ISSN: 0001-5113
AADRAY

ACTA ADRIAT.
33 (1/2): 67-74, 1992

UDC: 579.26 (262.3)
Original scientific paper

Stimulative effect of *Phaeodactylum tricornutum* on the growth of *Flavobacterium* sp.

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The effect of phytoplankton species Phaeodactylum tricornutum *Bohlin (Diatomeae) on the growth of bacterial strain* Flavobacterium *sp. was investigated in mixed culture at different temperatures, salinities and light intensities. Bacterial growth in the filtrate of algal culture medium was also observed. Stimulative effect of the alge* P. tricornutum *on the growth of bacteria* Flavobacterium *sp. was established. Stimulation of bacterial growth was stronger when the growth of algal population was more intensive.*

INTRODUCTION

There is a close relationship between phytoplankton as primary producers, and heterotrophic bacteria as decomposers of organic matter and regenerators of primary nutrients in marine ecosystems. Besides their mutual relationship in food chains, very important are also interactions between them by their products of excretion. It has been known that excreting substances produced by phytoplankton and bacteria may cause stimulative and inhibitory effects on other organisms in their environment (LUCAS, 1955).

Many authors have suggested that phytoplankton organisms producing antibacterial substances cause inhibitory and even lethal effects on certain bacteria (WAKSMAN *et al.*, 1938; JORGENSEN, 1956; SIEBURTH, 1959, 1964; BURKHOLDER *et al.*, 1960; DROOP and ELSON, 1966; DUFF *et al.*, 1966; BERLAND and MAESTRINI, 1969).

On the other hand, several authors have clarified the intensive development of bacteria in the presence of algae as a consequence of dying of algal cells. Namely, the dying algal cells enrich the environment with easily assimilated organic substances which appeared to be an excellent substratum for development of bacteria (WAKSMAN and HOTCHKISS, 1937; STEEMAN-NIELSEN, 1955; FUKAMI *et al.*, 1981). Moreover, it has been recognized that the products of phytoplankton metabolism may stimulate the growth of bacteria as their potential nutrients (HELLEBUST, 1965; BROCK, 1966; FOGG, 1966; VELA and GUERRA, 1966; BERLAND *et al.*, 1970; ALEXANDER, 1971; NIEWOLAK, 1971; SAMUEL *et al.*, 1971; THOMAS, 1971; WHITTAKER and FEENY, 1971; BELLY *et al.*, 1973; ALLEN and GARRETT, 1977). Several authors have found that the number of bacteria, as well as their activities, increase in the vicinity of the phytoplankton bloom zone (WOOD, 1963, VACCARO *et al.*, 1968). BELL and MITCHELL (1972) have used the term "Phycosphere" to mark this region.

The stimulative effect of the diatom on bacterial genus *Flavobacterium* has been observed by a number of authors. Thus, SIEBURTH (1968) has found the maximal growth of *Flavobacterium* during the diatom bloom.

The stimulative effect on the growth of different strains of *Flavobacterium* has been established for the diatom *Skeletonema costatum* (BELL *et al.*, 1974; KOGURE *et al.*, 1979), and the diatom *Navicula muralis* (JOLLEY and JONES, 1977).

In the present study we have investigated the effect of the diatom *Phaeodactylum tricornutum* Bohlin on the growth of *Flavobacterium sp.*

MATERIALS AND METHODS

Organisms

An axenic culture of the alga *P. tricornutum* was kindly supplied by Dr Viličić, Biological Institute, Dubrovnik, Croatia.

Flavobacterium sp. was isolated from the Kaštela bay (Adriatic sea). It was identified by using several identification schemes (SHEWAN *et al.*, 1960; HENDRIE *et al.*, 1964; GIBSON *et al.*, 1977). According to the Hayes'scheme of yellow pigmented Gram negative rods (HAYES, 1977) the isolated strain was most similar to Hayes'phenons 2, 3 and 4. Some morphological and biochemical characteristics of the isolated strain are listed in Table I.

Algal cells were counted under a light microscope using a hemocytometer. Bacterial counts were followed by the plate count method on ZoBells 2216 medium (ZO BELL, 1946).

The growth of algal and bacterial populations were expressed by a daily growth rate (k) during the exponential phase.

(Nt = No x e-kt)

Mixed culture

The culture of P. tricornutum was grown in Walne's medium with artificial sea water (WALNE, 1966) containing:

Table I. Morphological and biochemical characteristics of *Flavobacterium sp.*

1.	**Colony morphology**	
	Dimension	1-3 mm
	Shape	round
	Profile	convex
	Edge	smooth
	Periphery	smoth & shiny
	Pigment production	yellow
2.	**Cell morphology**	
	Shape	rods
	Dimension	1-3 μm
	Type of cells arrangement	single cells
	Motility	–
	Gram stain	–
3.	**Biochemical tests**	
	Nitrate reduction	–
	NH_3 formation	+
	Oxidase	+
	Catalase	+
	H_2S formation	+
	Indol formation	–
	Methyl red test	–
	Voges-Proskauer	–
	Glucose metabolism	oxid
	Acid from:	
	glucose	+
	galactose	–
	mannitol	–
	maltose	–
	lactose	–
	surcose	+
	Starch hydrolysis	–
	Gelatin liquefaction	–
	Na-citrate utilization	–
	Ammonium nitrate utilization	–
4.	**Antibiotic sensitivy**	
	Penicilin	–
	Tetracyline	–
	Streptomycin	–
	Erythromycin	+
	Chloramphenicol	+
	Kanamycin	–
	0/129	–

+ positive reaction
– negative reaction
oxid. oxidative reaction

$FeCl_3$ x $6H_2O$ - 1.3 mgl^{-1}, $MnCl_2$ x $4H_2O$ - 0.36 mgl^{-1}, H_3BO_3 - 33.6 mgl^{-1}, E.D.T.A. (Na salt) - 45 mgl^{-1}, NaH_2PO_4 x $2H_2O$ - 20 mgl^{-1}, $NaNO_3$ - 100 mgl^{-1}, supplemented with dissolved metals:

$ZnCl_2$ - 0.02 mgl^{-1}, $CoCl^2$ x $6H_2O$ - 0.02 mgl^{-1}, $(NH_4)_6Mo_7O_{24}$ x $4H_2O$ - 0.009 mgl^{-1}, $CuSo_4$ x $5H_2O$ - 0.02 mgl^{-1}, and dissolved vitamis: B^{12} - 0.005 mgl^{-1}, Thiamine - 0.1 mgl^{-1}, pH = 7.6 - 7.8.

The medium was enriched with 10 mgl^{-1} Na_2SiO_3 as silicium source for the growth of the diatom. When the algal population was in its exponential phase, the culture was subdivided into 100 ml portions in 300 ml flasks with cotton plugs. Algal cultures were then inoculated with bacteria previously grown to exponential phase in 2216 broth (ZO BELL, 1946), at 21°C. In controls, bacteria were inoculated in the same medium but free from algal cells. Just before the inoculation, glucose (50 mgl^{-1}) was added aseptically as carbon source.

Experiments

The experiments were carried out under different conditions of temperature, salinity and light intensity:

– temperature at 17°C, 21°C and 25°C, while other factors were kept constant (Salinity of 25 x 10^{-3} and light intensity at 2000 Lux)

– salinity of 15 x 10^{-3}, 25 x 10^{-3} and 35 x 10^{-3} (constant temperature at 21°C and light intensity at 2000 Lux)

– light intensity at 800 Lux, 2000 Lux and 4000 Lux (constant temperature at 21°C and salinity of 25 x 10^{-3})

Growth of Flavobacterium sp. in the culture filtrate of P. tricornutum

Exponential phase culture of *P. tricornutum* was filtrated under sterile conditions through Millipore HA filter 0.45 um. The filtrate was then inoculated with bacteria in which glucose was previously added.

The experiments were carried out under continuous light for 24 hours (Fluorescent bulbs, 40W, 5200K). During the experiment flasks were gently shaken to prevent the attaching of cells to its walls. All experiments were carried out in triplicate. Differences between bacterial growth in mixed culture and in the control free from alga statistically were tested by the t-test.

RESULTS

Effect of Phaeodactylum tricornutum on the growth of Flavobacterium sp.

The growth of *Flavobacterium sp.* in mixed culture with the diatom *P. tricornutum* indicates the stimulative effect of the alga on the growth of bacteria. In all initiated experiments the bacterial growth rate statistically was signficantly higher (P=0.01) in mixed culture with alga then in the control free from alga (Table II).

Table II. Growth rates (day^{-1}) of *Flavobacterium sp.* and *P. tricornutum* under different experimental conditions

Temperature (T°C)	Salinity (10^{-3})	Light intesity (Lux)	k_m	k_c	k_m/k_c	k_a
17	25	2000	1.67	1.44	1.16	0.30
21	25	2000	2.31	1.56	1.48	0.55
25	25	2000	2.56	1.66	1.54	0.66
21	15	2000	1.77	1.53	1.16	0.36
21	35	2000	2.48	1.55	1.60	0.73
21	25	800	2.04	1.89	1.08	0.19
21	25	4000	1.83	1.16	1.58	0.82

k_m – growth rate (day^{-1}) of *Flavobacterium sp.* in mixed culture with alga
k_c – growth rate (day^{-1}) of *Flavobacterium sp.* in the control
k_a – growth rate (day^{-1}) of *P. tricornutum*

The intensity of stimulative effect of the alga on bacterial growth, shown as k_M/k_C ratio (bacterial growth rate in mixed culture/bacterial growth rate in the control), varied with different experimental conditions (temperature, salinity and light intensity).

Temperature

The highest growth rate of *Flavobacterium sp.* was observed at 25°C and lowest at 17°C. The results in mixed culture coincided with those in controls (Fig. 2). Moreover, the stimulative effect of *P. tricornutum* on bacterial growth (k_M/k_C ratio) was stronger at higher temperatures. The k_M/k_C ratio coincided with the algal growth rate which also increased at higher temperatures.

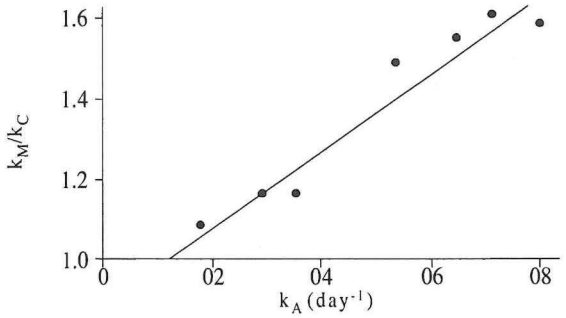

Fig. 1. The intensity of the stimulative effect of the diatom P. tricornutum on the growth of Flavobacterium sp. (expressed as k_M/k_C ratio) in the relation to the intensity of the algal growth (expressed as the algal growth rate - k_A). The coefficient of correlation was 0.97.

Salinity

In controls, the change of salinity showed no significant influence on the growth of *Flavobacterium sp.* (Fig. 3). However, in mixed culture, the increase of salinity influenced the bacterial growth. The lowest growth rate was observed at the salinity of 15 x 10^{-3}, and the highest of 35 x 10^{-3}. The increase of salinity caused stronger stimulative effect of the alga on bacterial growth (k_M/k_C ratio), which again was in correlation with the algal growth.

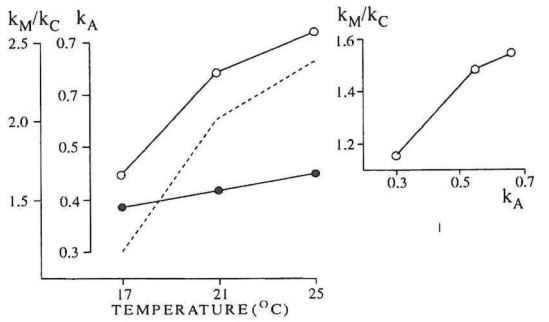

Fig. 2. Growth rates (day^{-1}) of Flavobacterium sp. in mixed culture, k_M (—○—) and in the control, k_C (—●—), and of the diatom P. tricornutum, k_A (– – –) at different temperatures. Smaller figure on the right shows the intensity of stimulative effect of the alga on bacterial growth (expressed as k_M/k_C ratio) in relation to the intensity of algal growth.

Light intensity

Light showed inhibitory effects on the growth of *Flavobacterium sp.* This was best observed in controls with the maximal growth rate at 800 Lux and minimal at 4000 Lux (Fig. 4). When in mixed culture, the inhibitory effect of light was reduced due to the stimulative effect of P. tricornutum on bacterial growth. Namely, the increase of light intensity stimulated the growth of the alga, what resulted in stronger stimulative effect of the alga on bacteria under those conditions.

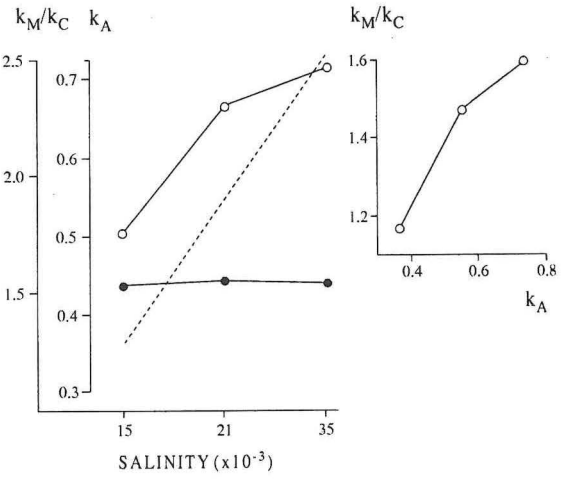

Fig. 3. Growth rates (day^{-1}) of Flavobacterium sp. in mixed cultura, k_M (—○—) and in the control, k_C (—●—), and of diatom P. tricornutum, k_A (– – –) with different salinities.

The experiment in which the cultures grew parallelly in the light and in the dark showed that the stimulative effect of the alga was stronger than the inhibitory effect of the light (Fig. 5). Thus, in controls the bacterial growth was greater in the dark, while in mixed culture, due to the algal influence, the growth was greater in the light.

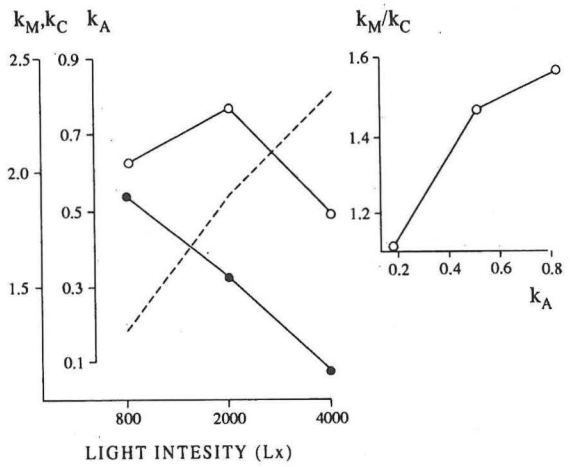

Fig. 4. Growth rates (day^{-1}) of Flavobacterium sp. in mixed culture, k_M (—○—) and in the control, k_C (—●—), and of the diatom P. tricornutum, kA (– – –) at different light intensities.

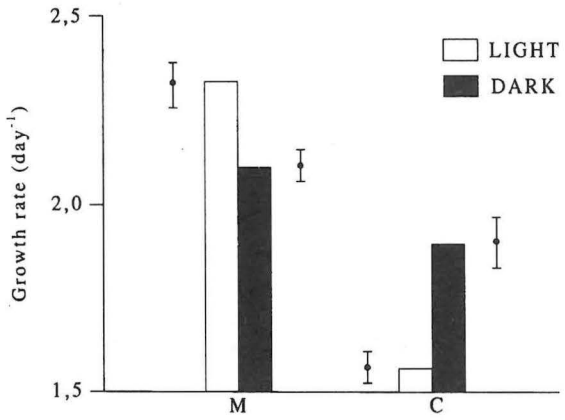

Fig. 5. Growth rates of Flavobacterium sp. in the control (C) and in mixed culture with the diatom P. tricornutum (M) in the light (2000 Lux) and in the dark. Temperature was 21°C and salinity 25 x 10^{-3}. Standard errors are for triplicate samples. The achieved differences of the growth rates statistically were significant (P=0.01).

Growth of Flavobacterium sp. in the culture filtrate of P. tricornutum

The culture filtrate of *P. tricornutum* also stimulated the growth of *Flavobacterium sp.* Figure 6 indicates that the bacterial growth rate statistically was significantly higher (P=0.01) in the culture filtrate of *P. tricornutum* than in the control.

DISCUSSION

The results of our study indicate the presence of the stimulative effect of the diatom P. tricornutum on the growth of *Flavobacterium sp.* The stimulation of bacterial growth primarily depended on the intensity of algal growth. It was observed that the more intensive algal growth was, the more intesive were the algal cell activities and, consequently, the producing of substances stimulative for the growth of bacteria. The temperature, salinity and light intensity, affecting the algal growth intensity, influenced the intensity of stimulation (shown as k_M/k_C ratio) on bacterial growth.

The results of all experiments are shown of Fig. 1. The data presented here indicate the

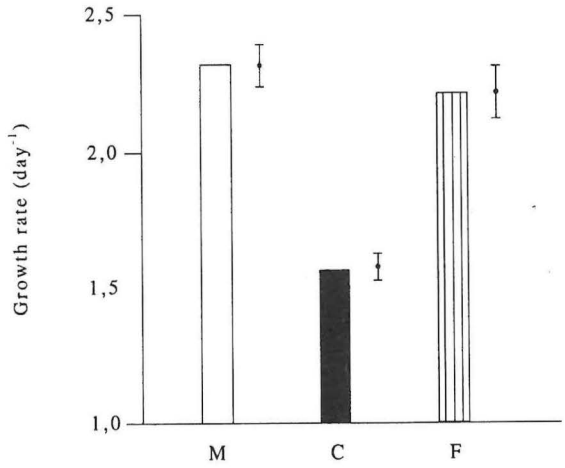

Fig. 6. Growth rates of Flavobacterium sp. in the control (C), in mixed culture with the diatom P. tricornutum (M) and in culture filtrate of P. tricornutum (F).
Bacterial growth rates statistically were significantly higher (P=0.01) in mixed culture and in the algal culture filtrate than in the control. Bacterial growth in mixed culture compared to that in the algal culture filtrate statistically shows no significant difference.

correlation between the intensity of the growth of *P. tricornutum* and the intensity of its stimulative effect on *Flavobacterium sp.* (k_M/k_C ratio).

Similar results have been suggested by KOGURE *et al.* (1979) who studied the effect of the diatom Skeletonema costatum on the growth of different bacterial genera. They have recognized the inhibitory effect of the alga on the growth of bacterial genera *Pseudomona, Vibrio* and *Acinetobacter,* and stimulative effect on the bacterial genus *Flavobacterium.* Moreover, the stimulation and inhibition were stronger when the algal growth was more intesive.

JOLLEY and JONES (1977) have clarified the stimulative effect of the diatom *Navicula muralis* on the genus *Flavobacterium.* They have found that the growth of *Flavobacterium,* when grown in mixed culture with algae, was better in the light then in the dark. The control showed opposite results. This kind of behaviour was similar to that observed in our experiments.

The stimulative effect of the culture filtrate of *P. tricornutum* on the growth of *Flavobacterium sp.* indicates that the alga releases into the environment certain substances stimulative for the bacterial growth. It is therefore conceivable that the cause of the stimulative effect probably lies in the nature of the algal extracellular products. It has been known that the algal extracellular products consist of various hydrocarbonates and nitrogenus compounds (GUILLARD and WANGERSKY, 1958; HELLEBUST, 1965; MARKER, 1965; WATT, 1969).

Moreover, vitamin B_{12}, thiamine and biotin have also been found in the algal culture filtrate (BEDNAR and HOLM-HANSEN, 1964; CARLUCCI and BOWES, 1970a,b). JOLLEY and JONES (1977) have found in the filtrate of the diatom *Navicula muralis* various vitamins and aminoacids of which some appeared to be essential for the growth of *Flavobacterium* species. The same authors have also suggested the existence of the simbiotic relationship between *Navicula muralis* and *Flavobacterium.* The existence of the simbiotic relationship has also been observed by several other authors (SIEBURTH, 1968; ALLEN, 1971; SHIBA, 1978).

It seems justifiable therefore to conclude that the experiments with another diatom species, *Phaeodactylum tricornutum* have confirmed the known literary facts of the influence of the diatoms on bacterial genus *Flavobacterium.*

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Accepted: November 10, 1991

Stimulativno djelovanje *Phaeodactylum tricornutum* na rast *Flavobacterium* sp.

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KRATKI SADRŽAJ

U radu je ispitivan utjecaj fitoplanktonske vrste *Phaeodactylum tricornutum* Bohlin (Diatomeae) na rast bakterijskog soja *Flavobacterium sp.* u mješovitim kulturama. Eksperimenti su rađeni u malim volumenima (100 ml) pod strogo kontroliranim uvjetima. Djelovanje alge na bakterijsku populaciju praćeno je kod različitih temperatura (17, 21 i 25°C), saliniteta (15, 25 i 35 x 10^{-3}) i intenziteta svjetlosti (800, 2000 i 4000 Lx), a također je ispitivan i rast bakterija u filtratu kulture alga. Svi eksperimenti rađeni su u tri replikacije.

Statistički značajno (P=0.01) veća stopa rasta bakterija u mješovitoj kulturi u odnosu na rast u kontroli utvrđena je u svim eksperimentima. Stimulativno djelovanje *P. tricornutum* na rast *Flavobacterium sp.* bilo je najjače izraženo kod temperature od 25°C, saliniteta od 35 x 10^{-3}, te intenziteta svjetlosti od 4000 Lx. Jačina stimulativnog djelovanja bila je u visokoj korelaciji sa stopom rasta populacije alga. Također je utvrđen znatno bolji rast bakterija u filtratu kulture alga u odnosu na kontrolu, što ukazuje da stimulativno djelovanje *P. tricornutum* na rast *Flavobacterium sp.* leži u prirodi ekstracelularnih produkata alge.