Microbial Ecology

Bacterial Population Association with Phytoplankton Cultured in a Bivalve Hatchery

J.-L. Nicolas[1], S. Corre[2] and J.-C. Cochard[1]

(1) Laboratoire de Physiologie des Invertébrés, DRV/RA, Ifremer, Brest BP70, 29280 Plouzané, France
(2) Micromer, ZI du Vernis, Technopole, 29200, Brest, France

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Abstract

Bacterial populations association with phytoplankton cultures used as food for bivalve larvae were enumerated and identified from their partial 16S rDNA gene sequences. Microalgae were provided from different European hatcheries during the larval production season. Average concentration (direct counts) of bacteria ranged from 1.3×10^5 to 5.3×10^8 mL^{-1} while culturable bacteria represented from 10% to >60% of total bacteria. In most cases, three to six representatives of each type of colony were collected on solid medium. The identity of isolates from the same colony type was checked by two different randomly amplified polymorphic DNA (RAPD) typing methods, after which the 16S rDNA gene of one to three isolates by colony type were partially sequenced. Algae harbored a large spectrum of bacteria belonging to the α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, *Cytophaga–Flavobacterium–Bacteroides* (CFB) group, Actinobacteria, and *Bacillus*. Members of the *Roseobacter* clade and CFB group were the most abundant. In the majority of cases one strain constituted 50% or more of the culturable bacterial flora. About half of the isolates were common to two hatcheries or at least two microalgal cultures. Several isolates were closely related to bacteria associated with harmful dinoflagellates in culture. Thus, the algal cultures seemed to favor certain bacterial species which belonged to distantly separated groups. As some of them could disturb the development of bivalve larvae, the control of bacterial populations would undoubtedly make it possible to reduce larval losses in bivalve rearing.

Correspondence to: J.-L. Nicolas; E-mail: jlnicola@ifremer.fr

Introduction

The stock species of algae used in aquaculture are maintained carefully to avoid contamination by bacteria, ciliates, or other algae. In spite of these precautions, the algal cultures are generally not axenic and unknown bacteria are often associated with them. In large-volume cultures other bacteria can be easily introduced through the air or by contact. However, bacteria are considered as innocuous except when *Vibrio* sp. are present. Such bacteria could have a harmful effect [10, 17, 20, 26] or, conversely, a beneficial influence [10, 34]. Few authors have studied the bacteria associated with algae used for larval rearing in aquaculture [10, 25, 31]. According to Prieur [31] the concentration of culturable bacteria varied from 10^6 to 2×10^8 mL^{-1} of algal culture and the species identified by phenotypic characters were diverse, apparently without any dominant bacterial type. However the method used, i.e., numerical taxonomy, did not allow affiliation of the bacteria to genera, groups or families. That bacteria could influence algal cultures was first demonstrated by Fukami et al. [10], who observed a positive effect with some bacteria, especially a strain of an *Alcaligenes* sp. on growth of the diatom *Chaetoceros ceratosporum*. Suminto and Hirayama [34] also noted a significant and beneficial effect of a *Flavobacterium* sp. on the culture of *Chaetoeceros gracilis*. However, this isolate did not influence *Isochrysis galbana* or *Pavlova lutheri* during the exponential phase of growth but it prolonged the viability of cells in stationary phase. On the other hand, a strain of a *Flavobacterium* sp., isolated during a red tide, exhibited an algicidal effect on *Gymnodinium mikimoto* [10], whereas a *Pseudomonas* sp. was reported to cause mortality of *Prorocentrum micans* [20]. Last, an isolate related to *Micrococcus* sp. caused mortality of the harmful dinoflagellate *Cochlodinium polykrikoides* [26]. Such adverse effect of bacteria on algae used in aquaculture could cause problems including poor growth and

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flocculation, although no definite reports are known of this.

When bivalve larvae show slow growth or do not feed, various causes are suspected such as the quality of seawater, eggs or microalgae. Microalgal culture are considered to have a high nutritional value when cells appear healthy. However, current observations indicate that this does not guarantee good nutritional value. For example, a culture of *Isochrysis galbana* regularly disrupted growth of scallop (*Pecten maximus*) larvae even through the algal culture did not exhibit any unusual features (René Robert, personal communication). This was considered to be due to a *Cytophaga* sp. which was present in the culture. The impact of bacteria on larvae may depend on their concentration, and bacteria associated with algae may be more numerous than those present in sea water. The inlet seawater in an aquaculture site surveyed by Prieur [31] generally contained between 10^2 and 10^3 culturable bacteria per mL, whereas bacteria associated with algal cultures ranged from 10^3 to 10^5 mL^{-1} in rearing tanks [31]. The impact of bacterial growth in larval rearing tanks can be substantial. But before determining the relationship between larval rearing problems and bacteria associated with the microalgae, an analysis of the microalgal bacterial flora is an essential step. In this study, bacteria associated with algal cultures from six hatcheries were enumerated and identified by molecular methods as part of a collaborative European programme.

Materials and Methods

Algal Cultures. Algal cultures were provided by commercial hatcheries located in Spain (Tinamenor S.A.), the United Kingdom (Seasalter Shellfish, Whitstable, from two hatcheries at Reculver and Walney), Guernsey (Guernsey Sea Farms), Ireland (Lissadel Shellfish Co.), and France (Satmar). The methods of algal cultivation differed at the different hatcheries. Algae for larval nutrition were produced in continuous cultures, without temperature regulation, and depending partially on solar light in the UK, Spain and Ireland, whereas in France algae were grown in batch cultures with only artificial light. Enriched seawater based on the Conway medium [35] was used in the Satmar and Guernsey hatcheries, and f/2 medium [16] in the other hatcheries. The concentration and duration of algal cultures varied according to hatcheries and seasons. Seawater used for culture medium was sterilized in large volumes with chlorine and neutralization with equimolar or excess sodium thiosulfate in the Satmar hatchery and by pasteurization in the other hatcheries. In small volumes (<20 L) seawater was autoclaved.

The diet distributed to bivalve (mainly oyster) larvae was composed of a mixture of two to three microalgae in equal quantities of cells mL^{-1}: one or two flagellates such as *Pavlova lutheri*, *Isochrysis galbana*, and one diatom, such as *Skeletonema costatum*, *Chaetoceros calcitrans*, *Chaetoceros muelleri* or *Chaetoceros gracilis*. *Tetraselmis suecica* and *Rhodomonas* sp. were given to larvae near metamorphosis in some hatcheries. The quantity of algae distributed daily to larvae depended on the rearing method and the age of larvae and ranged from 5×10^3 to 2×10^4 cells per larva.

In each hatchery, 1 L of algal culture used as larval food was sampled routinely and sent by mail at 4°C, taking a maximum of 48 h to arrive at the laboratory. On receipt, a portion of each algal sample was fixed with formaldehyde (1% v/v final concentration) for counting algal cells using a hemacytometer (Malassez cell) and bacterial cells by epifluorescence microscopy.

Testing Microalgae in Larval Bioassays. Tests were performed to ensure that transport did not cause deterioration of algal cultures. Tests were set up in triplicate in 2-L beakers, each containing 20,000 *Crassostrea gigas* larvae, previously obtained by stripping the gonads of mature animals. Seawater was filtered to 1 μm and maintained at 25°C.

Each sample of algae was sufficient to feed the larvae three times. During that period algae were kept in their original containers, illuminated but not aerated. A control culture was fed on algae grown at the Brest facilities.

One week later, at the end of the experiment, each batch of larvae was sampled and size (major length) measured automatically using image analysis processing software.

Counting of Bacteria. Bacteria were counted on a epifluorescence microscope following staining with 4′,6-diamidino-2-phenylindole (DAPI) according to [30]. In addition algal samples were serially diluted 10-fold and plated on Marine agar 2216 E (Difco) and TCBS (Thiosulfate Citrate Bile Sucrose) agar (Difco). Colonies were counted after incubation for 2 days for TCBS agar and 2 weeks for Marine agar at 20°C.

Isolation, Selection and Identification of Bacterial Strains. On plates containing 30 to 200 colonies, three to six single colonies per distinct morphological type were picked and purified for identification of bacterial strains. When it was impossible to distinguish different types of colony or when they were too diversified, 20 to 25 colonies were collected at random.

DNA was extracted and purified by a simple previously described method [12]. Briefly, bacterial cells from a overnight culture were collected and resuspended in 200 μL lysis buffer (10 mM tris/HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100) and boiled for 5 min. DNA was purified by a single chloroform extraction. DNA was

Table 1. Ratio between direct count (DC) and plate count (PC):DC/PC

Hatchery	*<1.0*	*From 1.5 to 2.5*	*>3.5*
Reculver		1.5 (Ts)[a]	4.3 (Ig), 7.5 (Sc)
Satmar	0.4 (PI)	1.5 (Ig)	3.7 (Cc),
Lissadel	0.7 (Ts), 0.26 (Ig), 0.4 (Cm), 0.7 (Sc)		
Walney	0.9 (Pl), 0.9 (Ts),	2.4(Rh), 2.3(Ig)	
Tinamenor	0.9 (Ig)		12.1 (Cg)
Guernesey		2.2 (Sc), 2..4 (Pl),	4.4 (Ig)
Total	8	6	5

[a]The algal species was indicated in parentheses. Ig: *Isochrysis galbana*; Pl: *Pavlova lutheri*; Cc: *Chaetoceros calcitrans*; Cp: *C. pumilum*; Cm: *C. muelleri*; Ts: *Tetraselmis suecica*; Rs: *Rhodomonas* sp.

amplified using a Ready-to-go RAPD (Randomly Amplified Polymorphic DNA) kit (Amersham) according to the manufacturer's instructions. To ensure that colonies with identical morphology corresponded to the same isolate, DNA was amplified twice via RAPD using two different primers (primer 1283: 5′-GCGATCCCCA-3′ and primer 3: 5′-GTAGACCCGT-3′). The RAPD products were separated by electrophoresis in 2% agarose gels in TAE buffer containing 0.5 μg mL^{-1} ethidium bromide. In most cases, colonies with the same morphology corresponded to only one fingerprint, but sometimes to two or even three different fingerprints. In these cases the percentage of these colony types was divided equally by the number of related fingerprints.

For each algal sample one to three bacteria per RAPD pattern were identified by partial sequencing of 16S rDNA (about 700 bases). Sequencing PCR reaction was performed in 50 μL final volume, with ~50 ng DNA, 250 μM of each dNTP, 1.5 M $MgCl_2$, 1.5 U *Taq* polymerase (Appligene), 100 ng of each primer, 342*f* (5′-TACGGGAGGCAGCA-3′) and 1114*r* (5′-AGGGTTG CGCTCGTTG-3′) or in some cases 8 *f* (5′-AGAGTTT GATCCATGGC-3′) and 925 *r* (5′-AAACTCAAATGAA TTGACGG-3′), and double-distilled water (Milli-Q). The PCR temperature profile used was 95°C for 3 min, followed by 30 cycles consisting of 94°C for 30 s, 52°C for 1 min, 72°C for 90 s with a final extension step at 72°C for 5 min. PCR products were purified according to Gauthier et al. [12]. Briefly, *Taq* polymerase was removed by extraction with chloroform (2 volumes). The amplicons were precipitated by adding 0.6 M polyethylene glycol (PEG) and 2 M NaCl, and rinsed with 70% ethanol. Sequencing was performed by the Genome Express Company with an automatic sequencer (Grenoble, France).

Phylogenetic Analysis. The 16S rRNA gene sequences were aligned with the nearest reference sequences, obtained from Blast analysis, with the ClustalW and Megalign programmes (Dnastar, Madison, WI). Phylogenetic trees were built with the Phylowin program [11] using the neighbor-joining method. A strain was affiliated into a group or genus if it was located by phylogenetic analysis inside this group or genus. An isolate was affiliated to a reference bacterium when their 16S rDNA sequences >97% identical.

Nucleotide Sequence Accession Numbers. The nucleotide sequences of the isolates in this study have been deposited in the EMBL and GenBank data banks. The accession numbers of the isolates and relevant bacterial sequences those found in the GenBank database are given in Figs. 2 and 3.

Results

Larval Bioassays. Tests showed that the 24 to 48 h transport did not cause deterioration in the algal quality. The algal culture did not collapse during storage and larval survival was >90% at the end of the experiments. Comparison of the oyster larval size on day 9 showed a slight, but not significant, reduction of growth with algae from Satmar, compared with the control, and a significant improvement in one case (Tinamenor).

Bacterial Counts. Plate counts and direct counts were closely related, with the ratio between direct counts and plate counts being <2.5 in 14 cases out of 19 (Table 1). In eight out of these 14 cases, plate counts were greater than direct counts, especially when bacteria were less numerous than algal cells. For the others (five out of 19) the ratios ranged from 3.7 to 12.1. Considering the highest counts between direct counts and plate counts, the bacterial concentrations were <10 times those of the algal cell concentrations in all except four cases. Bacterial concentrations and/or the algal:bacterial cell ratios were on average significantly less in *P. lutheri* and *I. galbana* than in the diatoms (Fig. 1), *T. suecica* and *Rhodomonas* sp. TCBS plate counts were often below the level of detection (10 CFU mL^{-1}), except for all algal cultures received from Reculver, where TCBS counts ranged from 9.0×10 to 5.2×10^4 CFU mL^{-1} (data not shown).

Characterization of the Bacterial Population of Algal Cultures. Estimates of the proportion of identified bacteria per algal strain (Table 2) depended on the ratio

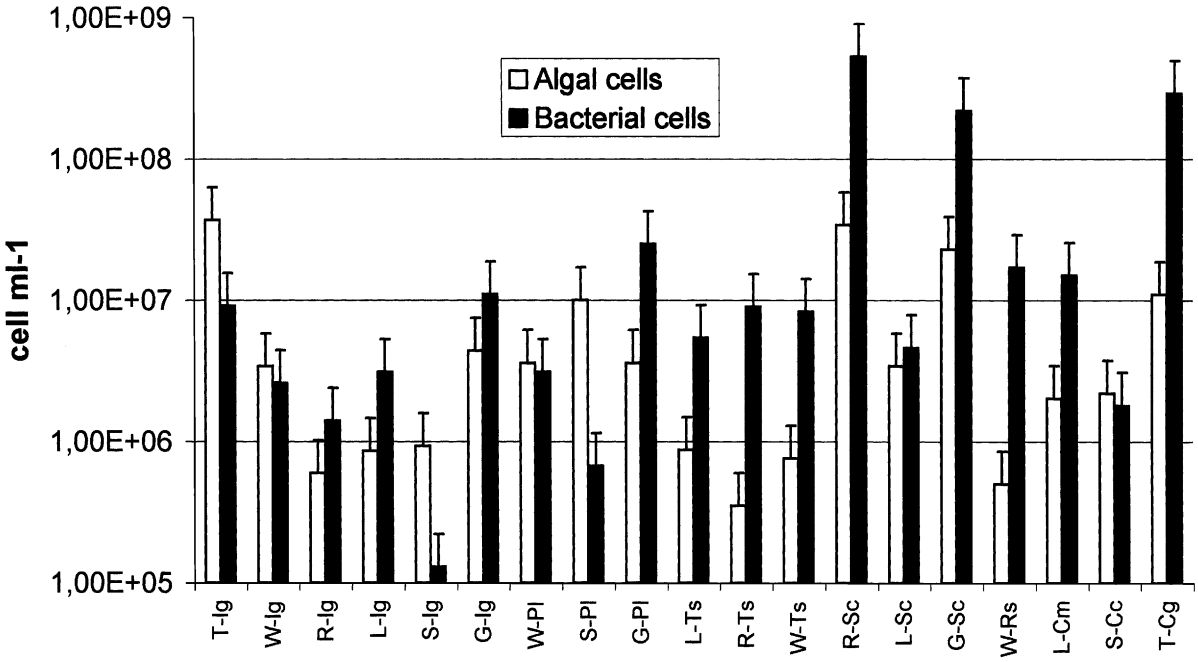

Figure 1. Concentrations of bacteria and algae in different algal cultures. The first letter indicates the hatchery, L: Lissadel; S: Satmar; R: Reculver; G: Guernsey; W: Walney; T: Tinamenor. The two following letters indicate the algal culture, Ig: *Isochrysis galbana*; Pl: *Pavlova lutheri*; Cc: *Chaetoceros calcitrans*; Cp: *C. pumilum*; Cm: *C. muelleri*; Ts: *Tetraselmis suecica*; Rs: *Rhodomonas* sp.

of culturable bacteria, the success of collection, amplification, and sequencing. The poor recovery of bacteria associated with *I. galbana* was mainly due to the inability to regrow many of the dominant isolates. For *C. gracilis*, *C. calcitrans*, and *S. costatum* only a small fraction of bacterial population was identified because culturable bacteria were a small proportion of the total bacteria.

In nine out of 19 cases one strain represented at least 50% of the bacterial flora, but in all algal cultures except one (*S. costatum*, Guernsey), there were at least three bacterial strains per algal culture, including strains which did not regrow. No relationship was found between diversity and bacterial concentration. The phylogenetic trees show the affiliation of representative bacteria isolated from the algal cultures (Figs. 2 and 3). The isolates belonged to a wide range of genera or groups.

The *Rhodobacter* group is well represented and most of isolates in this group are clustered in four genera: *Ruegeria*, *Roseobacter*, *Sulfitobacter* and *Prionitis* (Fig. 2). The other isolates placed inside α-Proteobacteria are scattered in four groups: *Hyphomonas*, *Zymomonas*, Rhizobiaceae, and *Azospiririllum* (Fig. 2). Two isolates belonging to the same species close to *Zooglea ramigera* were members of β-Proteobacteria (Fig. 2).

γ-Proteobacteria were weakly represented and very dispersed with only one isolate per genus (Fig. 3). Conversely in the CFB group, most isolates were grouped with, and closely related to, *Muricauda ruestringensis*. Some Gram-positive bacteria were also present. Three isolates had at least 99% similarity with reference bacteria: WRs-7 (*Bacillus horikoshii*), Tig-5 (*Agrobacterium sanguineum*), LCm-3 (*Alteromonas macleodii*); 13 were

Table 2. Percentage of identified bacteria

Algae	*Number of samples*	*% of culturable bacteria identified*	*Direct count/plate count ratio*[a]	*% of total bacteria identified*
I. galbana	6	54.2	4.1	13.2
P. lutheri	3	83.3	1.2	69.4
S. costatum	3	81.7	3.4	24.0
T. suecica	3	95.7	1.1	87.0
C. gracilis	1	94.1	12	7.8
C. muelleri	1	87.3	1	87.3
Rhodomonas sp.	1	85.2	2.4	35.5
C. calcitrans	1	87.4	4	21.8

[a]Average DC/PC ratios. Ratios below 1 were considered as equal to 1.

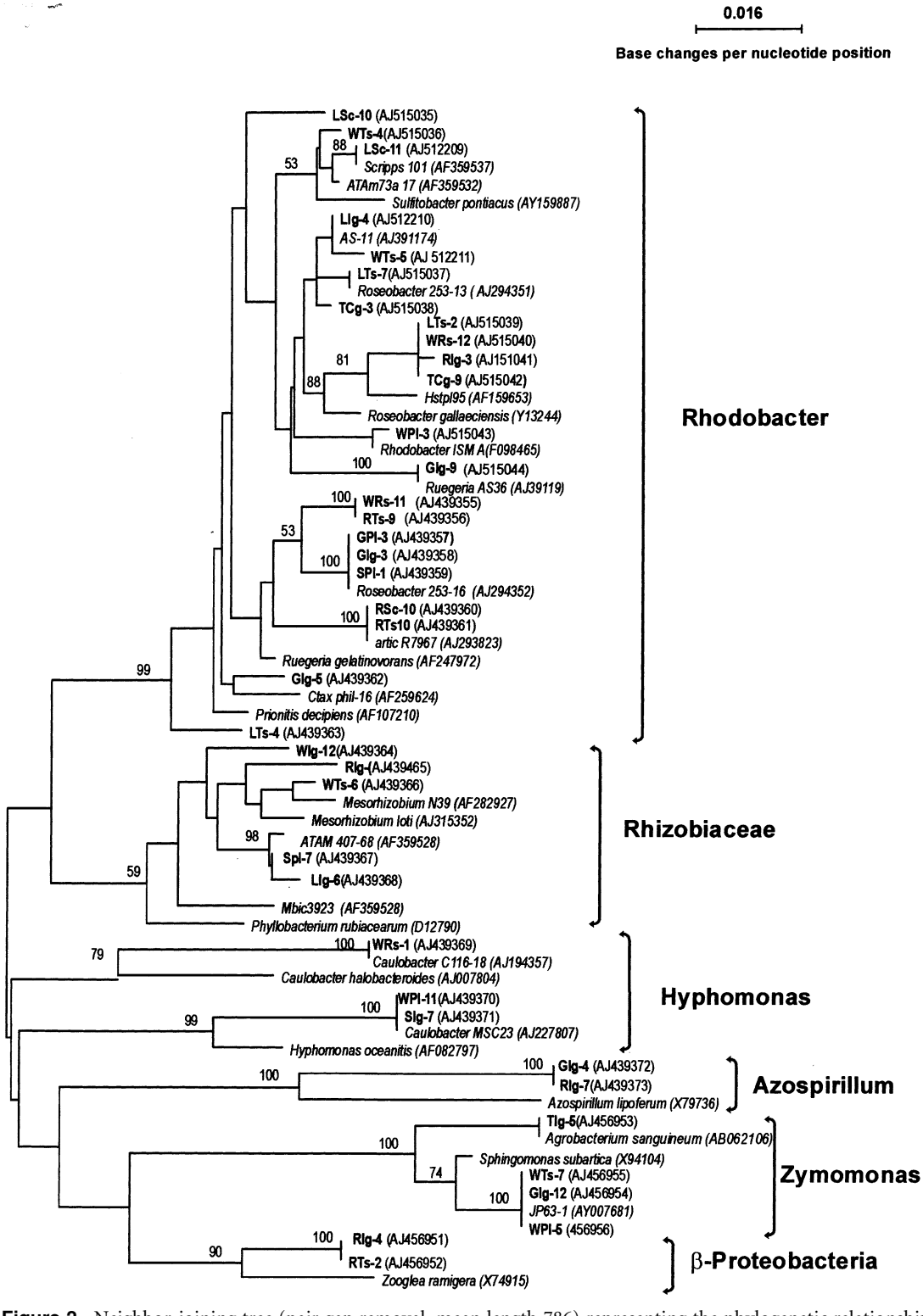

Figure 2. Neighbor-joining tree (pair gap removal, mean length 786) representing the phylogenetic relationship among partial sequences of bacteria (boldface) isolated on solid medium from the algal cultures and α- and β-Proteobacteria from the EMBL sequences data bank. Bootstrap values (500 replicates) >50% are indicated. Accession numbers of EMBL sequences are given following the name of the isolate. Isolates are identified by three letters and one figure. The first letter indicates the hatchery and the two following letters indicate the algal culture (see legend to Fig. 1). The number indicates the serial number of an isolate on agar plate.

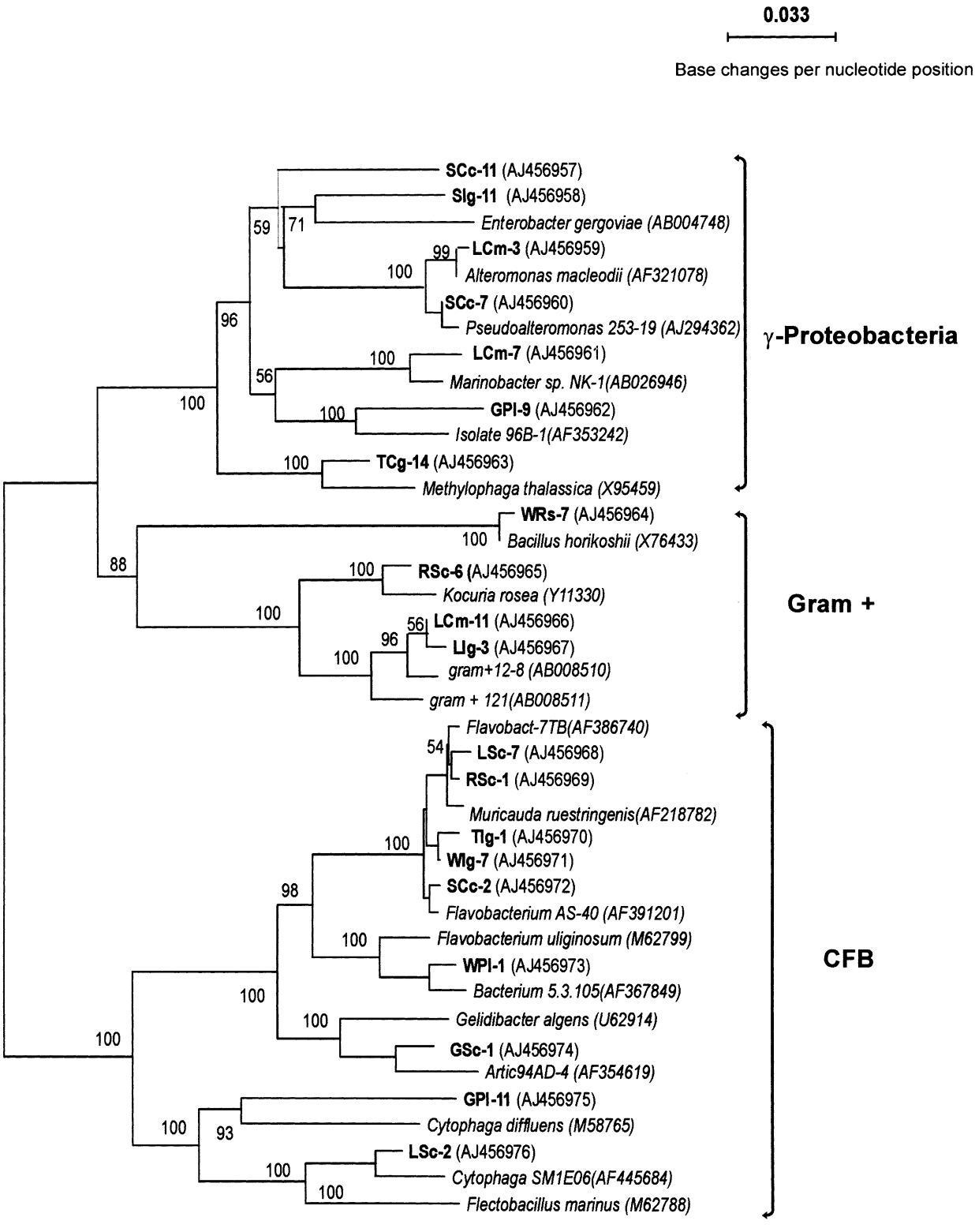

Figure 3. Neighbor-joining tree (pair gap removal, mean length 867) representing the phylogenetic relationship among partial sequences of bacteria (boldface) isolated on solid medium from the algal cultures and γ-Proteobacteria, CFB group, and Actinobacteria, from the EMBL databank sequences. For other legends see Fig. 2.

Table 3. Isolates from the EMBL data bank most closely to bacterial strains associated with microalgal cultures

Main species	*Taxons*	*Homology (%)*	*Nearest relative to isolate*	*Origin of bacteria*
	LSc-10	95	Scripps-101	Culture of *Scrippseilla trochoidea*
	LSc-11	99		
	WTs-4	98	ATAM173a_17	Culture of *Alexandrium tamarense*
	LIg-4	99	α-Bacterium AS-11	Adriatic sea
	WTs-5	98		
	TCg-3	96	*Rhodobacter* CtaxPhil-16	*Caulerpa taxifolia*
	LTs-7	99	*Roseobacter* 253-13	Culture of *Alexandrium lusitanicum*
	Wpl-3	98	*Roseobacter* ISM	Transformation of sulfur content
	GIg-9	99	*Ruegeria* AS-36	Adriatic sea
1	RIg-3, TCg-9; LTs-2; WRs-12;	97	Hstpl 95,	Sea grass, *Halophila stipula*
2	WRs 11, RTs-2	94.1	Isolate F190-32	Culture of *Chattonella marina*
	GIg-5	97	Prionitis decipiens	Algal-bacterial symbioses
3	Spl-1; GIg-3, GPl-3	99	*Roseobacter* 253-16	Culture of *Alexandrium* sp;
4	RSc-10, RTs-10	99	*Arctic bacterium* R7967	Polar sea
	LTs-4	98	Rhodobacter Ctax-Phil-16	Caulerpa taxifolia
	LIg-6	98	ATAM407 68	Culture of *Alexandrium tamarense*
	Spl-7	97	MBIC3923	Marine environment
	WIg-12	96	Clone COX 1	Forest soil
	RIg-11	96	Phyllobacterium rubiacearum	Plant
	WTs-6	97	*Mesorhizobium* N39	Arctic legumes
	WRs1	99	*Alphabacterium* C116-18	Culture of *Alexandrium* sp.
5	SIg-7, WPl-11	99	*Caulobacter* MCS23	Seawater, California
6	GIg-4, RIg-7	93	Alpha bacterium OM75	Marine coastal, North Carolina
7	WTs-7, WPl-5, GIg-12	99	*Alphabacterium* JP63.1	German Bight
8	RIg-4, RTs-2	94	*Zoogloea ramigera*	Lake
	TIg-5	99	*Agrobacterium sanguineum*	Marine environment
	SCc-7	99	*Alteromonas* 253-19	Culture of *Alexandrium*, sp
	LCm-3	99	*Alteromas macleodii*	Ubiquitous marine bacterium
	Sig-11	96	CKT1	Marine environment
	SCc-11	95	*Thalassomonas viridans*	Oyster
	LCm-7	96	*Marinobacter* NK-1	Marine environment
	GPl-9	92	Arctic 96B-1	Bacterioplankton Arctic ocean
	TCg-14	94	*Methylophaga thalassica*	Marine environment
9	LCm-11	99	Gram positive 12-8	Urban location
	Lig-3	96		
	Wig-7, Tig-1	98	*Flavobacterium* AS40	Adriatic sea
10	SCc-2	99		
	RSc-1	99	*Flavobacterium* 7BT	Picoplankton: *Aureococcus*
	LSc-7	98		*anophagefferens*
	WRs7	99	*Bacillus horikoshii* DSM	Terrestrial location
	RSc-6	97	*Kocuria* erythromyxa	Terrestrial location
	WPl-1	97	*Bacterium* (CLB) *5.3.10*	Diatom bloom
	GSc-1	94	Arctic *Cytophaga*	Arctic sea
	GPl-11	86	*Cytophaga diffluens*	Marine environment
	LSc-2	96	*Cytophaga* SM1E06	Hot spring

In the first column the species present in several algal cultures and/or hatcheries are numbered 1 to 10.

almost identical (99% homology) to environmental isolates. Several (Table 3) were clustered with bacteria associated with harmful dinoflagellates, *Alexandrium* spp. culture [6], *Scrippsiella trochoidea* [17], and *Aureococcus anophagefferens* [4]. Thus, LSc-10 and LSc-11-3 are closely related to Scripps-101; Spl-1, GIg-3, and GPl-3 to *Roseobacter* 253-16; WTs-4 to ATAM173a-17 (Table 4); LTs7 to *Roseobacter* 253-13; WRs1 to C116-18; SCc-7 to *Alteromonas* 253-19 and RSc-1; and LSc-7 to *Flavobacterium* 7BT (Fig. 3).

Some others were also closely related to recently identified marine bacteria. RSc-10 and RTs-10 were affiliated with Arctic R7697 and WRs-1 with *Caulobacter* C116-18. Some isolates may constitute new species and some others may be new genera when their branches are deep, for example GPl-9, LCm-7, SCc-11, SIg-11, and GPl-11. The Gram-positive bacteria, LCm-11, LIg-3, WRs-7 and RSC-6, were only related to terrestrial bacteria.

Isolates belonging to the *Rhodobacter* group represented 36% of bacterial flora and 50.6% of them were members of *Roseobacter* clade (Table 4). Ten bacterial species (numbered 1 to 10, Table 3) comprised half of the microbiota in terms of abundance. Four strains (LTs-2,

Table 4. Abundance of culturable bacteria associated with different algal species

	I. galbana	*P. luther*	*S. costatum*	*T. suecica*	*Rhodomonas sp.*	*C. gracilis*	*C. muelleri*	*C. calcitrans*
Number of samples	6	3	3	3	1	1	1	1
Rhodobacter	***	****	*	***	***	***	****	
Rhizobiaceae	*	*		**				
Hyphomonas group	*	*		*				
Rhodospirillaceae	*							
Sphingomonas group	**	*		*	***			
γ-Proteobacteria	*	*				*	****	**
Cytophagaceae	*	*	****					****
Gram +	*		*		*		*	
Unidentified	***	**	**	*	**	*	*	**

*: 1 to 10%; **: 11 to 30%; ***: 31 to 50%, ****: >50%.

WRs-12, RIg-3, TCg-9), species 1 (Table 3), grouped close to the isolate Hstpl 95 (Fig. 2, Rhodobacter group), were present in four hatcheries and associated with four different algal species. Another three isolates (GPl-3, GIg-3, SPl-1), species 6 (Table 3), similar to *Roseobacter* 253-16, colonized two algal species in two different hatcheries. Three strains (WTs-7, WPl-5, GIg-12), species 7 (Table 3), clustered with the isolate JP63-1, were present in three algal species and two hatcheries. Five strains (LSc-7, RSc-1, Tig-1, Wig-7, SCc-2), species 10 (Table 3), closely related to *Muricauda ruestringensis*, were associated with three diatom cultures and two *I. galbana* cultures. They were present in five out of six hatcheries. The other bacterial species were less ubiquitous but they were harbored by two algal species and/or by two hatcheries at least.

Analysis of Bacterial Population by Hatchery. (See Table 5).

Lissadel. Bacteria were moderately numerous and were at the same concentration or up to four times (*T. suecica*) greater than algal cells. Most of the dominant bacteria could be identified and showed little diversity. In all cultures two strains constituted >78% of the bacterial population identified. The bacterial populations were very different from one algal culture to another. The *C. muelleri* culture exhibited an atypical bacterial community, constituted by a strain of *Alteromonas macleodii* (LCm-3) and an Actinobacterium (LCm-11).

Satmar. In *C. calcitrans* and *I. galbana* cultures bacterial cells were at the same level as algal cells, while *P. lutheri* harboured very few bacteria. Bacterial communities of the first two were relatively diverse with at least three bacteria in the dominant flora. In *P. lutheri*, a *Ruegeria* sp. (SPl-1) constituted the vast majority of the bacteria.

Guernsey. The ratio of bacterial cells to algal cells was greater than in other hatcheries, except for Reculver, and the bacterial populations showed little diversity. *S. costatum* was colonised by only one bacterium, a *Gelidibacter* sp. (GSc-1), and at high concentration. The culturable bacteria associated with *P. lutheri* at the Satmar and Guernsey hatcheries were mainly composed of strains (SPl-1, GPl-3) belonging to the same cluster as *Ruegeria* 253-16 (99% similarity). However, strain GPl-3 was 39 times more numerous and the ratio of bacterial cells to algal cells (direct counts) was 112 times higher. A similar strain (Gig-3) and two others were also established in the *I. galbana* culture.

Tinamenor. In *I. galbana*, although algal cells were at high density, bacteria numbers remained moderate, in contrast to *C. gracilis* where they reached high concentrations. The predominant bacteria were *Agrobacterium sanguineum* (Tig-5) in *I. galbana* and a *Roseobacter* sp. (TCg 9) as also found in other hatcheries in *C. gracilis*.

Walney. The direct counts were very close to the plate counts. Concentrations of bacteria were average, except in *Rhodomonas* sp. and *T. suecica* cultures where they exceeded 10 bacterial cells per algal cell. However, in comparison with these algae which have large cells, bacteria represented little in terms of biomass. In *T. suecica*, a *Roseobacter* sp. constituted 60% of the bacterial population, and an unidentified bacterium colonized the *I. galbana* culture. No bacterium was common between the different algal cultures, except for a *Sphingomonas* in *T. suecica* (WTs-7) and *P. lutheri* (WPl-5). However, the algal cultures shared the highest number of identical bacteria with other hatcheries (Figs. 2 and 3).

Reculver. The bacterial population was high compared to algal concentration, and of the same magnitude as in the Guernsey hatchery. The bacterial population was diverse except in the *T. suecica* culture, which was dominated by a *Rhizobium* strain (RTs2) also isolated from the *I. galbana* culture. The bacterial population identified from *S. costatum* was different from the others with isolates RSc-1 (*Muricauda* sp.) and RSc-6 (*Kocuria*

Table 5. Bacterial populations by hatchery and algal culture

Hatchery	*Algae*	*Number of algal cells ML^{-1}*	*Bacteria CFU mL^{-1}*	*Bacteria: direct counts cell mL^{-1}*	*% bacterial species among identified bacteria*
Lissadel	*Tetraselmis suecica*	8.7×10^5	5.4×10^6	3.6×10^6	7% *Roseobacter* sp. (LTs-2) 44% *Rhodobacter* sp. (LTs-4) 48% *Roseobacter* sp. (LTs-7) } 99%
	Isochrysis galbana	8.6×10^5	3.1×10^6	8.2×10^5	78% *Roseobacter* sp. (LIg-4) 7% *Rhizobium* sp. (LIg-6) 4% *Clavibacter* sp. (LIg-3) } 89%
	Skeletonema costatum	$3.4. \times 10^6$	4.6×10^6	3.0×10^6	62% *Cytophaga* sp. (LSc-2) 16% *Sulfitobacter* sp. (LSc-11) 9% *Sulfitobacter* sp. (LSc-10) 9% *Muricauda* sp. (LSc-7) } 96%
	Chaetoceros muelleri	2.0×10^6	1.5×10^7	6.5×10^6	52% *Alteromonas macleodii* (LCm-3) 30% *Marinobacter* sp. (LCm-7) 5% *Clavibacter* sp. (LCm-11) } 87%
Satmar	*Chaetocerans calcitrans*	2.2×10^6	4.9×10^5	1.8×10^6	30% *Muricauda* sp. (SCc-2) 20% γ *Proteobacterium* (SCc-11) 22% *Pseudoaltermonas* sp. (SCc-7) } 72%
	Isochrysis galbana	9.3×10^5	8.7×10^4	1.3×10^5	8% *Enterobacter* sp. (SIg-11) 12% *Caulobacter* sp. (SIg-7) No regrowth of dominant strain } 20%
	Pavlova lutheri	1.0×10^7	6.7×10^5	2.8×10^5	80% *Ruegeria* sp. (SPl-1) 8% *Mesorhizobium* sp. (SPl-7) } 88%
Guernsey	*Skeletonema costatum*	2.3×10^7	1.0×10^8	2.2×10^8	99% *Gelibacter* sp. (GSc-1) 99%
	Pavlova lutheri	3.6×10^6	1.1×10^7	2.5×10^7	70% *Roseobacter* sp. (GPl-3) 18% *Pseudomonas* sp. (GPl-9) 5% *Cytophaga* sp. (GPl-11) } 93%

(Continued)

Table 5. Continued

Hatchery	*Algae*	*Number of algal cells ML^{-1}*	*Bacteria CFU mL^{-1}*	*Bacteria: direct counts cell mL^{-1}*	*% bacterial species among identified bacteria*
	Isochrysis galbana	4.4×10^6	2.5×10^6	1.1×10^7	32% *Prionitis* sp. (GIg-5) 28% *Ruegeria* sp. (GIg-3) 15% *Azospirillum* sp. (Gig-4) 8% *Ruegeria* sp.(GIg-9) 8% *Sphingomonas* sp. (GIg-12) } 91%
Tinamenor	*Chaetoceros gracilis*	1.1×10^7	2.4×10^7	2.9×10^8	71% *Roseobacter* sp. (TCg-9) 14% *Antarctobacter* sp. (TCg-3) 9% *Methylophaga* sp. (TCg-14) } 94%
	Isochrysis galbana	3.7×10^7	9.1×10^6	8.2×10^6	40% *Agrobacterium sangineum* (TIg-5) 11% *Muricauda* sp. (TIg-1) } 51%
Walney	*Rhodomonas* sp.	5.0×10^5	7.0×10^6	1.7×10^7	40% *Caulobacter* sp. (WRs-1) 37% *Roseobacter* sp. (WRs-12) 8% *Bacillus horikoshii* (WRs-7) } 85%
	Pavlova lutheri	3.6×10^6	3.1×10^6	2.8×10^6	23% *Sphingomonas* sp. (WPl-5) 21% *Roseobacter* sp. (WPl-3) 15% *Flavobacterium* sp. (WPl-1) 11% *Caulobacter* sp. (WPl-11) } 70%
	Tetraselmis suecica	7.6×10^5	8.3×10^6	7.1×10^6	59% *Roseobacter* sp. (WTs-5) 17% *Sphingomonas* sp. (WTs-7) 12% *Mesorhizobium* sp. (WTs-6) } 88%
	Isochrysis galbana	3.4×10^6	1.1×10^6	2.6×10^6	10% *Sinorhizobium* sp. (WIg-12) 8% *Muricauda* sp. (WIg-7) No regrowth of dominant strain } 18%

(Continued)

Table 5. Continued

Hatchery	*Algae*	*Number of algal cells ML^{-1}*	*Bacteria CFU mL^{-1}*	*Bacteria: direct counts cell mL^{-1}*	*% bacterial species among identified bacteria*
Reculver	*Tetraselmis suecica*	3.5×10^5	6.0×10^6	9.0×10^6	91% *Zoogloae* sp. (RTs-2) 5% *Ruegeria* sp. (RTs-10) 3% *Roseobacter* sp. (RTs-8) } 99%
	Isochrysis galbana	6×10^5	3.2×10^5	1.4×10^6	8% *Azospirillum* sp. (RIg-7) 20% *Zoogloae* sp. (RIg-4) 18% *Roseobacter* sp. (RIg-3) 16% *Phyllobacterium* sp. (RIg-11) } 62%
	Skeletonema costatum	3.4×10^7	7.1×10^7	5.3×10^8	39% *Muricauda* sp. (RSc-1) 17% *Ruegeria* sp. (RSc-10) 7% *Kocuria* sp. (RSc-6) } 63%

sp.) forming the dominant population. Two isolates, RSc-10 in *C. gracilis* and RTs-10 in *T. suecica*, were identical but low in abundance.

Discussion

In open and oligotrophic ecosystems, bacterioplankton communities are generally highly diverse, and this diversity often prevents any correspondence between unculturable and culturable bacteria at species level [9]. When a sample of seawater was incubated in a tank with added organic matter, the diversity of the bacterial populations dropped, the percentage of culturable bacteria increased markedly, and the similarity between unculturable and culturable bacteria increased [21]. Similar trends occurred naturally in biotopes enriched in organic matter. During an algal bloom, bacterial populations increased as well as the proportion of culturable bacteria [28]. In aquaculture systems, which are generally richer in organic matter than natural seawater, the proportion of culturable bacteria may be much more important [18] than in the open sea. In a closed volume such as a container of algal culture, a significant fraction of the unculturable bacterial cells might be identical to culturable bacteria. In this study, bacteria cultured on Marine Agar were estimated to represent from 10% to >60% of the total bacterial communities. In some cases, the poor recovery was not always due to lack of culturability. Collection of some dominant colonies, particularly those from the *I. galbana* culture, failed because of failure to regrow the tiny colonies.

Despite the imprecise direct counts and selectivity of the Zobell medium, recovery of bacteria appeared high. Better results might be obtained by using a medium poorer in organic matter as tested for the culture of bacterioplankton [15]. However, several authors used Marine Agar as a reference medium to study bacterioplankton [21]. Culturability of marine bacteria has been discussed for many years without reaching clear conclusions [9, 28]. Although methods such as dilution (MPN, most-probable-number) optimize the detection of viable cells, the ratio often remains below 1% [13]. Bernard et al. [5] reported a greater proportion of marine bacteria growing on Marine Agar, from 0.43 to 3.52% of total bacteria, whereas the viable cell concentrations reached 10.8 to 22.2%. Nevertheless, cultivation reduces the diversity of bacterial populations, and selection of bacterial genera by Marine Agar was clearly seen in DGGE analysis performed by these authors.

Direct counts of bacteria were probably underestimated, especially when the bacterial concentration was low, as some bacteria could be attached to algal cells. Up to 66% of the total bacterial biomass has been found attached to particles of >10 μm, including phytoplankton [36]. Algal cells can also mask bacteria because they

are larger and highly fluorescent. The concentration of algal cells could not be reduced by dilution since bacteria would not then be sufficiently concentrated for counting. Bacterial clumps, which were sometimes observed, could distort counting especially if they were dissociated during dilution. These different biasing factors could explain why in eight cases the plate counts were greater than direct counts. Consequently, it was impossible to define accurately the true concentration of bacteria associated with microalgae and the proportion of culturable bacteria. When bacterial cell concentrations were greater than those of algal cells and when direct counts were higher than the plate counts, this inaccuracy may be reduced. Although the direct counts were probably underestimated, bacterial concentrations appeared to fluctuate around 10^7 cells mL^{-1}, except in some cases where they reached concentrations as high as 5×10^8 cells mL^{-1}.

The bacterial flora associated with algal cultures, as identified by partial sequences of 16S rDNA, was of low diversity. Greater than 80% were composed of two or three strains, and in 9 out of 19 algal cultures one strain comprised >50% of the bacteria.

High concentrations of bacteria were not correlated with low species diversity. For example, at the Satmar hatchery the bacterial flora growing in the *P. lutheri* culture was of relatively low concentration (2.8×10^5 mL^{-1}) but it was dominated by one strain of *Roseobacter* (SPl-1). Conversely, bacterial populations of *S. costatum* cultures from Reculver were of high concentration (5.3×10^8 cell mL^{-1}), but they remained relatively diverse.

The strains which colonized the microalgae cultures belonged principally to the *Rhodobacter* and CFB groups, which are frequently encountered in seawater. *Roseobacter*, *Ruegeria*, *Sulfitobacter*, and *Antarctobacter* constitute an important part of the bacterioplankton found in marine ecosystems [14, 39, 40]. Members of the CFB group are also ubiquitous in open oceans and coastal seawater [3, 8, 9]. However, enrichment of these last clusters seemed to occur in algal cultures [32], in comparison with γ-Proteobacteria and β-Proteobacteria, which were poorly represented. Culturability alone can not explain this trend. Indeed, according to Eilers et al. [9], bacterial culture favors members of the γ–Proteobacteria and *Roseobacter*, compared to members of the CFB group.

Gram-positive bacteria, which occurred at low level in some algal cultures, were probably not native to the marine environment but are suspected of being conveyed by tap water used to rinse the algal culture transport vessel.

The spectrum of genera and species in flagellate cultures seemed to increase when the number of analyses increased (Table 4). All groups which were identified in this study were present in *I. galbana* cultures (six samples). In *P. lutheri* cultures (three samples) only two were missing. However no members of the γ-Proteobacteria and CFB group occurred in *T. suecica* (three samples). *S. costatum* (three samples) appeared still more selective since members the CFB group colonized the cultures almost exclusively. For other algae conclusions were difficult because only one analysis was done.

Among the bacterial groups that colonized the algal cultures, some species appeared particularly well adapted to the conditions of algal culture. For example the high similarity with sequences of bacteria isolated from potentially toxic flagellate/picoplankton cultures was remarkable [4, 6, 17]. The isolates clustered around *Muricauda ruestrengensis* colonized several algal cultures in the same hatchery or in different facilities. Several cultures of flagellate/picoplankton cultures harbored the same bacterial species. Diatoms had differing bacterial populations. Few isolates were shared with flagellate cultures. The bacterial species were less diverse and did not include Rhizobiaceae, Rhodospirillaceae, *Zymomonas*, and *Hyphomonas*. The *S. costatum* culture harbored members of CFB group γ- and a few α-proteobacteria. The absence of other groups might be caused by production of antibacterial substances [24] and/or by promoting, with exudates rich in polysaccharides [37], particular bacterial strains, such as members of Cytophagacaea. Indeed the latter can become dominant in ecosystems rich in polysaccharides, and they degrade them very efficiency [19]. The ability to produce extracellular polymeric substances appeared very widespread in all diatom species [37]. On the other hand, different factors such as light, CO_2, mineral salt concentration, and organic matter released by microalgae may favor the establishment of certain species and then their redundancy. Zubkov et al. [40] and Ansede et al. [1] showed that the dimethylsulfoniopropionate released by phytoplankton during blooms was mainly metabolized by *Roseobacter*-related species. That would explain the predominance of this group during algal blooms and in algal cultures.

The *Rhodobacter* group encompassed chemoautotroph and photosynthetic species and others which have widely differing metabolisms [14, 39]. Some isolates such as RIg-3, WRs-12, LTs-2, and TCg-9 clustered with HstpL95 (97% similarity) could be photosynthetic and others might fix nitrogen as suggested by Pereg et al. [27]. Bacteria can modify algal metabolism as demonstrated with harmful algae by Brinkmeyer et al. [6]. In this case the presence of bacteria increased the yield of toxin. The nutritional relationship is complex, but a synergism between algal and bacterial cells is generally observed [15].

The presence of certain strains could have negative consequences. Isolates LTs-7 and SCc-7 clustered, respectively, with strains 253-13 and 253-19, which were associated with toxic algae and were suspected to produce toxin [6]. Although no experiment has yet been per-

formed with these bacteria, they might have similar detrimental effect to those of *Gyrodinium aureolum* [22, 33] and *Alexandrium tamarense* [22] on bivalve juveniles and larvae.

Some bacteria, including members of the CFB and *Pseudomonas* spp., can significantly affect or stimulate algae, especially diatoms [10, 34]. Although in this study neither members of the CFB group nor γ-Proteobacteria were affiliated with these strains, it would be interesting to test these isolates one by one or in combination in axenic diatoms and flagellates to observe their effect on algal and larval cultures.

Bacteria could also promote larval rearing success. The role of bacteria in larval nutrition must be minor compared to algae since the bacterial biomass is low and the retention efficiency of larvae is low for particles below 1–2 μm. However, bacteria can complement the diet with growth factors, as reported by Moal et al. [23], and improve larval growth significantly, even at low concentrations [7].

There are few sources of algal strains for hatcheries, and the method of algal maintenance was not taken into account in analysis of the bacterial populations. This could represent a potential risk if associated bacteria affected larval or algal cultures. Although the suspected bacteria have yet to be tested, it is recommended that hatcheries check the bacterial diversity regularly on solid medium or change the source of algae frequently. In the future, algae could be deliberately associated with bacteria selected for their innocuous or beneficial effect. For example, Avendano et al. [2] used axenic algae combined with probiotic bacteria to improve the survival of scallop (*Argopecten purpuratus*) larvae. If this method is applied, the problem will be to maintain these beneficial bacteria in algal culture since different bacteria can easily colonize batches of algae in the actual conditions of culture.

Several features of bacterial populations can be drawn from this study. First a large proportion of bacterial populations associated with algal cultures were easily culturable. Use of specific media would probably allow culture of other groups including cyanobacteria and other autotrophs.

They are not so diverse, dominated by Roseobacter and CFB groups. Although a wide range of bacterial species could occur in algal culture the most frequently encountered belonged to 10 species, some of which were also found by other authors.

Bacteria showed little overlap between diatom cultures and flagellate cultures, i.e., they appeared to harbor distinct bacterial populations. That proved the importance of interaction between bacteria and microalgae. However, some bacterial species were present in both types of phytoplankton cultures, thus showing that their difference was not total.

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