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# Application of a Specific and Sensitive Radiometric Assay for Microbial Lipase Activities in Marine Water Samples from the Lagoon of Nouméa

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**Marine microbiologists commonly assay lipase activities by using a synthetic fluorescent analog, 4-methylumbelliferyl (MUF)-oleate. The technique is convenient, but it is considered to be unspecific because of the structure of this analog. This study reports the design of a new specific and sensitive lipase assay based on the use of a radiolabeled triglyceride, [$^3$H]triolein. Free fatty acids (FFA) resulting from its hydrolysis are isolated as a function of time in a one-step liquid-liquid extraction and then radioassayed. MUF-oleate and [$^3$H]triolein techniques were compared by measuring lipase activities at similar substrate concentrations along a trophic gradient in the Southwest Lagoon of New Caledonia, near Nouméa. Hydrolysis rates decreased from the nearshore station to the offshore station and showed similar trends regardless of the technique used. Rates decreased from 5.83 to 0.88 nmol of FFA · liter$^{-1}$ · h$^{-1}$ and from 0.76 to 0.23 nmol of $^3$H-FFA · liter$^{-1}$ · h$^{-1}$, respectively. These results appeared to be consistent with bacterial production results, which also decreased similarly (from 0.59 to 0.26 μg of C · liter$^{-1}$ · h$^{-1}$). However, the ratio of MUF-oleate activities to [$^3$H]triolein activities, which was constant at the offshore stations (3.8 ± 0.1), gradually increased at the nearshore stations (from 4.1 to 7.6). This result shows that the two assays respond in different ways to changes in environmental conditions and validates the need to set up more specific enzymatic assays.**

Because of their capacity to use dissolved organic matter as a carbon source for their biomass, bacteria play a key role in the degradation of organic matter in the marine environment. They constitute the essential step which leads to higher trophic levels in what is known as a microbial loop (4). This step is based on the ability of bacteria to take up organic compounds via specialized transport proteins. However, only small-molecular-mass compounds (<600 Da) can be directly transported (34), while higher-molecular-mass compounds, which represent 30 to 95% of total organic matter (2, 9, 15, 26), must be hydrolyzed prior to uptake.

Cell-associated bacterial ectoenzymes, along with extracellular enzymes dissolved in the aquatic medium, mediate this hydrolysis (14). This is a primary step, conditioning all subsequent steps of heterotrophic bacterial production.

Among these bacterial ectoenzymes, lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are defined as the enzymes which catalyze the hydrolysis of triacylglycerols, releasing long-chain fatty acids (10, 12, 23). Marine microbiologists generally assay lipase activities by using synthetic fluorescent analogs related to the coumarin fatty acyl ester family, such as 4-methylumbelliferyl (MUF) fatty acyl esters (22). One commonly used analog is MUF-oleate (24, 29, 30). However, major drawbacks have been reported concerning this technique. According to Hendrickson (20) and Beisson et al. (6), lipases should not behave toward triglycerides in the same way as they do toward MUF-oleate. Indeed, this analog, being chemically unrelated to triglycerides, is not a priori hydrolyzed specifically by lipases (7) and is likely to be nonspecifically hydrolyzed by carboxylesterases (6, 20). Such enzymes may be particularly abundant in a natural medium such as seawater. Thus, this substrate may not be fully relevant for measurements in natural seawater samples. Particularly, it is important for carbon cycle modeling to assess whether the hydrolysis rates estimated through MUF-oleate consumption reflect naturally occurring rates and their variability.

The aim of the present work was to design a new test based on the use of a radiolabeled triglyceride as a substrate for lipase hydrolysis. Such methods are reported to be specific, because lipases cannot distinguish a radiolabeled triglyceride from a natural one, and they are also very sensitive, due to the high specific activity of radiolabeled probes. Therefore, these methods appeared suitable for real lipase activity measurement in natural seawater samples (7). Our assay is based on the principle of Borgström's protocol as modified by Belfrage and Vaughan (8), in which fatty acids resulting from the hydrolysis of a radiolabeled triglyceride are isolated in a one-step liquid-liquid extraction and radioassayed over time.

The present paper describes the setup of this new assay and its application to the measurement of lipase activities along a trophic gradient in the Southwest Lagoon of New Caledonia

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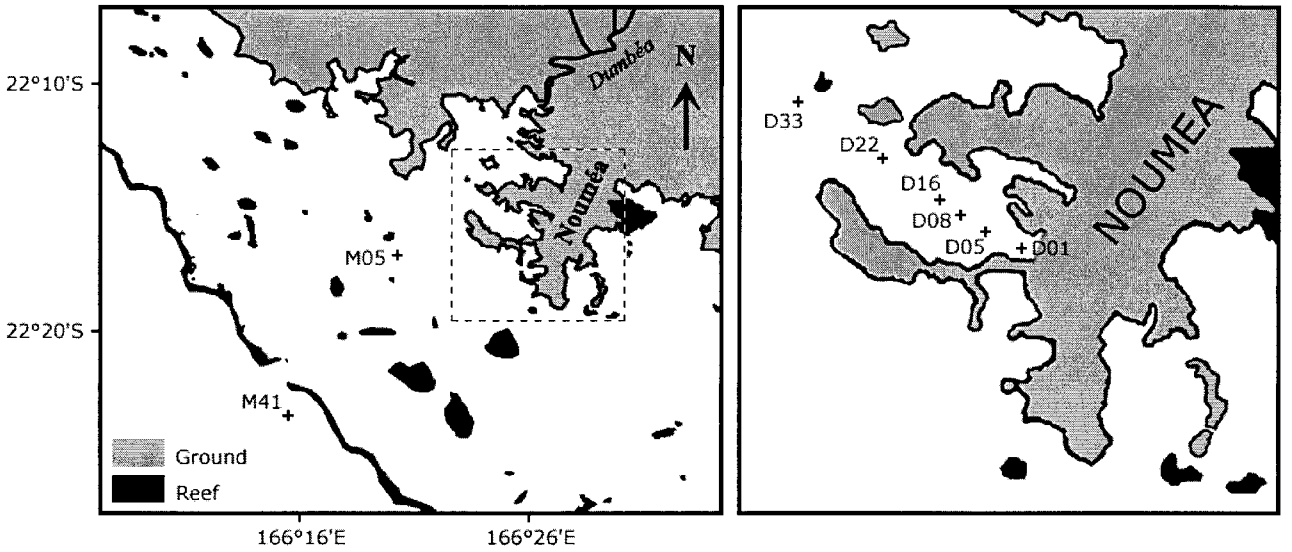


FIG. 1. Map of the lagoon offshore Nouméa city. +, locations of sampling stations.

near Nouméa (Grande Rade). One major goal was to compare the results of the [$^3$H]triolein assay to those obtained by using the common MUF-oleate technique.

## MATERIALS AND METHODS

**[$^3$H]triolein lipase assay.** [9,10-$^3$H(N)]oleic acid ([$^3$H]oleic acid, 15 Ci · mmol$^{-1}$) and [9,10-$^3$H(N)]triolein ([$^3$H]triolein, 22 Ci · mmol$^{-1}$) were obtained from Perkin-Elmer—NEN. Water samples were distributed into 50-ml polypropylene centrifuge tubes, and [$^3$H]triolein (<1 to 60 nM $^3$H-esterified fatty acid [EFA]) was added as a solution in ethanol (final concentration, 0.005 to <0.2%). Immediately after vortex homogenization, series (triplicates) of 0.8-ml subsamples were distributed into 15-ml polypropylene centrifuge tubes. The first series was stopped without delay by the addition of 0.5 ml of a 1 M solution of Tris (adjusted to pH 11 using NaOH) and 4.8 ml of a methanol-chloroform-heptane (38.6:34:27.4) solvent mixture, followed by vigorous vortex agitation. The other series were placed in the dark, in an incubation bath (in situ temperature, 25 ± 1°C), until the end of their incubation period and then treated as described above. Stopped samples were centrifuged (for 3 min at 5,445 × *g*) to allow separation of phases. A portion (2 ml) of the upper-aqueous phase was transferred to polypropylene scintillation vials containing 10 ml of Hionic-Fluor liquid scintillation counting cocktail (Packard Bioscience) for radioactivity counting. At time zero, the distribution of fatty acids between the two phases was estimated with the same protocol, using [$^3$H]oleic acid instead of [$^3$H]triolein. No incubation was performed in this case. Finally, the expression of lipase activity was calculated from the radioactivity in the total volume of the aqueous phase, the proportion of released [$^3$H]oleic acids contained in this phase, and the specific activity of the radiolabeled probe. It is noteworthy that the transformation products of the [$^3$H]oleic acid (including $^3$H-fatty acids and $^3H_2O$) also end up in the aqueous phase. Autoclaved samples were used as controls for abiotic substrate degradation.

**MUF-oleate lipase assay.** Samples (50 ml) were incubated in the dark (25 ± 1°C) with MUF-oleate (Sigma) fluorogenic substrate (22). At selected incubation times, substrate hydrolysis was assayed on triplicate aliquots by using a TD-700 fluorometer (Turner Designs) that was equipped with an ammonium filter set (excitation, 365 nm; emission, >410 nm) and calibrated with standard solutions of MUF (Sigma). Autoclaved samples were used as controls for abiotic substrate degradation.

**Expression of substrate [$^3$H]triolein and MUF-oleate concentrations and hydrolysis rates.** One mole of [$^3$H]triolein corresponds to 3 mol of ester bonds, which may be hydrolyzed by lipase enzymes, whereas 1 mol of MUF-oleate corresponds to 1 mol of ester bonds. We chose to express substrate concentrations as their EFA equivalence, and we described activity rates in terms of the release of free fatty acids (FFA) to facilitate comparison between the two techniques.

**Test for reproducibility of the [$^3$H]triolein lipase assay.** Lipase activity was measured at two different stations (M05 and M41) in five subsamples by using the [$^3$H]triolein lipase assay at a fixed substrate concentration. Substrate concentrations were 2.49 ± 0.25 (mean ± standard deviation [SD]) nM $^3$H-EFA and 2.23 ± 0.12 nM $^3$H-EFA at stations M05 and M41, respectively.

**Bacterial production.** Bacterial production was determined from [*methyl*-$^3$H]thymidine (45 Ci · mmol$^{-1}$; Amersham) incorporation (16). Duplicate 10-ml subsamples were incubated in the dark at in situ temperature (25 ± 1°C) for 60 min with 15 nM [$^3$H]thymidine and were then preserved with buffered formaldehyde and unlabeled thymidine (final concentrations, 2% and 1 mM, respectively), filtered through 0.2-μm-pore-size Nuclepore membranes, extracted by ice-cold 5% trichloroacetic acid (TCA), and rinsed four times with 5 ml of 5% TCA. Labeled TCA precipitates were heated with 0.5 ml of 0.5 N HCl at 100°C for 30 min to minimize self-absorption of beta radiation before determination of radioactivity. Blanks prekilled with 2% formaldehyde were processed in parallel. The biomass production rates were computed by using the average of empirically determined conversion factors at D01, D08, and M05 ($2.9 \times 10^{18}$ cells · mol$^{-1}$ of thymidine [unpublished data]) and 12.4 fg of C · cell$^{-1}$ (17).

**Station properties and sample collection.** Samples were collected in subsurface waters (depth, 3 m) in and outside the lagoon of Nouméa, New Caledonia, onboard the R/V *Coris*. Locations of the stations are presented in Fig. 1. Stations along a nearshore-to-offshore transect (D01 to D33; depth, 6 to 23 m) exhibited an increasing gradient of salinity (34.98 to 35.10 g · liter$^{-1}$), which was associated with a decreasing gradient of chlorophyll *a* concentration (0.83 μg · liter$^{-1}$ to 0.42 μg · liter$^{-1}$). A middle-lagoon station (M05; depth, 32 m) and an outside-lagoon reference station (M41; depth, >200 m) were also investigated. Seawater samples were collected in a 5-liter Niskin sampling bottle and processed back in the laboratory within 2 h after sampling. Whenever possible, hydrological data were collected along the whole water column.

**Bacterial activities along a trophic gradient.** Bacterial production and lipase activities evaluated by both [$^3$H]triolein and MUF-oleate assays at similar substrate concentrations (12.5 nM EFA) were measured along the trophic gradient (D01 to D33). Additionally, multiconcentration kinetics experiments were conducted at D01 and D33 using MUF-oleate (4 to 75 nM EFA) and [$^3$H]triolein (2.62 to 76.26 nM $^3$H-EFA). Kinetic parameters ($V_m$ and $K_m$) were determined from a Michaelis-Menten model by using the Marquardt Levenberg minimization in SB's Kinetics software package, version 3.0 (11).

## RESULTS

**[$^3$H]triolein hydrolysis.** The hydrolysis of the [$^3$H]triolein as a function of time was reflected by the appearance of ionized free [$^3$H]oleic acids in the alkaline aqueous phase of the extraction medium. Different assays on autoclaved seawater sam-

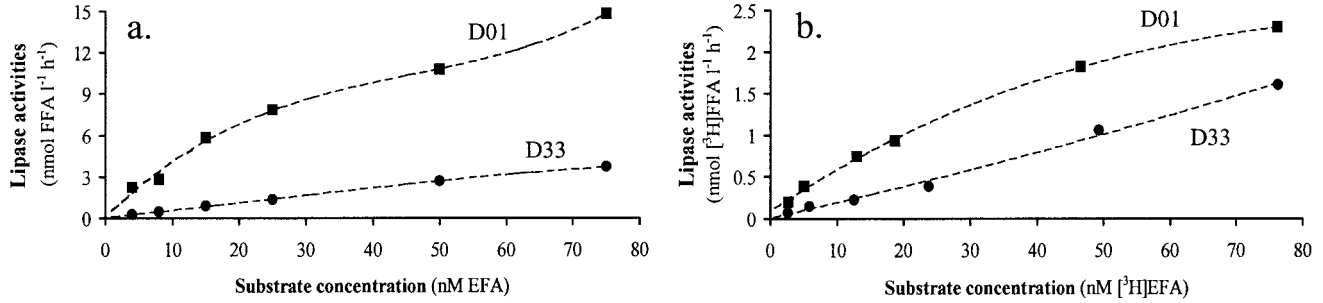


FIG. 2. Multiconcentration kinetics. The graphs show lipase activities at stations D01 (■) and D33 (●), which were determined by using the MUF-oleate lipase assay (a) and the [$^3$H]triolein lipase assay (b).

ples showed that neither contamination of the aqueous phase by the nonhydrolyzed radiolabeled substrate nor adsorption of the fatty acids on the inner wall of the tubes occurred during extraction. Tests performed at various incubation times (ranging from minutes to days) and various substrate concentrations led us to fix optimal conditions for the assay, i.e., with incubation times ranging from 1 h to several hours and substrate concentrations ranging from <1 to 25 to 50 nM $^3$H-EFA. The highest [$^3$H]triolein substrate concentration that could be used significantly was approximately 60 nM $^3$H-EFA (i.e., 20 nM [$^3$H]triolein). The use of higher substrate concentrations led to poor reproducibility in replicate samples and loss of linearity over time.

**Reproducibility of the [$^3$H]triolein lipase assay.** For each station, turnover rates were calculated as the percentage per hour of $^3$H-FFA released from the $^3$H-EFA substrate pool. Under these conditions, turnover rates ranged from 5.10 to 6.77% $^3$H-FFA · h$^{-1}$ at M05 and from 5.38 to 6.76% $^3$H-FFA · h$^{-1}$ at M41. Variability of the [$^3$H]triolein lipase assay at the indicated stations was 10.7 and 9.7%, respectively.

**Multiconcentration kinetics.** The multiconcentration kinetics profiles at the D01 and D33 stations, determined with both the MUF-oleate lipase and the [$^3$H]triolein assays, are presented in Fig. 2.

In the MUF-oleate assay (Fig. 2a), the $V_m$ of MUF-oleate hydrolysis was 24.5 ± 3.4 nmol · liter$^{-1}$ · h$^{-1}$ and the $K_m$ was 54.1 ± 13.9 nmol · liter$^{-1}$ (mean ± SD) at D01, while at D33 the $V_m$ was 20.3 ± 3.8 nmol · liter$^{-1}$ · h$^{-1}$ and the $K_m$ was 334 ± 74 nmol · liter$^{-1}$.

In the [$^3$H]triolein assay (Fig. 2b), the $V_m$ of [$^3$H]triolein hydrolysis was 4.09 ± 0.24 nmol · liter$^{-1}$ · h$^{-1}$ and the $K_m$ was 59.1 ± 6.5 nmol · liter$^{-1}$ (mean ± SD) at D01. Under our experimental conditions, results at D33 did not fit a simple Michaelis-Menten model, and thus it was not possible to calculate significant kinetic parameters.

**Bacterial activities along the trophic gradient.** Lipase activities measured along the trophic gradient by using MUF-oleate (Fig. 3a) and [$^3$H]triolein (Fig. 3b) substrates decreased from 5.83 to 0.88 nmol of FFA · liter$^{-1}$ · h$^{-1}$ and from 0.76 to 0.23 nmol of $^3$H-FFA · liter$^{-1}$ · h$^{-1}$, respectively. They varied similarly, with activity values decreasing from D01 to D33 (except for D16). Bacterial production (Fig. 3c) also similarly decreased from D01 (0.59 μg of C · liter$^{-1}$ · h$^{-1}$) to D33 (0.26 μg of C · liter$^{-1}$ · h$^{-1}$).

**Correlation of MUF-oleate and [$^3$H]triolein hydrolysis with bacterial production.** The ratio of MUF-oleate to [$^3$H]triolein activities (Fig. 4a) was constant at the three offshore stations (D16, D22, and D33; 3.8 ± 0.1 [mean ± SD]) but gradually increased from 4.1 to 7.6 at nearshore stations D08, D05, and

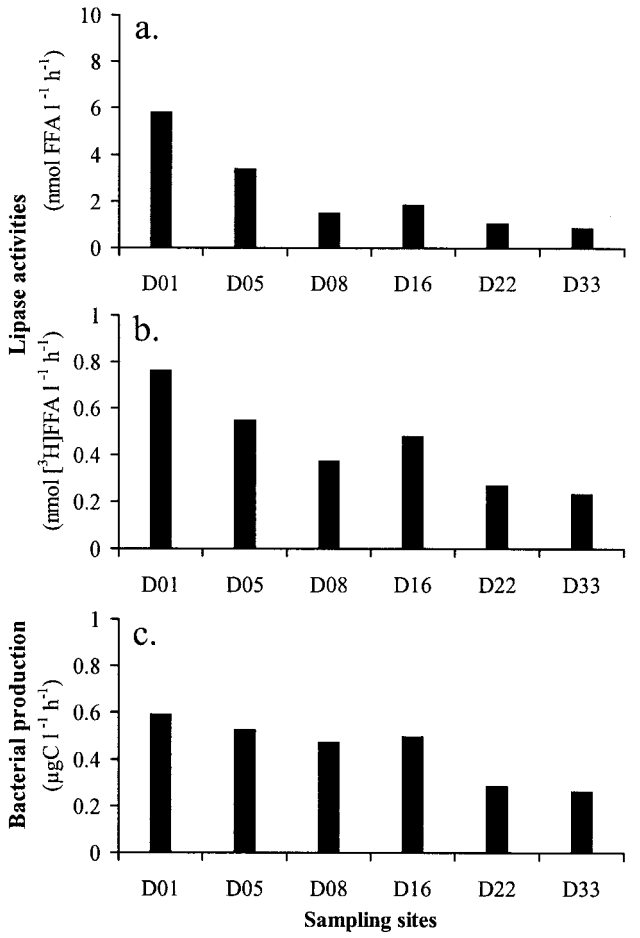


FIG. 3. Lipase activities and bacterial production along a trophic gradient (D01 to D33). Lipase activities were measured using MUF-oleate (a) and [$^3$H]triolein (b) as substrates (12.5 nM EFA substrate concentrations). Bacterial productions (c) are also presented.

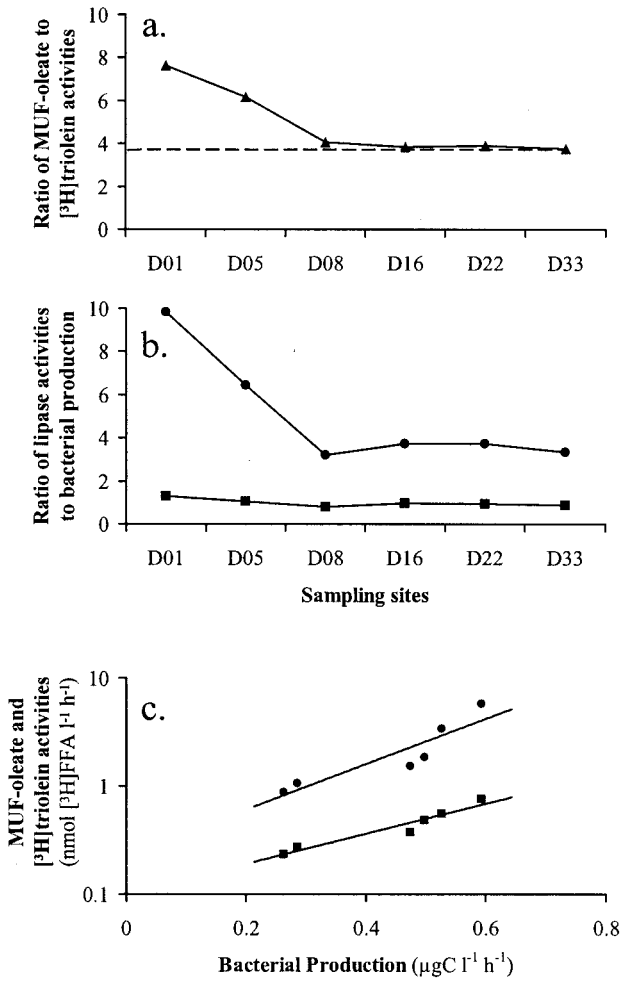


FIG. 4. Ratio of MUF-oleate activities to [$^3$H]triolein activities (a), ratio of MUF-oleate (●) and [$^3$H]triolein (■) activities to bacterial production (b), and scatter plot of MUF-oleate (●) and [$^3$H]triolein (■) ectoenzymatic activities versus bacterial production (c). Solid lines represent exponential-regression fits.

D01. As shown in Fig. 4b, the ratio of [$^3$H]triolein activities to bacterial production varied little between D33 and D01 [0.99 ± 0.17 nmol of [$^3$H]FFA · (μg of C)$^{-1}$], while the ratio of MUF-oleate activities to bacterial production increased considerably over the six stations [3.3 to 9.8; mean, 5.0 ± 2.6 nmol of FFA · (μg of C)$^{-1}$].

Both enzymatic activities were closely related to bacterial production by an exponential relationship (Fig. 4c). However, [$^3$H]triolein activities turned out to be better linked to bacterial production ($n = 6$; $P = 0.0016$) than MUF-oleate activities ($n = 6$; $P = 0.012$) were.

## DISCUSSION

Few environmental studies have focused on lipid-degrading enzymatic activities of marine bacteria. In contrast, numerous studies have investigated protein and carbohydrate enzymatic degradations (13, 18, 21), mainly because these compounds are considered to be primary carbon sources for bacterial growth. However, lipids constitute an important class of natural compounds that are frequently used in field studies as biomarkers to track the source and fate of organic matter (33). Most of these biomarkers are enzyme-mediated products derived from the hydrolysis of fatty acid esters. Estimation of the rates at which these compounds are released in the medium is a major goal for comprehensive environmental studies. For this purpose, several studies have attempted to identify substrates that would be more representative of natural molecules than the commonly used fluorogenic substrate. New ectoenzymatic activity assays are aimed to provide more realistic data regarding organic matter degradation rates in the marine environment and are based on the use of innovative substrates which are more specific and/or sensitive. For instance, fluorescent Lucifer Yellow derivatives of different peptides (27) and fluorescently labeled polysaccharides (3) have been proposed as alternatives to the commonly used L-leucine 7-amino-4-methylcoumarin (MCA-leucine) and MUF-monosaccharide dimers to estimate rates of polypeptide and polysaccharide hydrolysis. Although many techniques of lipase activity measurement have been described (7), few have been applied to the marine environment, where lipase activity is generally assayed by using MUF-fatty acyl esters, typically MUF-oleate (24, 29, 30). However, the method proposed here differs from these previous methods in that it measures the hydrolysis rate of radiolabeled triolein, a triglyceride that is widespread among marine organisms (1, 25, 28, 32).

As is the case for most methods developed to measure enzyme activities in seawaters, the proposed [$^3$H]triolein method is adapted from an existing lipase assay (8). Beisson et al. (5) adapted the same assay to measure lipase activities in human stratum corneum samples. These authors report the use of a reaction buffer that was designed to facilitate the mixing of the [$^3$H]triolein and to create favorable conditions for lipolysis, but this should be avoided when measuring lipase activity in marine samples. Indeed, we learned that the use of such a buffer inhibited the natural lipase activity (data not shown). When [$^3$H]triolein was added directly to the water sample as described herein, the intrinsic properties of seawater, probably due to the presence of natural organic compounds, enabled the mixing of the substrate in the range of [$^3$H]triolein concentrations useable for this test.

We identified several factors limiting the possible extent of the concentration range within which the [$^3$H]triolein kinetics experiment can be run. On one hand, the specific activity of the radiolabeled substrate can be limiting when trying to reach the lowest substrate concentrations. On the other hand, the [$^3$H]triolein solvent, ethanol, may disrupt bacterial activity if present in too-large proportions (final concentration, >0.2 to 0.3%). Concentrating [$^3$H]triolein in ethanol allows us to reach higher concentrations to some extent, but the success of this approach is limited due to the poor solubility of [$^3$H]triolein. We tested the direct dropping of substrate solution into the incubation vial with subsequent addition of the water sample after ethanol evaporation: most of the [$^3$H]triolein did not dissolve back into the seawater and stayed bound to the inner wall of the tubes, where it appeared to be inaccessible to the enzymes. Considering these limitations, significant substrate concentrations ranged from <1 to <60 nM $^3$H-EFA. Incubation time depends on both the local level of activity and the

substrate concentration. We found that the incubation time could generally be fixed between 1 h and a few hours to ensure linearity.

To investigate possible [$^3$H]FFA consumption within this short incubation time, samples were incubated by using the [$^3$H]triolein lipase assay with [$^3$H]oleic acid. There was no (or negligible) processing of released fatty acids that could alter the test, probably because they represented only a negligible fraction of the natural pool. This finding should be verified when studying a different environment. Any $^3H_2O$ formed by mineralization of the [$^3$H]oleic acid would end up in the aqueous phase as well (as would other transformation products). [$^3$H]triolein may potentially be used to estimate mineralization, although the comparison with the MUF-oleate suggests that not much mineralization occurs during the short incubation time. Under our test conditions, the intrinsic variability of the [$^3$H]triolein lipase assay was approximately 10%.

Similar concentration ranges of both [$^3$H]triolein and MUF-oleate substrates were used to allow comparison during kinetic experiments. The decrease from the productive nearshore stations to the oligotrophic offshore stations both in the $V_m$ values determined by the MUF-oleate assays and in the affinity of the lipolytic enzymes was consistent with previous observations in tropical lagoons (29). Using the [$^3$H]triolein lipase assay, we were unable to determine Michaelis-Menten parameters at offshore station D33. The concentration range for the multiconcentration kinetics was set up between minima and maxima that were determined by the MUF-oleate assay's sensitivity and the upper limit of triglyceride solubilization, respectively. It is obvious that these conditions were not optimum for any of the multiconcentration kinetics. In fact, the [$^3$H]triolein assay allows researchers to use substrate concentrations as low as $<1$ nM [$^3$H]EFA, while natural triglyceride concentration in seawaters is generally found to be between 2 and 45 nM EFA (up to $>100$ nM) (15, 19, 31). Assuming that triolein is representative, this finding enables us to run the assay at trace concentrations and to measure naturally occurring hydrolysis rates and turnover, which could then be extrapolated to the whole triglyceride pool.

Lipase activities recorded along the trophic gradient using both the [$^3$H]triolein and MUF-oleate lipase assays appeared to be consistent with bacterial production as they all covaried. Similar to previous observations (29), both hydrolytic and production activities of bacteria were much higher at the productive nearshore station than at the oligotrophic offshore station. The [$^3$H]triolein activities were inferior to MUF-oleate activities by at least a factor of four, suggesting that MUF-oleate was more easily hydrolyzed by lipases. In spite of this trend of similarity, it was not possible to apply a constant conversion factor to convert lipase activities measured with the MUF-oleate assay into rates measured with the [$^3$H]triolein assay, which suggested a different response of the two assays to changes in trophic characteristics. This could be due to the presence of nonspecific enzymes that can hydrolyze MUF-oleate bonds but are unable to hydrolyze the [$^3$H]triolein substrate. However, one should note that the 12.5 nM EFA concentration used for comparison of the two techniques was too high for trace concentration and below saturation conditions: patterns in the results might have been affected by the presence of natural triglycerides.

The ratio of [$^3$H]triolein activity to bacterial production varied little. Using the triolein carbon content ($684\ g \cdot mol^{-1}$) for lipase activity conversion, this ratio averaged 0.23 (SD, 0.04) in the lagoon. Therefore, the hydrolysis of triolein at the concentration used represents 23% of bacterial carbon production in lagoon waters. Since bacterial carbon growth efficiency in oligotrophic to mesotrophic waters is typically low ($<20\%$), bacterial carbon demand is at least five times greater than bacterial production. Yet triglyceride hydrolysis could supply a small but significant part of bacterioplankton carbon demand.

Both MUF-oleate and [$^3$H]triolein activities were closely correlated with bacterial production. However, [$^3$H]triolein activities appeared to be better linked to bacterial production than MUF-oleate activities, suggesting that this new assay could better reflect bacterium-specific hydrolysis. Such observations encourage the development of specific enzymatic assays to better approach the response of bacteria to environmental growth conditions.

In conclusion, while there is no doubt that MUF assays provide interesting data to examine hydrolysis within marine systems, the [$^3$H]triolein lipase assay appears to be more reproducible, sensitive, and specific. It may therefore provide a better estimate of in situ lipolytic activities. Our study suggests that developing similar assays on target components of marine organic matter, which appear to be potentially important to fulfill bacterioplankton carbon requirements, would lead to significant progress in understanding changes in organic carbon turnover times in marine systems as well as in the general cycling of organic carbon in the marine environment.

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## REFERENCES

1. **Albers, C. S., G. Kattner, and W. Hagen.** 1996. The compositions of wax esters, triacylglycerols and phospholipids in Arctic and Antarctic copepods: evidence of energetic adaptations. Mar. Chem. **55:**347–358.
2. **Amon, R. M. W., and R. Benner.** 1994. Rapid cycling of high-molecular-weight dissolved organic matter in the ocean. Nature **369:**549–552.
3. **Arnosti, C.** 1996. A new method for measuring polysaccharide hydrolysis rates in marine environments. Org. Geochem. **25:**105–115.
4. **Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad.** 1983. The ecological role of water-column microbes in the sea. Mar. Ecol. Prog. Ser. **10:**257–263.
5. **Beisson, F., M. Aoubala, S. Marull, A. M. Moustacas-Gardies, R. Voultoury, R. Verger, and V. Arondel.** 2001. Use of the tape stripping technique for directly quantifying esterase activities in human stratum corneum. Anal. Biochem. **290:**179–185.
6. **Beisson, F., N. Ferte, J. Nari, G. Noat, V. Arondel, and R. Verger.** 1999. Use of naturally fluorescent triacylglycerols from *Parinari glaberrimum* to detect low lipase activities from *Arabidopsis thaliana* seedlings. J. Lipid Res. **40:** 2313–2321.
7. **Beisson, F., A. Tiss, C. Rivière, and R. Verger.** 2000. Methods for lipase detection and assay: a critical review. Eur. J. Lipid Sci. Technol. **2:**133–153.
8. **Belfrage, P., and M. Vaughan.** 1969. Simple liquid-liquid partition system for isolation of labeled oleic acid from mixtures with glycerides. J. Lipid Res. **10:**341–344.
9. **Benner, R., J. D. Pakulski, M. McCarthy, J. I. Hedges, and P. G. Hatcher.**

1992. Bulk chemical characteristics of dissolved organic matter in the ocean. Science **255:**1561–1564.
10. **Brockerhoff, H., and R. G. Jensen.** 1974. Lipolytic enzymes. Academic Press, Inc., New York, N.Y.
11. **Brooks, S. P. J.** 1992. A simple computer program with statistical tests for the analysis of enzyme kinetics. BioTechniques **13:**906–911.
12. **Brune, K. A., and F. Götz.** 1992. Degradation of lipids by bacterial lipases, p. 243–266. *In* G. Winkelmann (ed.), Microbial degradation of natural products. VCH Verlagsgesellschaft mbH, Weinheim, Germany.
13. **Christian, J. R., and D. M. Karl.** 1995. Bacterial ectoenzymes in marine waters: activity ratios and temperature responses in three oceanographic provinces. Limnol. Oceanogr. **40:**1042–1049.
14. **Chróst, R. J.** 1991. Microbial enzymes in aquatic environments. Springer-Verlag, New York, N.Y.
15. **Derieux, S., J. Fillaux, and A. Saliot.** 1998. Lipid class and fatty acid distributions in particulate and dissolved fractions in the north Adriatic sea. Org. Geochem. **29:**1609–1621.
16. **Fuhrman, J. A., and F. Azam.** 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. **66:**109–120.
17. **Fukuda, R., H. Ogawa, T. Nagata, and I. I. Koike.** 1998. Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environments. Appl. Environ. Microbiol. **64:**3352–3358.
18. **Fukuda, R., Y. Sohrin, N. Saotome, H. Fukuda, T. Nagata, and I. Koike.** 2000. East-west gradient in ectoenzyme activities in the subarctic Pacific: possible regulation by zinc. Limnol. Oceanogr. **45:**930–939.
19. **Gérin, C., and M. Goutx.** 1994. Iatroscan-measured particulate and dissolved lipids in the Almeria-Oran frontal system (Almofront-1, May 1991). J. Mar. Systems **5:**343–360.
20. **Hendrickson, H. S.** 1994. Fluorescence-based assays of lipases, phospholipases, and other lipolytic enzymes. Anal. Biochem. **219:**1–8.
21. **Hoppe, H. G.** 1983. Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methylumbelliferyl-substrates. Mar. Ecol. Prog. Ser. **11:**299–308.
22. **Hoppe, H. G.** 1993. Use of fluorogenic model substrates for extracellular enzyme activity (EEA) measurement of bacteria, p. 423–431. *In* P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (ed.), Handbook of methods in aquatic microbial ecology. Lewis, Boca Raton, Fla.
23. **Jaeger, K. E., S. Ransac, B. W. Dijkstra, C. Colson, M. Van Heuvel, and O. Misset.** 1994. Bacterial lipases. FEMS Microbiol. Rev. **15:**29–63.
24. **Martinez, J., D. C. Smith, G. F. Steward, and F. Azam.** 1996. Variability in ectohydrolytic enzyme activities of pelagic marine bacteria and its significance for substrate processing in the sea. Aquat. Microb. Ecol. **10:**223–230.
25. **Mayzaud, P., P. Virtue, and E. Ibessard.** 1999. Seasonal variations in the lipid and fatty acid composition of the euphausiid *Meganyctiphanes norvegica* from the Ligurian Sea. Mar. Ecol. Prog. Ser. **186:**199–210.
26. **Munster, U.** 1993. Concentrations and fluxes of organic carbon substrates in the aquatic environment. Antonie Leeuwenhoek **63:**243–274.
27. **Pantoja, S., C. Lee, and J. F. Marecek.** 1997. Hydrolysis of peptides in seawater and sediment. Mar. Chem. **57:**25–40.
28. **Parrish, C. C., G. Bodennec, and P. Gentien.** 1992. Separation of polyunsaturated and saturated lipids from marine phytoplankton on silica gel-coated Chromarods. J. Chromatogr. A **607:**97–104.
29. **Rath, J., C. Schiller, and G. J. Herndl.** 1993. Ectoenzymatic activity and bacterial dynamics along a trophic gradient in the Caribbean Sea. Mar. Ecol. Prog. Ser. **102:**89–96.
30. **Riemann, L., G. F. Steward, and F. Azam.** 2000. Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. Appl. Environ. Microbiol. **66:**578–587.
31. **Van Wambeke, F., M. Goutx, L. Striby, R. Sempéré, and F. Vidussi.** 2001. Bacterial dynamics during the transition from spring bloom to oligotrophy in the northwestern Mediterranean Sea: relationships with particulate detritus and dissolved organic matter. Mar. Ecol. Prog. Ser. **212:**89–105.
32. **Volkman, J. K., S. W. Jeffrey, P. D. Nichols, G. I. Rogers, and C. D. Garland.** 1989. Fatty acid and lipid composition of 10 species of microalgae used in mariculture. J. Exp. Mar. Biol. Ecol. **128:**219–240.
33. **Wakeham, S. G.** 1995. Lipid biomarkers for heterotrophic alteration of suspended particulate organic matter in oxygenated and anoxic water columns of the ocean. Deep-Sea Res. Part I Oceanogr. Res. Pap. **42:**1749–1771.
34. **Weiss, M. S., U. Abele, J. Weckesser, W. Welte, E. Schiltz, and G. E. Schulz.** 1991. Molecular architecture and electrostatic properties of a bacterial porin. Science **254:**1627–1630.