Ecological Implications of an Improved Direct Viable Count Method for Aquatic Bacteria

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The direct viable count method first described by Kogure et al. (Can. J. Microbiol. 25:415–420, 1979) was improved by using an antibiotic cocktail instead of nalidixic acid alone. We screened 100 marine isolates from two coastal areas for their sensitivities to five replication-inhibiting antibiotics, including four quinolones (nalidixic, piromidic, and pipemidic acids and ciprofloxacin) and one β -lactam (cephalexin). It was shown that growth inhibition of all isolates cannot be readily achieved by using a single antibiotic. Inhibition was much more efficient when all the antibiotics were combined, making it possible to use this method with natural communities. In combination, the concentration of each antibiotic could be lowered and the incubation time could be increased without any growth. Under such conditions, it was shown that the fraction of substrate-responsive cells within natural marine communities is much greater (1 to 2 orders of magnitude) than those reported by traditional procedures. Furthermore, the new procedure made substrate-responsive cells more clearly distinguishable. These improvements resulted in an increased incubation time and were related to metabolic expression of slow-growing cells and/or to the recovery of starved cells. The increased fraction of viable cells within marine communities has ecological implications on the metabolic role of nonculturable cells.

The accurate enumeration of living bacteria has long been a challenge to aquatic microbiologists. The introduction of the concept of viable but nonculturable cells in the 1980s by R. Colwell's group has led to important research work concerning the existence and significance of these kinds of cells within natural bacterial communities. These cells may be more representative of metabolically active bacteria in aquatic systems than those determined by plate count or direct count methods. Their enumeration is also of great assistance in understanding the influence of microorganisms on a given ecosystem as well as in quantifying the cells which are responsible for bacterial activities.

Considering the ability to reproduce or grow as the most stringent proof of life, the presence of reproducible or growing viable cells may be considered the primary measure of bacterial viability. The following methods related to cellular viability have been proposed to overcome the limitations of traditional cultures: dilution cultures (6), microautoradiography (30), microcolonies (3), and direct viable counts (DVC) (17). Among these, microautoradiography, dilution cultures, and microcolonies (although they are very promising) involve complex and time-consuming procedures. On the other hand, the DVC method has received much more attention and has been applied in many autoecological studies (7, 9, 20, 27), but its application to natural samples suffers from methodological limitations (1, 8, 25).

The original DVC method is based on the incubation of samples with a single antibiotic (nalidixic acid) and nutrients (yeast extract). Nalidixic acid acts as a specific inhibitor of DNA synthesis and prevents cell division without affecting other cellular metabolic activities (14). The resulting cells can continue to metabolize nutrients and become elongated after incubation. One of the main limitations in applying the DVC

procedure to complex communities is the presence of natural bacteria which are resistant to the antibiotic used and are able to grow before elongated cells can be formed. This limitation has led to important modifications of the initial procedure in terms of antibiotics and incubation times (8, 18, 31). To overcome this difficulty, short incubation times, corresponding to the limits of detectable growth, are generally used, but this does not allow the detection of slow-growing bacteria and/or dormant cells.

One possible way of improving this procedure is to combine different antibiotics to ensure good growth inhibition of most cells and to increase the incubation time. Some combinations proposed in the literature have been to add pipemidic and piromidic acids (18) or isopropyl cinodine (28), but their application to marine bacterial communities generally results in growth after at least 8 h of incubation (18).

The objective of this study was to optimize the combination and concentrations of antibiotics to improve the detection of viable cells within marine communities. We compared the antibiotic sensitivities of 100 bacterial strains isolated from different marine coastal environments to five replication-inhibiting antibiotics which prevent DNA synthesis (nalidixic, piromidic, and pipemidic acids and ciprofloxacin) or cytoplasmic membrane septum formation (cephalexin). Here we propose a new DVC procedure which combines these antibiotics at low concentrations and allows increased incubation time. This procedure resulted in an increased and more clearly distinguishable fraction of substrate-responsive cells, suggesting the existence of a large and unexpected number of viable cells in coastal marine areas.

MATERIALS AND METHODS

Bacterial isolation from coastal waters. A set of 100 bacterial isolates were selected from spread marine agar 2216 (MA; Difco Laboratories, Detroit, Mich.) plates of bacteria from two marine locations. Seawater samples were collected (i) in the northwestern Mediterranean Sea (Bay of Banyuls-sur-Mer [France]) in June 1996 ($n = 56$) and (ii) in the northeastern Mediterranean Sea (Adriatic Sea [Bay of Trieste in Italy]) in April and June 1996 ($n = 44$). For each location,

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TABLE 1. Bacterial counts and activity measurements at different sampling stations and times by various methods

Type of sample (location)	Date (mo/ day/yr)	Temp (°C) (salinity [‰])	TC (10 ⁵ /ml) ^a	CTC (%) ^{b,c}	CFU (%) ^{b,d}		DVC _{NA}		DVC _{MIX}	
					MA	YE	TC (10 ⁵ /ml) ^a	DVC ⁺ (%) ^e	TC (10 ⁵ /ml) ^a	DVC ⁺ (%) ^e
Coastal seawater (Mediterranean Sea)	03/13/96	10 (ND) ^f	15.21 (3.55)	ND	ND	ND	ND	ND	12.02 (1.90)	24.2*
	03/18/96	10 (ND)	7.78 (2.52)	ND	0.32	ND	ND	ND	7.63 (1.31)	36.4*
	03/27/96	12 (ND)	6.41 (1.17)	ND	1.63	0.02	5.44 (1.22)	0.5*	6.31 (1.32)	17.7*
	04/05/96	11 (ND)	8.36 (1.31)	ND	ND	ND	ND	ND	8.35 (1.58)	36.8*
	04/11/96	12 (38)	8.32 (1.72)	4.7	0.17	0.07	ND	ND	9.10 (1.90)	36.7*
	06/06/96	19 (35)	8.66 (2.27)	1.7	0.46	0.05	8.41 (2.09)	0.3**	6.90 (1.26)	6.9**
	06/07/96	20 (36)	5.15 (1.35)	5.2	0.20	0.11	4.92 (0.72)	0.2**	4.92 (0.46)	7.5**
	10/29/96	17 (36)	5.84 (0.87)	5.0	0.28	0.29	5.03 (0.90)	0.6**	4.39 (0.87)	9.9**
	04/28/97	15 (ND)	2.78 (0.87)	3.5	0.75	ND	2.13 (0.62)	3.8**	3.22 (0.78)	41.2**
	04/30/97	15 (ND)	8.30 (1.38)	4.8	0.39	ND	8.50 (1.46)	0.1**	8.61 (2.08)	17.6**
	05/23/97	19 (ND)	5.59 (0.80)	2.8	2.92	ND	5.44 (1.42)	0.6**	4.47 (0.86)	44.4**
	06/09/96	23 (27)	57.23 (16.68)	3.2	1.57	5 × 10 ⁻³	57.43 (9.20)	0.3*	65.48 (10.57)	31.9**
Lagoon (Leucate, France)	11/08/96	14 (33)	44.72 (21.80)	1.6	0.33	5 × 10 ⁻³	48.28 (14.35)	0.2**	49.81 (14.69)	16.8***

^a Data are mean total counts (TC), with standard deviations noted parenthetically.

^b Data are percentages of total cell counts, as determined by DAPI staining.

^c CTC, respiring cells, i.e., cells that are able to reduce CTC.

^d CFU, culturable cells on MA and yeast extract (YE).

^e DVC⁺, substrate-responsive cells. *, one replicate; **, two replicates; ***, three replicates.

^f ND, not determined.

isolates were chosen on the basis of colony morphology to increase the chance of picking different species. All isolates were purified twice on MA plates.

Antibiotic sensitivities. All isolates were screened for Gram staining and for their sensitivities to nalidixic acid, pipemidic acid, piromidic acid, ciprofloxacin, and cephalixin by the standard disk-agar method. For each isolate, two or three colonies were picked and resuspended in 5 ml of marine broth (Difco). This suspension was diluted to approximately 1 × 10⁶ to 5 × 10⁶ cells/ml, and MA plates were swabbed with 2 ml. The plates were air dried for a few minutes, and five antibiotic disks (Diagnostic Pasteur) containing nalidixic acid (30 µg), pipemidic acid (20 µg), piromidic acid (25 µg), ciprofloxacin (5 µg), or cephalixin (30 µg) were placed on the gel. Plates were incubated for 2 to 3 days at room temperature (ca. 20 to 22°C). The zones of inhibition produced by antibiotics were measured and scored as resistant, intermediate, or sensitive by the method of Courvalin et al. (10).

Natural samples from coastal areas. Seawater samples were taken from the Bay of Banyuls-sur-Mer at stations located between 1.0 and 5.0 km offshore. Lagoon water samples were collected in the Leucate lagoon (Mediterranean coast of France). All samples were collected at approximately 1 m below the surface in glass bottles, and activity measurements were assayed within 2 h of sampling.

Total cell counts. Bacterial numbers were determined from formaldehyde-fixed (2% [final concentration]) samples stained with 2.5 µg of 4',6-diamidino-2-phenylindole (DAPI; Sigma) per ml for 30 min. Each sample was filtered onto 0.2-µm-pore-size Nuclepore filters. Cells were counted at a magnification of ×1,000 with an Olympus BH2 epifluorescence microscope equipped with a 100-W Hg lamp and a UV filter set (filter set U [UG-1, DM400, and L420]). Standard deviations (Table 1) were determined with a minimum of 1,200 cells, corresponding to at least two replicated samples.

DVC procedures. Antibiotics were dissolved in 0.05 M NaOH (nalidixic, piromidic, and pipemidic acids [all from Sigma]) or sterile distilled water (cephalexin [Sigma] and ciprofloxacin [Bayer Pharmacie]). All antibiotic solutions were filter sterilized through 0.2-µm-pore-size membrane filters (Sartorius). Sterile 15-ml glass tubes were filled with 9 ml of sample and aseptically enriched with 1 ml of yeast extract (50 mg/liter [final concentration]; Difco). Then nalidixic acid alone (20 µg/ml) (DVC_{NA}) or the antibiotic cocktail (nalidixic acid, 20 µg/ml; piromidic acid, 10 µg/ml; pipemidic acid, 10 µg/ml; cephalixin, 10 µg/ml; ciprofloxacin, 0.5 µg/ml) (DVC_{MIX}) was added (final concentrations given parenthetically). Incubation was performed statically in the dark at 20°C, and samples were formaldehyde fixed (2% [final concentration]) after 6 h of incubation for DVC_{NA} and at time intervals (18 to 24 h) for DVC_{MIX}. Samples were assayed as reported above for total direct counts. For each sample, minima of 30 enlarged substrate-responsive cells (DVC⁺ cells) and 600 total cells (DAPI⁺ cells) were enumerated. The proportion of DVC⁺ cells was calculated as the ratio of DVC⁺ to DAPI⁺ cell counts. Enumerations were performed with variable numbers of replicates (Table 1).

CFU. Samples were serially diluted in autoclaved seawater, and 100 µl of each dilution was plated on two agar media. CFU counts were determined on MA and low-nutrient yeast extract medium (yeast extract, 50 mg; Bacto Agar, 15 g; filtered [0.45-µm pore size] aged seawater, 1,000 ml). Colonies were counted

after 4 weeks of incubation at room temperature (ca. 20 to 22°C). The percentage of CFU was determined as the ratio of CFU to total cell counts. The mean coefficient of variation for all samples was 16.2% (range, 0.4 to 38.1%) for CFU.

Respiring-cell counts. Respiring bacteria were defined as cells able to reduce 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences Europe, Eppenheim, Germany) in red fluorescent formazan (26). Their counts were determined by a modification of the original method (11). Briefly, samples (2 ml) were incubated with 5 mM CTC (fresh stock solution, 50 mM) for 10 h in the dark at in situ temperature, formaldehyde fixed (2% [final concentration]), stored at 4°C, and counted for up to 2 days. Samples were counterstained with DAPI for total counts (see above). For each microscopic field, respiring cells (CTC⁺) were enumerated with filter set B (BP490, DM500, and AFC+O515). Then total cells were counted by switching to a UV filter set. Minima of 30 CTC⁺ cells and 600 DAPI⁺ cells were enumerated, and the proportion of CTC⁺ cells was calculated as the ratio of CTC⁺ to DAPI⁺ cell counts. The CTC⁺ fraction was calculated as the mean of two or three replicates. The mean coefficient of variation for all samples was 13.1% (range, 0.2 to 20.9%) for CTC⁺ cells.

RESULTS AND DISCUSSION

Susceptibilities of natural isolates to antibiotics. An analysis of the antibiotic sensitivities of culturable cells may be of great interest for optimizing the DVC procedure, although such cells do not represent more than 1% of total cell counts. It has been suggested that culturable cells are responsible for the growth that occurs during incubation of confined marine samples due to their rapid growth rate (12). We compared the sensitivities of 100 isolated strains to various antibiotics (Table 2). A large fraction of isolates was resistant to each antibiotic (41 to 65%), except ciprofloxacin (12%). The antibiotic sensitivities at the two sampling locations were not different. However, gram-positive isolates were much more sensitive to cephalixin and ciprofloxacin. Considering resistant and intermediate antibiotic susceptibility as antibiotic resistance, the great percentage of nalidixic acid-resistant strains (64%) illustrates the limitation of the standard procedure. Some strains had multiple resistances to nalidixic, piromidic, and pipemidic acids (62%) (data not shown). The multiple resistances of natural isolates to these antibiotics explain the low interest in this combination, as previously reported (18). The combination of all antibiotics resulted in the inhibition of 96% of isolates (data not shown). These results suggest that because of multiple antibiotic resistances of natural isolates, replication inhibition cannot be

TABLE 2. Antibiotic sensitivities of 100 gram-positive and -negative isolates from two sampling locations in the Mediterranean Sea

Antibiotic	Antibiotic sensitivity	% of isolates from:		% of isolates staining		Total (%) (n = 100)
		Location 1 (n = 56) ^a	Location 2 (n = 44) ^b	Gram negative (n = 85)	Gram positive (n = 15)	
Nalidixic acid	Sensitive	42.9	27.3	37.6	26.7	36.0
	Intermediate	10.7	13.6	12.9	6.7	12.0
	Resistant	46.4	59.1	49.4	66.7	52.0
Pipemidic acid	Sensitive	25.0	15.9	22.4	13.3	21.0
	Intermediate	17.9	9.1	15.3	6.7	14.0
	Resistant	57.1	52.3	62.4	80.0	65.0
Piromidic acid	Sensitive	26.8	13.6	22.4	13.3	21.0
	Intermediate	12.5	18.2	16.5	6.7	15.0
	Resistant	60.7	68.2	61.2	80.0	64.0
Ciprofloxacin	Sensitive	92.9	79.5	90.6	66.7	87.0
	Intermediate	1.8	0.0	0.0	6.7	1.0
	Resistant	5.4	20.5	9.4	26.7	12.0
Cephalexin	Sensitive	48.2	52.3	47.1	66.7	50.0
	Intermediate	7.1	11.4	9.4	6.7	9.0
	Resistant	44.6	36.4	43.5	26.7	41.0

^a Northwestern Mediterranean Sea (Bay of Banyuls-sur-Mer [France]).^b Adriatic Sea (Bay of Trieste [Italy]).

readily achieved by the use of a single antibiotic but only by their combination in the hope that cells which do not respond to a particular antibiotic do respond to another. Although nalidixic, piromidic, and pipemidic acids and ciprofloxacin prevent cell division in a similar manner by inhibiting DNA gyrase, differences in their effectiveness against species (34) and natural communities (16) have previously been reported. Differences in sensitivities to β -lactam antibiotics (e.g., cephalexin) are partly due to outer membrane permeability (23). Cephalexin inhibits transpeptidase and transglycosylase by binding to penicillin-binding protein 3 and blocks the formation of the septum during cell division.

Antibiotic concentrations. The sensitivity of a strain to a given antibiotic should be considered in terms of the antibiotic concentration. For the DVC procedure, the antibiotic concentration may be determined by the MIC which inhibits cell replication but allows other synthetic pathways to continue (4). However, within natural communities, the MICs of a given antibiotic for different species are not similar and the highest MIC is required to ensure growth inhibition. As a consequence, this high concentration may lead to metabolic inhibition and sometimes to cellular death or disruption of more sensitive cells (data not reported). Inversely, growth inhibition can be obtained by using lower concentrations when multiple antibiotics are combined. In this study, we combined five antibiotics and preliminary assays were performed to optimize antibiotic concentrations (data not shown). The concentrations reported by Kogure et al. (18) for nalidixic acid (20 μ g/ml), piromidic acid (10 μ g/ml), and pipemidic acid (10 μ g/ml) were used, whereas 0.5 and 10 μ g/ml were used for ciprofloxacin and cephalexin, respectively.

Incubation time. The inhibiting effect of the mixture was compared to that of nalidixic acid (DVC_{NA} [the traditional procedure]). The comparison was performed with natural seawater samples taken from Banyuls-sur-Mer Bay in March 1996 and with yeast extract (50 mg/liter) as the nutrient source. The temporal evolution of total counts during incubation is reported in Fig. 1. Growth was detectable by 6 h in the control and by 8 h when nalidixic acid was added. Similar results have

previously been reported for nalidixic acid (17, 18). Growth inhibition was much more efficient when the antibiotic mixture was used, allowing incubation to be increased up to 18 h without any detectable replication. A 3-h generation time was estimated for the control with an incubation of between 6 and 18 h, based on the increase in total counts. It was 1.5 h when it was based on the increase in culturable counts, with the assumption that growth was due to the initial culturable fraction of cells. This generation time is in good agreement with

FIG. 1. Response of a natural marine sample to the addition of yeast extract (50 mg/liter). Bars represent total bacterial abundances in the absence of antibiotics (solid bars), in the presence of nalidixic acid at 20 μ g/ml (high bars with stripes), and in the presence of the proposed antibiotic cocktail (dark bars with stripes). Samples for which growth was detected are identified by asterisks.

those reported for some culturable marine cells by Ferguson et al. (12). The low growth reported between 18 and 24 h with the antibiotic mixture resulted in a 1.25-fold increase in total counts. As incubation times may vary between samples, incubation times of 18, 21, and 24 h were used for subsequent experiments with the antibiotic mixture. However, the absence or presence of growth should be estimated with caution. On one hand, growth can be balanced by predation or cell disruption due to antibiotics or viral infection (29). On the other hand, growth can be overestimated when we consider that the initially very small cells which are not held on 0.2- μ m-pore-size filters (21) may be held after incubation by enlargement of cells.

Substrate concentration. Different nutrient concentrations were tested (data not shown). The best results were obtained when yeast extract was used at a final concentration of 50 mg/liter, as previously reported (19). The use of a higher concentration did not yield more elongated cells, although contradictory results have been reported in the literature (24). Furthermore, this low concentration may be more adapted to oligotrophic bacteria (6).

Application to natural samples. The two DVC procedures (DVC_{NA} and DVC_{MIX}) were applied to various natural samples (Table 1). The incubation time for the DVC_{MIX} procedure was defined as the longest incubation during which growth was not detected, whereas it was 6 h for the DVC_{NA} procedure. The mean percentage of elongated cells with the DVC_{MIX} procedure was 50 times higher than that with the DVC_{NA} procedure. The combination of antibiotics resulted in higher viable counts for all water samples. Surprisingly, the DVC_{NA} procedure resulted in viable counts that were lower than the culturable counts, as determined on MA plates (except for two samples taken on 29 October 1996 and 28 April 1997), and respiring-cell counts. These results are very different from those reported for different aquatic systems for which the percentages of substrate-responsive cells were higher than those of culturable counts (17–19, 25). Similarly, other reports have shown a good agreement between respiring-cell and substrate-responsive cell fractions (22, 25, 31). The low percentages of substrate-responsive cells reported in this study can be explained by our difficulty in discriminating between elongated and nonelongated cells after a short incubation period. This difficulty has previously been reported for natural samples showing size polymorphisms (1, 31). More recently, the use of cell volumes was suggested for better discrimination between cellular coccoid morphologies (2). This problem was overcome by the use of an antibiotic mixture, resulting in substrate-responsive cells that are more clearly distinguishable (Fig. 2). There was no confusion between substrate-responsive and dividing cells because elongated cells were longer than growing cells and did not contain any dividing septum.

The differences between culturable and substrate-responsive cells detected by DVC_{MIX} reveal a large fraction of viable but nonculturable cells (Table 1). These differences cannot be related to the use of a liquid environment in the DVC procedure since the use of liquid medium did not result in higher culturable counts (32). The recovery of marine bacteria may also be influenced by nutrient source composition (5). However, when spread plates were made with the same nutrient source as that used in the DVC procedure, culturable counts were much lower or sometimes closer to those detected on MA plates, representing less than 0.3% of total counts (Table 1). This suggests that the nutrient source and concentration are not sufficient to explain the low plateability of nonculturable cells. The large DVC^+ fraction reported for the antibiotic cocktail can be explained by the detection of some cells that may

FIG. 2. Fluorescence micrographs of DAPI-stained bacteria from a marine sample before incubation (A), after 6-h incubation with nalidixic acid (20 μ g/ml) (B), and after 18-h incubation with the antibiotic cocktail (C). Substrate-responsive cells are identified by arrows. Bar, 10 μ m.

undergo a few divisions but do so in insufficient numbers to produce bacterial colonies or any trouble within cell suspensions. The detection of these cells can be achieved by other methods, such as microcolonies and dilution cultures (3, 6, 33).

It has previously been reported that some marine bacteria have very long generation times (sometimes more than 1 week [6]) and are probably not detectable during a 24-h incubation period, suggesting that even the new DVC procedure underestimates the real fraction of viable cells within marine communities. Another hypothesis to explain the high differences between culturable and viable counts is the fact that quinolone antibiotics act as chelating agents on the divalent cations of the cellular wall (13), resulting in the passive entrance of nutrients into starved cells, which facilitates their recovery (27).

The antibiotic cocktail resulted in high percentages of viable cells (7 to 44%), underlining the high metabolic potentialities of natural communities. The differences between respiring cells (i.e., in situ active cells) and substrate-responsive cells detected by DVC_{MIX} (Table 1) may represent a fraction of inactive but viable cells and/or slow-growing cells for which respiration is not detectable. These two cell states may have been detected by the DVC_{MIX} procedure due to increased incubation time in the presence of nutrients. Under these conditions, the percentages of substrate-responsive cells observed within marine oligotrophic communities (Mediterranean Sea) were much higher than those reported previously for other nutrient-depleted marine environments (17, 19, 24). These results are of great interest for characterizing the physiological states of bacterial communities. The fraction of cells undetected as substrate responsive by the new DVC procedure can result from the inhibition of some cells due to the bactericidal effect of one antibiotic. It can also be due to the presence of dead cells within the community. In support of this last hypothesis, it was recently reported that phage-infected bacteria (29), as well as non-DNA-containing bacteria (35) and cells with damaged intracellular structures (15), may represent a large fraction of bacterial communities.

We conclude that the DVC procedure can provide very useful information about the metabolic potentialities of natural communities under optimized conditions. However, the DVC procedure should be improved for each ecosystem since antibiotic concentrations are strain dependent. The combination of the DVC method with in situ hybridization techniques is very helpful in determining the phylogenetic affiliations of substrate-responsive cells (16). This approach may be improved by using our new DVC procedure with natural communities.

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REFERENCES

- Al-Hadithi, S. A., and R. Goulder. 1989. An alternative approach to the yeast extract-nalidixic acid method for determining the proportion of metabolically active aquatic bacteria. *Lett. Appl. Microbiol.* **8**:87-90.
- Barcina, I., I. Arana, P. Santorum, J. Iriberrí, and L. Egea. 1995. Direct viable count of gram-positive and gram-negative bacteria using ciprofloxacin as inhibitor of cellular division. *J. Microbiol. Methods* **22**:139-150.
- Bianchi, A., and L. Giuliano. 1995. Enumeration of viable bacteria in the marine pelagic environment. *Appl. Environ. Microbiol.* **62**:174-177.
- Buchrieser, C., and C. W. Kaspar. 1993. An improved direct viable count for the enumeration of bacteria in milk. *Int. J. Food Microbiol.* **20**:227-236.
- Buck, J. 1974. Effects of medium composition on the recovery of bacteria from seawater. *J. Exp. Mar. Biol. Ecol.* **15**:25-34.
- Button, D. K., F. Schut, P. Quang, R. Martin, and B. R. Roberston. 1993. Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. *Appl. Environ. Microbiol.* **59**:881-891.
- Byrd, J. J., H.-S. Xu, and R. R. Colwell. 1991. Viable but nonculturable bacteria in drinking water. *Appl. Environ. Microbiol.* **57**:875-878.
- Coallier, J., M. Prévots, and A. Rompré. 1994. The optimization and application of two direct viable count methods for bacteria in distributed drinking water. *Can. J. Microbiol.* **40**:830-836.
- Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer. 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for the release of genetically engineered micro-organisms. *Bio/Technology* **3**:817-820.
- Courvalin, P., F. Goldstein, A. Phillipon, and J. Siroit. 1985. L'antibiogramme. Videom, Paris, France.
- del Giorgio, P. A., and G. Scarborough. 1995. Increase in the proportion of metabolically active bacteria along gradients of enrichment in freshwaters and marine plankton: implications for estimates of bacterial growth and production rates. *J. Plankton Res.* **17**:1905-1924.
- Ferguson, R. L., E. N. Buckley, and A. V. Palumbo. 1984. Response of marine bacterioplankton to differential filtration and confinement. *Appl. Environ. Microbiol.* **47**:49-55.
- Furet, Y. X., and J. C. Pechere. 1990. Usual and unusual antibacterial effects of quinolones. *Antimicrob. Agents Chemother.* **26**:7-15.
- Goss, W. A., W. H. Deitz, and T. M. Cook. 1964. Mechanism of action of nalidixic acid on *Escherichia coli*. *J. Bacteriol.* **88**:1112-1118.
- Heissenberger, A., G. G. Leppard, and G. J. Herndl. 1996. Relationship between the intracellular integrity and the morphology of the capsular envelope in attached and free-living marine bacteria. *Appl. Environ. Microbiol.* **62**:4521-4528.
- Kalmbach, S., W. Manz, and U. Szewzyk. 1997. Dynamics of biofilm formation in drinking water: phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and in situ hybridization. *FEMS Microbiol. Ecol.* **22**:265-279.
- Kogure, K., U. Simidu, and N. Taga. 1979. A tentative direct microscopic method for counting living marine bacteria. *Can. J. Microbiol.* **25**:415-420.
- Kogure, K., U. Simidu, and N. Taga. 1984. An improved direct viable count method for aquatic bacteria. *Arch. Hydrobiol.* **102**:117-122.
- Kogure, K., U. Simidu, N. Taga, and R. R. Colwell. 1987. Correlation of direct viable count with heterotrophic activity for marine bacteria. *Appl. Environ. Microbiol.* **53**:2332-2337.
- Lebaron, P., V. Roux, M. C. Lett, and B. Baleux. 1993. Effects of pili rigidity and energy availability on the conjugative transfer of plasmids in aquatic environments. *Microbiol. Releases* **2**:127-133.
- Li, W. K. W., and P. M. Dickie. 1985. Growth of bacteria in seawater filtered through 0.2 µm Nuclepore membranes: implications for dilution experiments. *Mar. Ecol. Prog. Ser.* **26**:245-252.
- Maki, J. S., and C. C. Remsen. 1981. Comparison of two direct-count methods for determining metabolizing bacteria in freshwater. *Appl. Environ. Microbiol.* **41**:1132-1138.
- Michel-Briand. 1986. Mécanismes moléculaires de l'action des antibiotiques. Mason, Paris, France.
- Peele, E. R., and R. R. Colwell. 1981. Application of a direct microscopic method for enumeration of substrate-responsive marine bacteria. *Can. J. Microbiol.* **27**:1071-1075.
- Quinn, J. P. 1984. The modification and evaluation of some cytochemical techniques for the enumeration of metabolically active heterotrophic bacteria in aquatic environment. *J. Appl. Bacteriol.* **57**:51-57.
- Rodriguez, G. G., D. Phipps, K. Ishiguro, and H. F. Ridgway. 1992. Use of fluorescent redox probe for direct visualization of actively respiring bacteria. *Appl. Environ. Microbiol.* **58**:1801-1808.
- Roszak, D. B., and R. R. Colwell. 1987. Metabolic activity of bacterial cells enumerated by direct viable count. *Appl. Environ. Microbiol.* **53**:2889-2893.
- Servis, N. A., M. S. Lytle, D. B. Midthun, R. A. Leake, and J. C. Adams. 1993. Comparison of isopropyl cinoxime with nalidixic acid in the direct viable count. *J. Appl. Bacteriol.* **75**:583-587.
- Suttle, C. A. 1994. The significance of viruses to mortality in aquatic microbial communities. *Microb. Ecol.* **28**:237-243.
- Tabor, P. S., and R. A. Neihof. 1982. Improved microautoradiographic method to determine individual microorganisms active in substrate uptake in natural waters. *Appl. Environ. Microbiol.* **44**:945-953.
- Tabor, P. S., and R. A. Neihof. 1984. Direct determination of activities for microorganisms of Chesapeake Bay populations. *Appl. Environ. Microbiol.* **48**:1012-1019.
- Tan, T. L., M. Reinke, and H.-J. Rüger. 1996. New dilution method in microtiter-plates for enumeration and enrichment of copiotrophic and oligotrophic bacteria. *Arch. Hydrobiol.* **137**:511-521.
- Torrella, F., and R. Y. Morita. 1981. Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in seawater. *Appl. Environ. Microbiol.* **41**:518-527.
- Wise, R., J. M. Andrew, and L. J. Edwards. 1983. In vitro activity of Bay 09867, a new quinolone derivative, compared with those of other antimicrobial agents. *Antimicrob. Agents Chemother.* **23**:559-564.
- Zweifel, U. L., and Å. Hagström. 1995. Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (ghosts). *Appl. Environ. Microbiol.* **61**:2180-2185.