Applicability of the Reverse-Flow Filter Technique to Marine Microbial Studies

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The use of the reverse-flow filtration technique to quantitatively concentrate marine bacteria was evaluated using both a pure culture and seawater samples. Our data indicate that the cells are altered during the filtration procedure and that a significant and inconsistent number of cells are lost on the membrane filter. The results obtained indicate that data on marine bacteria concentrated in this manner should be interpreted with caution.

In a recent study (2) the particulate matter in seawater was concentrated by use of the reverse-flow filter technique of Pomeroy and Johannes (5, 6). Various types of analyses were performed on these concentrates including enumeration and substrate uptake kinetics by the heterotrophic bacteria. Other investigators have also used this method in the determination of the relative abundance of bacteria in seawater (5). Ideally, the concentration of bacteria should be proportional to the reduction in volume and the function of the organisms should not be altered by the filtration process. To approach this ideal situation, no cells should be lost by adhesion to the filter and/or sides of the concentrator and few (if any) cells should be permitted to pass through the filter.

Since studies on the occurrence and function of natural marine microbial populations have been made by use of the reverse-flow filter technique, the validity of this technique was investigated. (A preliminary account of this work was presented at the 73rd Annual Meeting of The American Society for Microbiology.)

MATERIALS AND METHODS

Cell preparation and water sample collection. A suspension of washed cells of Vibrio marinus MP-1 (ATCC 15381) (an obligate psychrophile) in Rila Marine Mix solution and a sample of surface water (8 C) taken approximately 27 km off Newport, Oregon, were used in this investigation. The seawater sample was transported to the laboratory within 24 h and maintained at or below the in situ water temperature. V. marinus was grown at 15 C in SDB medium which was composed of the following: polypeptone (BBL), 5.0 g; yeast extract (Difco), 3.0 g; Rila Marine Mix (Rila Products, Teaneck, N.J.), 5.0 g; sodium chloride, 15.0 g; glucose, 0.5 g; succinic acid, 0.2 g; ferrous sulfate, 0.01 g; distilled water, 1 liter. The pH was adjusted to 7.5 with NaOH. The medium was sterilized by autoclaving at 2 atm for 15 min. The medium was cooled to 15 C and inoculated with a 24-h culture of V. marinus.

Cells from the late log phase of growth were harvested by centrifugation in a refrigerated Sorvall Centrifuge RC-2B at 16,300 × g (5 C), and washed with Rila Marine Mix solution (3.3%, wt/vol). This procedure was repeated twice more. The cells were then diluted with Rila Marine Mix solution so that the final cell concentration was 3 × 10^6 cells/ml.

Filtration and collection of various fractions. A one-stage, reverse-flow concentrator similar to that reported by Holm-Hansen, Packard, and Pomeroy (3) was used during this investigation. Two concentrators were used simultaneously drawing water samples from the same source (a 4-liter plastic beaker reservoir which was continuously mixed with a magnetic stirrer to insure a homogenous suspension at all times). The entire procedure was conducted in a refrigerated laboratory maintained at 5 C. Both concentrators were fitted with 38-mm membrane filters (Millipore Corp., type HA, 0.8 μm pore size) held in modified Millipore "Field Monitor" units. At the end of the filtration process (4 to 6 h) the volume of the filtrate and the combined concentrates were measured to determine the concentration factor, and analyses were made on all fractions. The filters were removed from the apparatus and rinsed with 50 ml of cold Rila Marine Mix solution (V. marinus studies) or with filtrate (seawater sample). The filter washings from both membrane filters were combined and were analyzed. During the washing process, the filters were carefully brushed with a fine camel-hair brush to remove as much material as possible (5).

Assay procedures. The spread-plate technique employing Lib-X medium was used to determine the number of colony-forming units (CFU) in the various fractions resulting from the reverse-flow filtration process as well as in the unfiltered samples. This

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medium contained the following: yeast extract (Difco), 1.2 g; trypticase (BBL), 2.3 g; sodium citrate, 0.3 g; l-glutamic acid, 0.3 g; sodium nitrate, 0.05 g; ferrous sulfate, 0.005 g; Rila Marine Mix, 33 g; distilled water, 1 liter (pH adjusted to 7.5). For V. marinus, the plates were incubated at the organism's optimum temperature for growth (15°C) for 24 h. Spread plates made on natural seawater were incubated at 8°C for 5 days.

To obtain uptake kinetic data, the technique of Hobbie and Crawford (1) was followed. Subsamples (10 ml) were taken from each of the fractions studied. Duplicate subsamples were used for each of the four substrate concentrations tested. Total substrate uptake was determined from the amount of radioactivity found in both the cells and the CO2 released from the cells. Both the maximum velocity of glutamate uptake ($V_{max}$) and the transport constant and the natural substrate concentration ($K_t + S_s$) were calculated by use of the equations of Wright and Hobbie (7). Levels of $^14$C activity were determined by assaying both CO2 and cells (trapped on HA 0.45-μm Millipore filters) in 10-ml portions of toluene scintillation fluor (Omnifluor, New England Nuclear) by use of a Mark I Nuclear Chicago scintillation counter. In the study of glutamate uptake at one concentration (21.4 μg/liter), 10-ml subsamples from the various fractions were analyzed. In addition, the uptake by cells associated with the filters was determined by incubating the filters in 250-ml serum bottles. The CO2 was trapped as described by Hobbie and Crawford (1). The radioactivity associated with the cells was determined by washing these filters onto larger membrane filters (Millipore, 47 mm, HA, 0.45 μm) and assaying these after drying.

The bacterial biomass was followed by pulse labeling a pure culture of V. marinus with $^14$C proline for 1 h, washing the cells, and resuspending them in Rila Marine Mix solution. The cell suspension, containing $3 \times 10^8$ cells per ml, was then concentrated by use of the reverse-flow concentrator. The total biomass in various fractions was determined by assaying radioactivity in the cells after filtration.

**RESULTS AND DISCUSSION**

To use the reverse-flow filtration technique to obtain quantitative data on marine bacteria, one must assume that the concentration is proportional to the reduction in volume and that this process does not significantly alter the function of the organisms present. We tested the first assumption by assaying the V. marinus biomass in the concentrate, filtrate, membrane filters, and filter washings after concentrating a water sample (Table 1). If all the cells remained in the concentrate after filtration, then all of the biomass should have been recovered from that fraction. The percentage recovered was approximately 0.7 to 2.4 instead of the expected 100. In terms of actual biomass, this figure represents approxi- mately the same biomass that would be found if no concentration had taken place at all (the concentration factor was 1.0 in one experiment and 1.5 in another as opposed to the expected 126 and 86, respectively).

We also measured the number of colony-forming units (CFU) found in the concentrate relative to what should have been present. Here again only about 2.0% of the total CFU put into the system was recovered in this fraction (Table 2).

The $V_{max}$ was also studied in these fractions during the same experiments (Table 3). In this case, the percentage of potential glutamate

| Table 1. Recovery of V. marinus from various fractions after reverse-flow filtrationa |
|---------------------------------------|--------|----------------|----------------|
| Expt | Fraction        | Bacterial biomass (%) | Volume concn factor | Bacterial biomass concn factor (A) | Bacterial biomass concn factor (B) |
|------|-----------------|-----------------------|---------------------|-------------------------------------|-------------------------------------|
| 1    | Concentrate     | 0.7                   | 126                 | 1.0                                 | 39.5                                |
|      | Filtrate        | 0.4                   |                      |                                     |                                     |
|      | Membrane Filter | 98.9                  |                      |                                     |                                     |
|      | (unwashed)      |                       |                      |                                     |                                     |
| 2    | Concentrate     | 2.4                   | 86                  | 1.5                                 |                                     |
|      | Filtrate        | 2.4                   |                      |                                     |                                     |
|      | Membrane Filter | 34.6                  |                      |                                     |                                     |
|      | (washed)        |                       |                      |                                     |                                     |
|      | Filter Wash     | 60.6                  |                      |                                     |                                     |


*a Bacterial biomass concentration factor (A) was calculated in terms of increased biomass in the concentrate relative to that found in the unconcentrated sample. The bacterial biomass concentration factor (B) was calculated in terms of combined biomass in the concentrate and filter wash.

b It should be noted that our use of the term “concentrate” refers only to the concentrated water sample that remains in the unit after filtration. This should not be confused with the same term used by other investigators which may also include filter wash material.

| Table 2. Colony-forming units recovered from various fractions after reverse-flow filtrationa |
|---------------------------------------|--------|----------------|----------------|
| Fraction     | V. marinus | CFU per fraction | Percent of total CFU | Seawater | CFU per fraction | Percent of total CFU |
|---------------|-----------|------------------|----------------------|---------|------------------|----------------------|
| Unconcentrated| 1.84 x 10⁶ | 2.0              | 3.65 x 10⁶           | 2.5     |
| Concentrate   | 3.66 x 10⁷ | 1.1              | 6.65 x 10⁷           | 1.8     |
| Filtrate      | 2.12 x 10⁷ | 22.0             | 1.56 x 10⁷           | 42.7    |
| Filter Wash   | 4.04 x 10⁸ |                  |                      |         |

*a Percentage of total CFU were calculated in terms of the number of CFU found in the original water sample before concentration.
The uptake for the concentrate was about 2.0% instead of the expected 100%. If there had been no alteration in the cell's ability to take up glutamate, the $V_{\text{max}}$ should have been proportional to the cell numbers. A similar pattern was also seen when glutamate uptake was studied at one substrate concentration (Table 4).

The fact that the low recovery in the concentrate was not an artifact of the artificial seawater system was established in similar studies made on a natural seawater sample (Tables 2, 3, and 4). The results were essentially the same. This same observation was also made on concentrates prepared during a recent Antarctic cruise (Gillespie and Jones, personal communication).

It is obvious from the biomass data that the vast majority of the bacteria remained on the filter after the concentration process (approximately 95 to 99%). This problem has apparently been recognized by others using this technique because some method of removing material from the filters is normally employed (2, 5, 6). Indeed, the term “concentrate” usually refers to the concentrated water sample and the material washed from the filters. Since so much of the bacterial biomass became trapped on the filter, we wanted to evaluate the efficiency of cell recovery during the filtration and recovery process.

Holm-Hansen, Packard, and Pomeroy (3) reported that significant quantities of material apparently adhered to the filter even when the filter was brushed or backwashed. They noted that up to 60% of the total deoxyribonucleic acid remained with the filter. While studying bacterial biomass, we found that about 35% of the total remained on the filter after washing with 50 ml of cold Rila Marine Mix solution and careful brushing (Table 1). During several pilot studies, this figure varied from 26 to 63%. Since a wide variation was observed in the number of bacteria adhering to the filter, it seems unlikely that the number of organisms lost in this manner can be accounted for by applying a constant. The loss of bacteria adhering to the filter would be further complicated if several stages were employed (2). If, as we have observed, the amount of material retained on the filter after washing varied and if several filters were used during the filtration process, very little quantitative data could be obtained from the concentrate and/or filter wash which could be applied to the original seawater sample. This is the conclusion of Hobbie and Pomeroy (4) in their comparative study of three water sample concentrators, one of which was a reverse-flow filtration unit.

The biomass, CFU, and kinetic data all showed that (for bacterial forms) almost all of the organisms that were actually concentrated came from the filter wash rather than the original concentrate. This presents the possibility that the organisms might be altered in some way by the filtration process. Microscope examination showed that there was a large amount of clumped debris in the wash water and that almost all of the bacteria present were

| Fraction                  | V. marinus | Seawater |
|---------------------------|------------|----------|
| Uptake (µg/fraction/h)    | Percent recovered | Uptake (µg/fraction/h) | Percent recovered |
| Nonconcentrated           | 3.98       | 1.7      | 6.55 x 10^-1  | 0.7       |
| Concentrated              | 6.70 x 10^-3  | 1.6      | 4.88 x 10^-4  | 5.8       |
| Filtrate                  | 6.34 x 10^-3  | 0.7      | 3.71 x 10^-4  | 20.6      |
| Membrane filters          | 2.72 x 10^-4  | 8.6      | 1.35 x 10^-2  | 20.6      |
| Filter wash               | 3.44 x 10^-1  | 8.6      | 1.35 x 10^-3  | 20.6      |

*The uptake of glutamate (micrograms per fraction per hour) was the actual amount taken up by the organisms in that fraction during 3 h of incubation. Percent recovery was calculated relative to the uptake observed at the same substrate concentration in the unconcentrated water sample.

Table 4. Glutamate uptake in various fractions that were incubated in 21.4 µg/14C glutamate

| Fraction | V. marinus | Seawater |
|----------|------------|----------|
| $V_{\text{max}}$ (µg/fraction/h) | Percent recovered | $K_{i} + S_{o}$ (µg/liter) | Percent recovered | $K_{i} + S_{o}$ (µg/liter) |
| Unconcentrated | 8.37 | 19 | 0.35 | 17 |
| Concentrated  | 0.15 | 27 | 0.0065 | 1.8 | 68 |
| Filtrate     | 0.26 | 66 | 0.097 | 27.6 | 31 |
| Filter Wash  | 1.90 | 95 |  |  |

*The $V_{\text{max}}$ micrograms per fraction per hour was calculated as the potential maximum velocity of glutamate uptake for all organisms in the indicated fraction. The percent recovered was calculated relative to the total potential uptake of the organisms in the water sample before concentration.
associated with this debris. This was observed in both the V. marinus system (where the organisms should be well dispersed in the unconcentrated water sample) and in the seawater samples. The clumping may, in part, explain the relatively low recovery of CFU in the filter wash (Table 2). To determine whether or not an alteration in the organism’s ability to take up glutamate had occurred, we measured the kinetics of glutamate uptake in the unconcentrated sample, the concentrate, filtrate, and filter wash (Table 3). The relatively low \( V_{\text{max}} \) in the recovered fractions may reflect either a reduction in cells lost by removal on the filter, a reduction in the ability to take up glutamate, or both. If only 20 to 30% of the cells were recovered from the filter, then the observed \( V_{\text{max}} \) values would reflect only the level of cells present. On the other hand, if the number of cells recovered in the filter wash was much greater than this, then injury to glutamate uptake potential would be observed. Unfortunately, the logistics involved in assaying the \( V_{\text{max}} \) of cells associated with the filter makes it impossible to obtain this measurement and thus an accounting for all of the cells.

Indirect evidence indicates that bacterial function is altered by the filtration process. In the seawater sample, at least 42.7% of the cells (as measured by CFU) were recovered in the filter wash, yet only 27.6% of the potential to take up glutamate was recovered in this fraction.

In a recent on-board study in which the reverse-flow technique was used, Hobbie et al. (2) presented data (although not discussed) in their tables which showed that the concentration factor (as determined by increased \( V_{\text{max}} \)) did not correlate well with the concentration factor as measured by volume reduction. Not only were the \( V_{\text{max}} \) concentration factors lower (up to one-tenth of that expected from volume reduction), but they were also inconsistent. They also reported data that support the idea that the bacteria were injured during the filtration process. In one of their samples (station 2 depth 13 m) the calculated concentration factor (\( V_{\text{max}} \)) varied with the substrate tested. If the reported decrease was due only to a loss in cell numbers, then the concentration factor (\( V_{\text{max}} \)) should have been the same regardless of the substrate tested. Since a large variation was seen, it must be concluded that various functions associated with substrate uptake were altered.

Further evidence for an alteration in function can also be drawn from our kinetic data, namely the \( K_t + S_u \) data. If we assume that the natural substrate concentration (\( S_u \)) was the same in all fractions tested for a given water sample (it should in fact be 0 in the V. marinus experiments), then any change in the \( K_t + S_u \) figures should be a function of the cell’s ability to take up glutamate since the transport constant (\( K_t \)) reflects the ability of a population to take up substrate regardless of the cell numbers involved. There was a significant difference in the calculated \( K_t + S_u \) values for all of the recovered fractions tested (Table 3). It is of interest to note that the \( K_t \) for the natural population (unconcentrated) was almost identical to that of our test organism MP-1. The higher \( K_t \) values seen in the recovered fractions indicate that the organisms had become less efficient at taking up glutamate at lower substrate concentrations. While studying the effects of reverse-flow filtration on respiration in ultraplankton, Pomeroy and Johannes (6) reported that, at unspecified “very high concentration factors,” there was a depression in respiration rates. This may well reflect injury in this group of organisms as well.

We can thus conclude from our data that bacteria concentrated in this way are not functionally equivalent (in their ability to take up glutamate) to those found in untreated water samples. The variations observed from sample to sample preclude the likelihood that a constant can be applied to substrate uptake data that would convert data collected on concentrates to meaningful in situ uptake rates. These data also show that the bacterial forms are to some degree inactivated by these procedures. This is in contrast to the work of Holm-Hansen, Packard, and Pomeroy (3) who concluded from adenosine 5'-triphosphate data, that no significant inactivation took place for any organisms present in seawater that had been concentrated in this manner. It also indicates that the data presented by Hobbie et al. (2) should be interpreted with caution.

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