51

Competition of marine psychrophilic bacteria at low temperatures

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The occurrence of obligately and facultatively psychrophilic bacteria in the marine environment suggests that environmental conditions exist which can favour each of these groups in competitive processes. Differences were found in the way in which temperature affected the growth rates of obligate and facultative psychrophiles. Maximum specific growth rates of a number of obligately and facultatively psychrophilic bacteria were determined in batch culture and competition experiments were carried out in a chemostat at growth-limiting substrate concentrations. From the results the relation between the specific growth rate and the concentration of the growth-limiting substrate for both types of organisms at different temperatures was deduced. Both at low and high substrate concentrations obligate psychrophiles grew faster than facultative psychrophiles at the lower temperature extreme ($\leqslant$ 4 C). These results suggest that obligately psychrophilic chemoorganotrophs are responsible for mineralization processes in cold natural environments such as ocean waters and the arctic and antarctic regions. In these environments they can successfully compete with facultative psychrophiles because they can grow faster.

INTRODUCTION

Microorganisms which are able to grow at low temperatures have been known for a long time (Ingraham and Stokes, 1959; Morita, 1966; Farrell and Rose, 1967). These so called psychrophilic organisms have been classified in 2 groups (Stokes, 1963): organisms which show an optimum growth temperature $\leqslant$ 20 C are known as obligate psychrophiles, whereas the term facultative psychrophile is used for organisms having an optimum growth temperature > 20 C.

At the time this classification was proposed the vast majority of psychrophiles isolated had to be placed in the group of facultative psychrophiles. In fact, only one obligately psychrophilic bacterium meeting the requirements of Stokes' definition had been isolated (Eimhjellen, cf. Stokes, 1963). However,

when it was recognized that obligate psychrophiles not only showed relatively low maximum growth temperatures but generally died rapidly at (moderate) supermaximal temperatures, appropriate techniques resulted in the isolation of a large number of these organisms (Morita, 1966; Harder and Veldkamp, 1966; Stanley and Rose, 1967; Sieburth, 1967). Results obtained so far indicate that facultative psychrophiles are found in environments in which the temperature varies but does not reach high values. Obligate psychrophiles on the other hand have only been found in permanently cold natural environments.

Among the permanently cold areas on earth the marine environment is of major importance. It has been estimated (cf. Morita, 1966) that more than 90% (by volume) of the marine water masses are colder than 4 C and it is therefore not surprising that reports of the isolation of marine psychrophilic bacteria are numerous (for a review, see Morita, 1966). Although for reasons stated above marine obligately psychrophilic bacteria have only been isolated in the last few years, it has already been suggested (Morita, 1966; Harder and Veldkamp, 1968) that these organisms constitute a major microbial population in the marine environment. If this were so, their predominance would be likely to result from an ecological advantage in microbial competition at low temperatures.

In a previous paper (Harder and Veldkamp, 1968) we reported the isolation of obligately and facultatively psychrophilic bacteria from North Sea water samples. The finding that representatives of both groups of organisms able to utilize a similar range of organic substrates for growth are easily isolated, indicates that environmental conditions exist which can favour each of these groups in competitive processes. The outcome of such processes under natural conditions which allow growth, is determined by differences in growth rates. This led us to postulate that selection in cold natural environments operates in favour of the obligate psychrophiles as a result of their capacity to grow faster at low temperatures. An experimental approach designed to test this hypothesis was made by comparing growth rates of representatives of both groups of organisms at low temperatures. As the concentration of organic carbon compounds in sea water is generally very low (Duursma, 1960), these experiments were carried out at low substrate concentrations. This was achieved by growing the organisms in a chemostat and advantage was taken of the fact that this method allows a study of microbial competition (Pfennig and Jannasch, 1962; Tempest, 1970). However, there have been reports of higher concentrations of organic compounds in sea water (Riley, 1963) and consequently a comparison of the growth rates of some obligate and facultative psychrophiles at high substrate concentrations was made as well. The results of these experiments are discussed in the present paper.

MATERIALS AND METHODS

Organisms. The medium and procedures for the isolation and growth of psychrophilic bacteria have been described previously (Harder and Veldkamp, 1968; Harder, 1969). The obligately psychrophilic pseudomonads used in the present investigation were maintained in artificial sea water with 0.2% (w/v) of sodium lactate as carbon and energy source, and were subcultured twice weekly at 4 C. The facultatively psychrophilic pseudomonads and spirilla were maintained similarly except that these organisms were transferred only once a week.

Growth conditions. Maximum specific growth rates at different temperatures were determined as described previously (Harder and Veldkamp, 1968). Cultures used as inocula for the mixed culture experiments in the chemostat were grown in 500 ml conical flasks containing 200 ml of medium (0.2% (w/v) sodium lactate in artificial sea water). Flasks were incubated on a rotary shaker (G–27 Gyrotory shaker, New Brunswick, New Jersey, U.S.A.) at the temperature at which the chemostat was operated. Cultures in the late exponential growth phase were transferred to the fermenter.

Selection in the chemostat. The apparatus used in the mixed culture experiments was similar to the chemostat described by Harder and Veldkamp (1967) and Harder (1969). The working volume of the fermenter was 1.5 liter and sodium lactate was used as the growth-limiting substrate. Selection at a number of different temperatures was followed at 2 substrate concentrations in the inflowing medium, namely S_R = 1 g/liter and S_R = 0.05 g/liter, respectively. The fermenter fed with medium in which S_R = 1 g/liter was inoculated with 700 ml of a culture of *Pseudomonas* L12 and 700 ml of a culture of *Spirillum* L5. When operating at the lower S_R the fermenter was inoculated with 70 ml of either of the cultures. The liquid volume in the fermenter was then adjusted to 1500 ml by adding sterile synthetic sea water, temperatures and dilution rates being adjusted to the values chosen for that particular experiment. Samples were taken from the chemostats at regular time intervals and the numbers of each of the organisms present were determined. The experiments were continued until the culture volume in each chemostat had been changed at least 5 times. In all cases one of the organisms was then dominant and the result obtained did not change on prolonged cultivation.

Determination of bacterial numbers. The colonies of *Pseudomonas* L12 and *Spirillum* L5 could be distinguished using an agar medium of the following composition: synthetic sea water, 0.2% (w/v), tryptone (Difco, bactotryptone), 0.2% (w/v) peptone (Difco bactopeptone) and 1% (w/v) agar (Difco bactoagar). A 5 ml sample was taken from the chemostat, diluted with sterile synthetic sea

water and then to 2 ml of an appropriate dilution was added 1 drop of a 30%
(v/v) aqueous solution of formaldehyde to immobilize the cells. An estimate of
total bacterial numbers was made using a counting chamber (Bürker-Türk,
depth 0.02 mm) and the original sample was then diluted with sterile synthetic
sea water to give approximately 300 colonies on the plates. Two independent
series of dilutions were made and from every final dilution 0.1 ml was spread
on the surface of precooled agarplates. The plates were incubated at 10 C for
5–7 days and the colonies of each of the two organisms were counted. The stan-
dard deviation of the count was less than 10% of the mean value and the num-
ber of colonies observed did not change on prolonged incubation.

Analytical procedures were as described previously (Harder and Veldkamp,
1967).

RESULTS

Maximum specific growth rates of obligately and facultatively
psychrophilic bacteria at different temperatures

An explanation for the occurrence or even predominance of obligately
psychrophilic bacteria in permanently cold natural environments is likely to be
found in the fact that these organisms show higher growth rates at low tempera-
tures than facultative psychrophiles. An experimental demonstration of such
a difference can be made by comparing the maximum specific growth rates of a
number of obligate and facultative psychrophiles. If any significance is to be
attached to the results of such experiments a large number of accurate measure-
ments is required. However, a less tedious approach is possible by examining
the effect of temperature on the growth rate of some obligate and facultative
psychrophiles using an Arrheniusplot. Considering Arrheniusplots of obligate
and facultative psychrophiles one may encounter one of the two possibilities
shown in Fig. 1:

A. there is a difference in the slope (= temperature characteristic of growth)
of the linear part of the plots, or

B. there is difference in the temperature range in which the plots are appro-
ximately linear.

Ingraham (1962) demonstrated the occurrence of the former possibility when
he found an appreciable difference in the temperature characteristics of growth
for psychrophiles and mesophiles. The psychrophiles tested showed much lower
temperature characteristics than mesophiles and as a result the decrease in the
growth rate of psychrophiles at decreasing temperatures is much less than
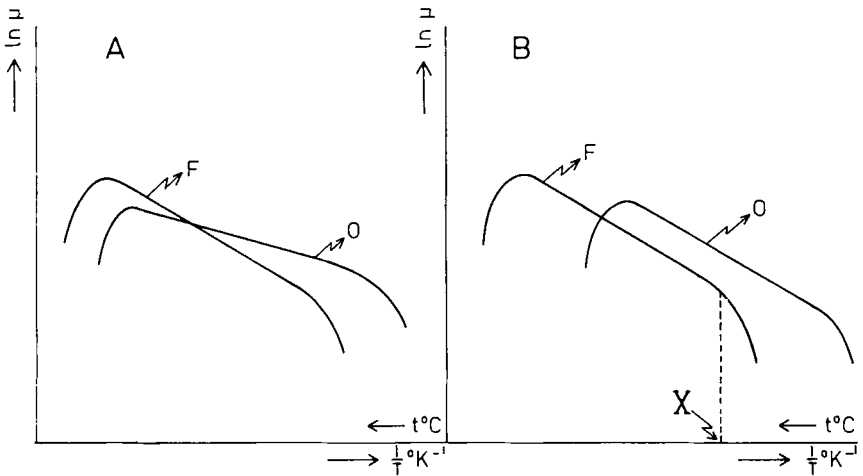that of mesophiles. If such a difference in temperature characteristics could also

Fig. 1. Hypothetical Arrheniusplots for two psychrophilic microorganisms (O = obligate and F = facultative) showing maximum specific growth rates at different temperatures.

be demonstrated in obligate and facultative psychrophiles, it would indicate that obligate psychrophiles are able to grow faster at the lower temperature extreme. Although there is some doubt as far as the general validity of Ingraham's observation is concerned (Shaw, 1967; K. E. Eimhjellen, 1967, personal communication; Harder and Veldkamp, 1968; Hanus and Morita, 1968; Baig and Hopton, 1969), it was of interest to see whether a difference in the temperature characteristics could account for the postulated differences in growth rates at low temperatures.

Another interesting feature of an Arrheniusplot of the specific growth rate of a microorganism is that it shows a temperature range in which the plot is approximately linear (Fig. 1B). This linear part of the plot extends from a temperature slightly below the optimum growth temperature towards one several degrees above the minimum temperature for growth. A temperature increase above the higher temperature at which the plot starts to deviate from linearity or a temperature decrease below the lower temperature (X for the facultative psychrophile F in Fig. 1B), results in a progressively decreasing growth rate. If the linear part of the graph extends towards lower temperatures (O in Fig.1B) then organism O will show higher growth rates than organism F at temperatures below X, provided the growth rates of O and F are comparable at intermediate temperatures.

To establish whether either of the above possibilities could give a clue to differences in the growth rates of obligate and facultative psychrophiles at low
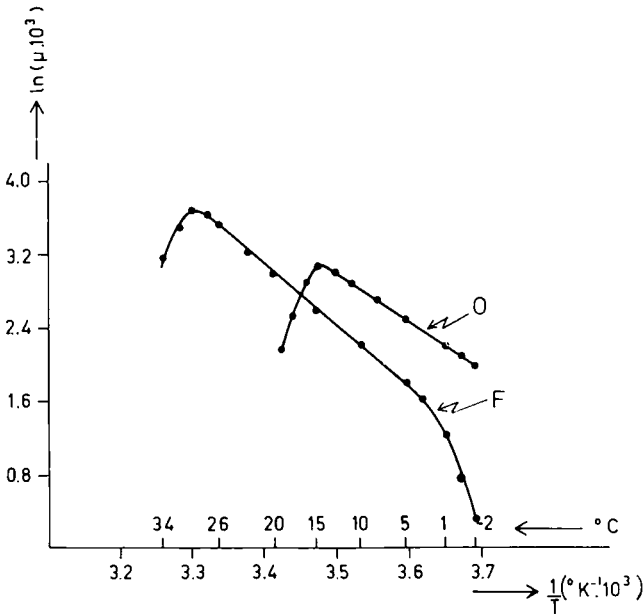
Fig. 2. Arrheniusplot of the maximum specific growth rate of an obligately (O) and a facultatively (F) psychrophilic *Pseudomonas* species (L12 and L9 respectively) at different temperatures.

temperatures, maximum specific growth rates of 4 obligately and 4 facultatively psychrophilic bacteria were determined at different temperatures. The results shown in Fig. 2, obtained with an obligately and a facultatively psychrophilic *Pseudomonas* species, are representative for the organisms tested. Similar results have been obtained by Eimhjellen (1967), who reported that the graphs for obligate psychrophiles start to deviate from linearity at–4 to–5 C.

The slopes of both curves are similar, showing a temperature characteristic of ca. 11 kcal/mole. This value is in good agreement with the results obtained by Shaw (1967), Brownlie (cf. Shaw, 1967) and Eimhjellen (1967, personal communication). These data show that the difference between obligate and facultative psychrophiles is found in the temperature range in which the Arrheniusplots are linear, rather than in different temperature characteristics of growth. The deviation from linearity at 4–5 C observed in facultative psychrophiles indicates that a decrease in temperature below ca. 5 C results in a decrease in growth rate, much greater than can be expected from a temperature effect on the rate of chemical reactions in the cell alone. Such a deviation is not found in obligate psychrophiles in the temperature range from + 4 to at least –2 C and as a result these organisms show a higher maximum growth rate at these temperatures.

Table 1. Maximum specific growth rates of an obligately and a facultatively psychrophilic *Pseudomonas* species (L12 and L9 respectively) at low temperatures

Temperature °C	Maximum specific growth rate (hr^{-1})	
	Obligately psychrophilic *Pseudomonas* L12	Facultatively psychrophilic *Spirillum* L9
–2	0.073	0.014
–0.5	0.081	0.018
1	0.090	0.033
3	0.104	0.050
5	0.120	0.059
10	0.174	0.090
14.5 (optimum)	0.208	N.D.
30 (optimum)	–	0.391

N.D. = not determined

This is illustrated in Table 1 in which the maximum specific growth rates of an obligately and a facultatively psychrophilic *Pseudomonas* species at different temperatures are presented.

Selection experiments at low substrate concentrations

The results discussed above indicate that microbial competition at low temperatures will favour obligate psychrophiles when all growth factors are in excess. However, it does not necessarily follow that these organisms will predominate at low temperatures when the carbon- and energy source is growth-limiting. As this situation occurs very often in the marine environment (Duursma, 1960), a meaningful explanation of a possible advantage of obligate psychrophiles must be based on a comparison of submaximal growth rates of obligate and facultative psychrophiles at different temperatures and very low substrate concentrations. Such conditions can be obtained when the organisms are grown in a chemostat under carbon limitation. Using this technique, it is possible to take advantage of the fact that a continuous culture can translate differences in growth rates into differences in the outcome of microbial competition: when two organisms are competing in a chemostat for the same growth-limiting substrate, the organism showing the highest (submaximal) growth rate at the particular substrate concentration will become predominant and eventually displace the other completely (Pfennig and Jannasch, 1962; Tempest, 1970). As it is possible to obtain very low substrate concentrations
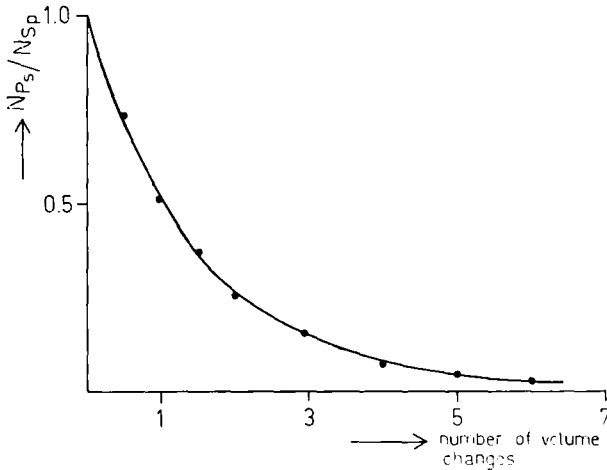
Fig. 3. Ratio of viable counts of *Pseudomonas* L12 (N_{P_s}) and *Spirillum* L5 (N_{S_p}) as a function of the number of volume changes in the chemostat. The dilution rate was 0.02 hr^{-1}, temperature 4 C and the substrate concentration in the inflowing medium 50 mg/liter.

by choosing a low dilution rate, the conditions in a chemostat imitate microbial selection in an open environment such as ocean waters (Jannasch, 1965). Studying the fate of a mixed culture of an obligate and a facultative psychrophile in a continuous culture at different temperatures and low dilution rates can then give a clue to the outcome of microbial competition under natural conditions. Such a study was undertaken using organisms of different morphology, thus facilitating the recognition of each individual species in the mixed culture. The chemostat was inoculated with approximately equal numbers of an obligately psychrophilic *Pseudomonas* species (L12) and a facultatively psychrophilic *Spirillum* species (L5). Temperature and dilution rate were adjusted to predetermined values; at different time intervals samples were taken and the numbers of each organism were estimated. The ratio of viable numbers of the organisms plotted against the number of volume changes was used to indicate the outcome of the competition.

   Fig. 3 shows a typical example of such a plot. The experiments were performed at two concentrations of the growth-limiting substrate (S_R = 1 g/liter and 0.05 g/liter respectively) in order to exclude effects of population density. At both concentrations the results of the competition were similar; selection was slightly faster at the lower S_R. At each temperature two dilution rates were applied: a low D of ca. 0.1D_c and a high D of ca. 0.7 D_c (D_c is the critical dilution rate of the obligate psychrophile at the particular temperature). The con-

Table 2. Competition between the obligately psychrophilic *Pseudomonas* L12 (O) and the fac- ultatively psychrophilic *Spirillum* L5 (F) at different dilution rates and different temperatures in a chemostat. The organism that was dominant after at least 5 volume changes is shown. D_c is the critical dilution rate of the obligate psychrophile at the different temperatures.

Dilution rate D (hr^{-1})	Temperature ($^\circ$C)			
	–2	4	10	16
LOW: ca. 0.1 D_c	O	F	F	F
HIGH: ca. 0.7 D_c	O	O	O	F

centration of the growth-limiting substrate was determined in samples which had been taken to estimate bacterial numbers, in order to ensure that selection at low values of D took place at low substrate concentrations. In all cases a very low S was found ($<$ 10 µg substrate/ml); this concentration is of the same order as that found for dissolved organic carbon in open ocean water (Duursma, 1960). The possibility that interactions between the two species may have in- fluenced the observed selection cannot be completely excluded. No indications of such an interaction could be found in mixed batch cultures: the overall growth rate of the mixed population as calculated from the optical density increase was the sum of each individual growth rate observed when the orga- nisms were grown in pure cultures. The results of the selection experiments are shown in Table 2.

At –2 C the obligate psychrophile was outgrowing the facultatively psychro- philic *Spirillum* irrespective of the dilution rate. At 4 and 10 C the outcome of the competition was dependent on the dilution rate applied. At low values of D the *Spirillum* spec. outgrew the obligate psychrophile, whereas the *Pseudo- monas* spec. became dominant at high values of D. At 16 C the facultative psychrophile was selected at all dilution rates.

These results can be interpreted with the aid of diagrams in which the growth rate at different temperatures is plotted as a function of the substrate concen- tration. The µ–S curves shown in Fig. 4 are schematic, and have been drawn according to the equation.

$$\mu = \mu_{\text{max}} \left(\frac{S}{K_s + S} \right) \qquad \text{(Monod, 1942)}$$

on the basis of 1) the maximal specific growth rate at the different temperatures as determined in batch culture and 2) the outcome of the competition experi- ments. The dilution rate at which selection was studied is indicated by arrows. At higher temperatures (16 C) the facultative psychrophile (F) shows a higher growth rate at all substrate concentrations. When the temperature decreases,
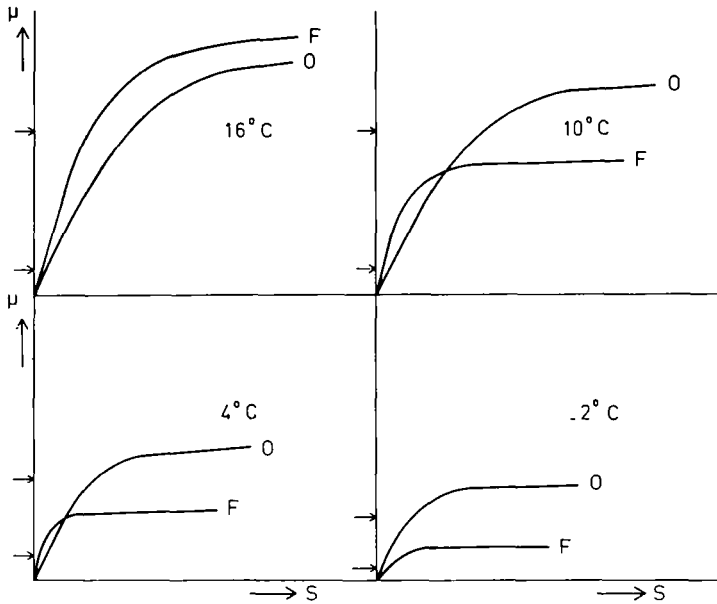
Fig. 4. Specific growth rate (μ) of an obligately psychrophilic *Pseudomonas* species (L12, O) and a facultatively psychrophilic *Spirillum* species (L5, F) as a function of substrate concentration (S) at different temperatures (schematic).

the maximum specific growth rate of *Pseudomonas* L12 (O) becomes higher than that of *Spirillum* L5 (F) and as a result the graphs show an intercept which moves towards lower substrate concentrations as temperature decreases. Selection will then depend on the substrate concentration: at values of S lower than that corresponding to the intercept the facultative psychrophile will become dominant, whereas the obligate psychrophile comes to the fore at higher substrate concentrations. Between 4 and –2 C the intercept disappears and at –2 C the obligate psychrophile shows a higher growth rate at all substrate concentrations and consequently will win the competition. The situation reported here is likely to represent one of the least favourable that an obligate psychrophile can encounter in microbial competition in view of the reports by Jannasch (1965, 1967) that the K_S values (i.e. the substrate concentration at which the growth rate is half-maximal) for *Spirillum* species are generally much lower than those found for *Pseudomonas*. Although the reason for this difference is obscure the result is that spirilla tend to show much higher growth rates at very low substrate concentrations than representatives of the genus *Pseudomonas*. It is therefore very likely that the intercept in the μ–S curves for an obligately and a facultatively psychrophilic *Pseudomonas* species will occur at higher temperatures than those reported here.

Evidence for this was obtained when it was found that the K_S-value of the facultatively psychrophilic *Pseudomonas* L9 was higher than that of the obligately psychrophilic *Pseudomonas* L12 at 10 C. Consequently competition between these pseudomonads will favour the obligate psychrophile at temperatures up to 10 C.

DISCUSSION

Many chemoorganotrophic obligately and facultatively psychrophilic marine bacteria show similar growth requirements with respect to the chemical composition of the growth medium. The fact that both types of organisms are found in nature can therefore not be explained on the basis of differences in their response to the chemical composition of their environment. It seemed likely therefore that physical factors are involved in microbial competition. When the effect of temperature on the growth of obligate and facultative psychrophiles was studied, it was found that obligately psychrophilic bacteria can outgrow facultative psychrophiles because of a higher growth rate at lower temperatures. Maximum specific growth rates found at low temperatures and excess of nutrients (Fig. 2; Table 1) as well as submaximal growth rates at growth-limiting substrate concentrations were higher for obligate psychrophiles than those found for facultative psychrophiles (Fig. 4; Table 2). Consequently when obligate psychrophiles are exposed to competition with facultative psychrophiles, they will have an advantage at the very low temperatures common in the marine environment. Although caution should be exercised in drawing conclusions from experiments with a limited number of organisms, it seems likely that it is impossible for an organism to grow relatively rapidly at low temperatures and at the same time show a high optimum growth temperature. In this respect it is important to note that the temperature range in which Arrheniusplots for growth are approximately linear, is covering a similar temperature interval for obligate and facultative psychrophiles, mesophiles and thermophiles (Fig. 2; Eimhjellen, 1967, personal communication; Ingraham, 1962; Allen, 1953). It seems likely therefore that there is a correlation between the factors involved in determining both the minimum- and the maximum temperature for the growth of microorganisms. It is not yet possible to indicate the nature of this correlation.

The finding that obligately psychrophilic bacteria grow faster at low temperatures and the assumption that this probably is a general phenomenon, suggests that there will be a continuous selection towards temperature throughout the year in environments showing seasonal temperature changes. An example of such a selection has been reported by Sieburth (1967). In samples taken

from the Narragansett Bay off the coast of Rhode Island in which the water temperature varies from –2 C (end of January) to + 23 C (August) the optimum growth temperature of the dominant chemoorganotrophic bacterial population together with the viable numbers of obligately and facultatively psychrophilic, and mesophilic bacteria were determined. In the summer the optimum growth temperature of the dominant population was considerably higher (ca. 10 C) than during the winter. Furthermore a high percentage of the winter population was obligately psychrophilic, whereas in the summer the majority of the organisms were mesophilic. Facultative psychrophiles were found in summer and winter, reaching maximum numbers in spring and autumn when the water temperature was moderate. The ratio of the numbers of representatives of the different bacterial genera were similar for summer and winter populations.

Our results and those of Sieburth (1967) indicate that in permanently cold environments such as ocean waters and the arctic and antarctic regions, mineralization processes are carried out by obligately psychrophilic microorganisms. In these environments they can successfully compete with facultative psychrophiles because they can grow faster. The price to be paid for this advantage at low temperatures apparently is that obligate psychrophiles are unable to grow at temperatures above ca. 25 C. It is not yet clear why this should be an inescapable consequence of an organism's capability to grow relatively fast at low temperatures.

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