustain all or a large proportion of the in situ viable cells? Do plate counts grossly underestimate in relation to direct counts and which is ecologically more significant? Collins (1963) and Overbeck (1964) briefly discuss the latter question.

Although spread plates are more convenient than pour plates, the standard recommended 0.1 ml. inoculum restricts effective counting to populations of 300 or more cells/ml (Clark 1971). He found that drying the plates to an optimum loss of 3 g. increased the possible inoculum size and rate of absorption. The recovery of viable colony numbers was independent of the time delay prior to spreading (10 minutes) and of the method of spreading. That is, spreading can be equally effectively continued until all of the inoculum is absorbed or residual wetness can be left to absorb on its own. In the latter case the spreading rod itself retained less than 4% of the colony forming organisms, after spreading for a mere 15 seconds.

Buck etal (1960 and 1961) concluded that the spread plate is superior to the poured plate and that the spreader retained an insignificant proportion of the inoculated bacteria. Jones (1970) found that the proportion of residual bacteria on the spreader amounted to less than 5% ($\bar{x}$ = 2%).

Rodina (1963) mentions bacterial cell clumping. Seki (1971) concluded that the plate count method is not valid as an indicator of microbial biomass due to the large variation in the total number of cells and of heterotrophic bacteria alone per microbial clump.

Surface active agents such as "Tween 80" (Buck etal 1961) have been tested as a means of dispersing bacterial clumps. At 20 ppm it was found to be inhibitory and at 10 ppm there were count increases, but not uniform. Jones and Jannasch (1959) comparing 4 surface active agents showed that "Tween 80" used in conjunction with pour plates produced maximal numbers (range of 1 - 10 ppm). The proportion of clumped bacteria was generally quite low and varied from depth to depth, but usually less than 13% of the total clumped cells were removed. Christensen and Cook (1972) using "Tween 20" found the most effective final concentration in the inoculating solution to be 0.1 ul/l. Counts of cytophagas were 2- 3 1/2 times higher with "Tween 20" than without. Between 1 and 1000 ul/l "Tween 20" had an inhibitory effect.

The use of a plate count procedure raises the question as to how many bacterial colonies per plate constitute a biologically and statistically valid number? Plate counts of milk, water and food (Murphy and Tucker 1970) have a precision of $\pm$ 25%. The standard range of acceptable counts has frequently been stated as being 30 to 300/plate. Counts of less than 30 may be biased due to a high proportion of contaminants, while counts above 300/plate are too difficult to count. An excess of colonies per plate will create competitive forces due to overcrowding (Cooper etal 1968) and may literally eliminate the growth of some. Collins and Willoughby (1962) restricted the range to the limits of 40 to 60 colonies per plate. Buck etal (1960) disputed the assertion that

spread plates routinely contain excessive aerial contaminants.

Jannasch and Jones (1959), using pour plates, found that direct counting produces counts as much as 3 approaching 4 orders of magnitude higher. Atsuki and Hanya (1972) state that plate counts underestimate by 1 to 2 orders of magnitude. According to Jannasch and Jones (1959), the discrepancy is the result of aggregated bacteria, selective effects of the media and the presence of inactive cells.

The length of the incubation period is also an area of discussion. Strzelczyk etal (1968) found that plate counts were still increasing after 10 days and that 14 days was optimal. Jones (1970) observed counts increasing until 23 days of incubation, but along with Strzelczyk etal (1968) found that increased incubation temperature resulted in decreased maximum counts, but a shorter interval preceeding the maximum.

It is preferable to use a medium which will consistently produce maximal numbers. Work done on the effect of altering both inorganic and organic constituents of media (Jones 1970) indicated that the inorganic alterations are more significant. The medium yielding maximum counts in this instance was CPS (casein-peptone-starch medium) as used by Collins and Willoughby (1962). Fonden (1968) testing YPA (yeast extract peptone agar) found that varying the concentrations of the yeast extract or peptone produced insignificant number changes. Comparisons of various media suggested that nutrient poor media are more efficient with respect to sustaining the growth of the bacteria of oligotrophic waters. Strzelczyk etal (1967) tested 7 media and observed that the iron peptone agar yielded the highest counts.

Cytophaga agar (Anderson and Ordal 1961) supports the growth of both eubacteria and myxobacteria. Christensen and Cook (1972) tested

Cytophaga agar and although not judged to be the best medium is a good medium for the enumeration of cytophagas. The addition of SLS (sodium lauryl sulfate) to some of the media inhibited the growth of both cytophagas and non-cytophagas. It was concluded that as a differential counting tool for cytophagas this medium (SLS) was unsuccessful .

2. Aquatic bacteria

The bacterial community consists of different groups of organisms of which plate counts may enumerate only a small proportion. Sorokin (1961) states that different groups of bacteria are distinguishable on the basis of external biosynthetic carbon dioxide requirements. Heterotrophs utilize external carbon dioxide as 3% to 5% of their total carbon metabolism. They are responsible for dark metabolism and assimilation of carbon dioxide in oligotrophic lakes and near the surface of eutrophic and mesotrophic lakes. Bacteria intermediate between true heterotrophs and true chemoautotrophs utilize between 30% and 90% external carbon dioxide in biosynthesis. They oxidize low molecular weight compounds such as methane, methanol, and formic acid. The true chemoautotrophs utilize the oxidation energy of reduced inorganics such as; H_2, H_2S, NH_4, and Fe^{++}. Photosynthetic bacteria may operate in the deeper layers of the euphotic zone if anaerobic conditions exist.

Of the groups mentioned above plate count methods may select for only a small proportion of the community presumed to exist in the lake. Drabkova (1965) enumerating heterotrophic bacteria by means of MPA (meat extract peptone agar) found that they constituted only 0.05% to 0.3% of the total number of bacteria estimated by direct counts.

Collins (1960) classifies lake bacteria into 3 categories:

1. They may be of allochthonous origin and may expire or adapt and

survive the new environment.

2. They may be of autochthonous origin existing in the lake's dilute nutrient solution, that is, planktonic forms using low concentrations of soluble organics as substrates.

3. They may exist on solid surfaces such as soil particles, mud detritus or plankton.

They may be active or inactive, reactivated by the stimulus of a medium. These statements provide the basis for possible discrepancy between actual occurrences in situ and that revealed by plate counts.

The active bacteria (Straskrabova - Prokesova 1966) are defined as being "bacteria capable of reproduction within 3 hours and the bacteria divided a short time before sampling." This number was found to vary from 10.1% to 68.5% of the total direct counts. Seki (1971) found that bacterial biomass as estimated by direct counts agrees well with biomass estimates by the glucose uptake method. He is insinuating that the viable heterotrophic micro-organisms either compose a major proportion of the total number and/or that the heterotrophs remain a constant fraction of the total. Sorokin (1968) in a lake situation estimates the number of saprophytes as being 15% of the total number of bacteria, the remaining 85% being autotrophs.

3. Pigment determinations and phytoplankton biomass

The procedure of pigment determination is subject to many sources of error. The presence of myxophyceae and their attendant phycobilin pigments maybe co-extracted and will interfere with all but the determination of chlorophyll a (Strickland and Parsons 1968). The use of 90% acetone may not completely extract all cellular pigments. During extraction some species may retain 50% of their pigment (Strickland and

Parsons 1968). In a marine situation most values are acceptable except for some chlorophyta and some benthic species. In freshwater situations Strickland and Parsons (1968) recommend checking the efficiency of extraction, if it were possible.

The presence of chlorophyll degradation products will also interfere in the precise determination of pigments (Strickland and Parsons 1968). Corrections can be made for these pheo-pigments (Moss 1967, Strickland and Parsons 1968, Lorenzen 1967, and Banse and Anderson 1967), whose presence is particularly significant in freshwater (Moss 1967).

Long and Cooke (1971) in a comparison of GF-A, GF-C and HA membrane filters found that glass fibre filters were superior with respect to filtering time, time of extraction and the quantity of pigment extracted. Their results show that quite possibly membrane filters may retain more detritus and extracellular products. After treatment by maceration the membrane filters apparently tended to retain some unbroken and partially fragmented cells with undissolved pigment, while glass fibre filters were void of pigmented cellular remains.

Due to the equation of chlorophyll a and biomass a discussion of the validity of this is necessary.

Chlorophyll a can vary in algae by a factor of 5 (Strickland 1960) dependent solely on the prehistory of nutrition and illumination. Chlorophyll a:carbon ratios vary from species to species by at least an order of magnitude. The "F" values vary from 10 to values larger than 100, but these values are quoted from various authors using different methods. There appears to be no correlation between biomass of algae and their constituents, particularly chlorophyll a (Pavoni 1969). Mullin etal (1966) showed no linear correlation between chlorophyll a

and carbon. Evans and McGill (1970) found a good positive correlation between chlorophyll a and carbon.

Thomas (1972) observed that as the growth rate was increasing the carbon:chlorophyll a ratio decreased and began to level off at about 50% of the maximum growth rate. McAllister etal (1961) and Antia etal (1963) noticed that as the culture aged, the carbon:chlorophyll a ratio increased.

Steele and Baird (1962) found a carbon:chlorophyll a ratio in winter of 213:1 and a maximum in autumn of 47:1. In July and August in Loch Nevis the carbon:chlorophyll a ratio was 74:1. During the spring peak in the northern North Sea the carbon:chlorophyll a ratio was 23:1. This variation may be due to species composition differences.

Chlorophyll to biomass conversions may vary with depth (Steele 1964) as a result of algal cells low in the euphotic zone having a higher proportion of chlorophyll a. Lorenzen (1968) states that the carbon:chlorophyll a ratio decreases with increasing %Io. The ratio ranges from 39 to 64, with the final mean value being 40.5.

In a comparison of various methods of determining microbial biomass (Holm-Hansen 1969), it was found that there was good agreement of direct examination of biomass, the ATP method and chlorophyll a determinations converted to carbon for 0 through 100 meters.

Strickland (1960) suggests that carotenoids are a better biomass estimator, because they have a more constant relationship to organic matter than does chlorophyll a.

4. Bacterial seasonal dynamics

Although numbers of inactive and/or active bacteria need not correlate with generation time (Straskrabova-Prokesova 1966), the latter is

shortest in spring and fall. Beliatskaia (1958) using total bacterial counts reports summer and winter minima in June and July and January respectively. In all 3 lakes examined the spring increase took place in May. The autumn maximum varied, beginning in August or September and ending in November. Kozhova and Kazantseva (1961) noted that in one year maximal bacterial numbers in Lake Baikal occurred in August and September and the next year the latter half of July and August. The summer maximum being larger than the spring maximum.

Drabkova (1965) mentions that the longest generation times in Red Lake occurred in June, August and February at which times bacterial numbers decreased. The maximum number of bacteria occurred from April to June. The heterotrophs measured using MPA (meat extract peptone agar) relative to the total number of bacteria increased faster in summer and slower from October to March. The heterotrophic number maximum occurred in April and from October to November. Potaenko (1968) demonstrated that bacterial growth was maximal in spring and autumn and minimal in summer and winter. Although the pattern remained the same the actual bacterial numbers as determined by direct counts varied from year to year. Aerobic heterotrophic bacteria (Overbeck 1967) have been reported as reaching a maximum in summer coincident with the phytoplankton peak.

The patterns of bacterial populations are generally very variable both chronologically and geographically. Overbeck and Babenzien (1964) observed that in a small artificial water body, bacterial numbers obtained by direct counts were at a maximum in April, May and in June and September and October. A pronounced summer minimum was a common feature noted by a number of authors. Other authors mentioned by Overbeck etal

(1964) did not detect a significant annual cycle, while others noted a maximum in summer and a minimum in winter.

5. Reasons for bacterial fluctuations

There is evidence for harmonic fluctuations of algae (Steven and Glombitza 1972), and possibly bacterial cycles are due to some direct or indirect influence of the algal fluctuations. These may be long term cycles and/or, as proposed by Saunders and Storch (1971), the total reaction sequence of light, phytoplankton, extracellular soluble organic matter and bacteria may be a linearly coupled fluctuating control system cycling on a daily basis.

It has been demonstrated by Potaenko and Mikheeva (1969) that bacteria and algae may possess 3 possible relationships:

1. Maximum bacterial growth accompanied by minimum algal growth or the inverse.
2. The growth of the 2 may be parallel.
3. Both of the above may occur in one lake.

Bacterial periodicity demonstrates various seasonal patterns as reviewed earlier. Jones (1971) presented a brief review of factors influencing the fluctuations of the bacterial community. Algal periodicity has been well reviewed by Lund (1964 and 1965).

That a relationship of algae and bacteria exists can be shown circumstantially by with depth studies. Fonden (1969) noted that chlorophyll a decreased with depth as did bacterial plate counts on YPA (yeast extract peptone agar). Overbeck (1967) reached similar conclusions and more specifically stated that bacteria depend on substrates released by autolysis and release and/or excretion of soluble organics. Substrates, in turn, exert their influence by increasing growth and

normally the associated reproduction rates of the bacterial community. The number of bacteria present at any one time is a direct function of prior reproduction rates (Straskrabova-Prokesova 1966). Of importance here are active bacteria which were found to vary from 10.1% to 68.5% of the total direct counts. The availability of substrate appears to be more important than temperature with respect to changes of generation time. Drabkova (1965) using direct counts obtained similar results. In February reproduction rates decreased due to a lack of available substrate, while in summer the phytoplankton had a bactericidal effect. In April heterotrophs grown on MPA (meat extract peptone agar) increased at the expense of allochthonous microflora, while at other times increases occurred in response to algal die-offs. Beliatskaia (1958) has also shown that numbers of bacteria are directly related to the amount of organic matter in the water or the trophic state of the system.

The allochthonous microflora (Drabkova (1965) and/or other organic matter may enter as a result of rainfall. The microflora dies quickly resulting in potential substrate material. Collins (1960) and Collins and Willoughby (1962) observed similar effects of rainfall at least down to the thermocline.

In a brief review Potaenko and Mikheeva (1969) mention how algae can influence bacterial communities by altering the environment. Activities include:

1. Competition for soluble organics.
2. Antibacterial effects.
3. Alkalization of water to pH 9-10 by algae, assimilating carbon dioxide in the free and bicarbonate forms.
4. An increase in the redox potential that results from saturation of