

CHAPTER 48

Use of Fluorogenic Model Substrates for Extracellular Enzyme Activity (EEA) Measurement of Bacteria

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INTRODUCTION

The bulk of organic substances in aquatic environments is macromolecular and not ready for incorporation into the bacterial cell. These materials have to be preconditioned by extracellular enzymes, so that they can be available for bacterial growth and nutrient cycles. The activity of these enzymes is, in many cases, a limiting factor for substrate decomposition and bacterial growth. As demonstrated by size fractionation experiments,¹ extracellular enzymes are mainly produced by bacteria. To a minor extent, they may also originate from autolytic processes and from other organisms. According to the definition given by Priest,² extracellular enzymes are generally located outside the cytoplasmic membrane. In order to distinguish between enzymes which are still associated with their producers and those which occur dissolved in the water or adsorbed to particles, Chr6st³ suggested calling the former "ectoenzymes" and the latter "extracellular" enzymes.

Substrates for hydrolysis are generally proteins, carbohydrates, fats and organic P- or S-compounds. Mechanisms of decomposition of individual compounds within these groups may be studied by *in vitro* experiments. However, aquatic microbial ecologists require, in many cases, a more general measurement of the *in situ* hydrolytic capacity of the prevailing bacterial community. This has led to the adaptation of biochemical methods for determination of overall bacterial extracellular enzyme activities (peptidases, α - and β -glucosidases, chitinases, etc.) in natural waters (Table 1). These methods enable us to study the impact of extracellular enzyme activity (EEA) on bacterial substrate uptake, bacterial growth, and water chemistry. The quantitative estimates of total bacterial extracellular enzyme activity are completed by rapid and sensitive tests for the detection of enzymatic properties of bacterial isolates. The methods used for these purposes are based on the application of fluorogenic model substrates. These substrates have some characteristics in common: (1) they contain an artificial fluorescent molecule and one or more natural molecules (e.g., glucose, amino acids), linked by a specific binding (e.g., peptide binding, ester binding); fluorescence is observed after enzymatic splitting of the complex molecule (Figure 1); (2) the hydrolysis of model substrates is competitively inhibited by a variety of natural compounds with the same structural characteristics; (3) hydrolysis of model substrates follows first order enzyme kinetics; and (4) application of those model substrates allows enzyme activity measurements under natural (*in situ*) conditions within short incubation periods. The latter is highly important for microbial ecologists, because the process of enzymatic hydrolysis is fully

Table 1. Catalog of Fluorogenic Analog (Model) Substrates used for the Study of Extracellular Enzyme Activity in Aquatic Habitats and of Natural or Hygienically Important Bacteria

Substrate	Extracellular enzyme	Application	Ref.
MUF-N-acetyl-glucosaminide	N-acetyl-glucosaminidase (chitinase)	Brackish water	11
L-Leucyl-p-naphthylamide	Aminopeptidase	Natural waters	17
		North Sea	5
L-Leucine-4-methylcoumarinyl-7-amide (Leu-MCA)	Aminopeptidase	Lakes	4
		Marine sediment	18
		Brackish water	19
		Coral reef	19
MUF-p-D-galactopyranoside	β -D-galactosidase	Natural waters	20
MUF-a-D-glucopyranoside	a-D-glucosidase	Brackish water	11
		Eutrophic water	21
MUF-p-D-glucopyranoside	p-D-glucosidase	Brackish water	11
		Eutrophic water	21
		Marine sediment	22
		Lake water	23
MUF- β -D-glucuronide	p-D-glucuronidase	Fecal coliform test	15,26
MUF-butyrate-heptanoate-palmitate	Lipase	Brackish water bacterial colonies	13
MUF-phosphate	Phosphatase (acid, alkaline)	Lake water	9
		Lake water bacteria	24
Methylfluorescein-phosphate	Phosphatase	North Pacific	25
MUF-sulfate	Sulfatase	River epilithon	8

MUF = 4-methylumbelliferone; MCA = 4-methylcoumarinyl-7-amide; the fluorochrome included in MCA-substrates is AMC = 7-amino-4-methylcoumarin.

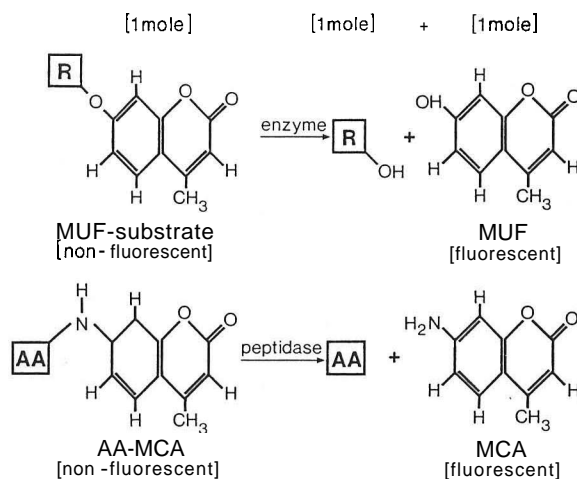


Figure 1. Molecular structure and enzymatic hydrolysis products of 4-methylumbelliferyl (MUF) substrates and 4-methylcoumarinyl-7-amide (MCA) substrates.

integrated into the dynamics of the substrate pool in question (e.g., input by primary production, output by bacterial substrate uptake), which should not be changed or interrupted. Short incubation times avoid or minimize changes of the microbial community structure and the substrate regime (which are due to the bottle effect) during the experiment. Fluorogenic model substrates involving methylumbelliferone (MUF) or 7-amino-4-methylcoumarin (AMC) as a fluorescing agent are not toxic and they are supplied to natural water in low quantities for measurement of their hydrolysis rate (HR), which is dependent on the concentration of

their natural competitors, and in high or increasing quantities for the measurement of the maximum velocity of hydrolysis (V_{\max}).

The method of determining bacterial EEA is valid for all types of natural waters and also, for waste water and drinking water. Using special application techniques, it can also be applied to undisturbed sediment cores.⁴

MATERIALS REQUIRED

Equipment

- Epifluorescence microscope (used with low magnification objective lens or without objective for specific UV excitation of bacterial colonies)
- Fluorometer (type: filter, spectral, cuvette, or microtiter plate)
- Temperature-controlled incubator
- Deep-freezer (storage of stock solutions and incubation devices inoculated with model substrate for later use)

Supplies

- Micropipettes
- Incubation devices (optional scintillation vials, plastic cuvettes, or microtiter plates)

Solutions

See Table 1 for list of substrates. Concentrations of model substrate solutions and specifications of buffers are provided in the section Preparation and Application of Substrate Solutions.

PROCEDURES

Quantitative Determination of EEA in Water

Sample Preparation

In general, water samples are taken by sterile bacteriological samplers and subsamples are distributed to sterile flasks for incubation with the model substrate. Alternatively, water samples may also be taken from oceanographic samplers and distributed in clean scintillation vials, plastic cuvettes, or microtiter plates for incubation. The risk of contamination is, of course, greater in the latter case.

The measurement of EEA is independent of water volume, because the fluorescent end-product of hydrolysis is not bound to particles or incorporated by them, but exists dissolved in the water. The organic compound linked to the fluorescent tracer may be incorporated by bacteria after hydrolysis of the combined molecule; however, this does not influence the measurement.

Sediment cores are treated with injections of the fluorogenic model substrate in order to establish an even distribution of substrate in the core and not to disturb the sediment structure. For details see Meyer-Reil.⁴ Special sample preparations and methodical details for the determination of EEAs of bacteria attached to surfaces (plant, detritus, stones, artificial

surfaces) submerged in natural water are supplied by Rego et al.,⁵ Goulder,⁶ Hoppe,⁷ and Chappell and Goulder.⁸

Model Substrates

A variety of fluorogenic model substrates is in use for the most common types of natural macromolecules. Occasionally, two or more types of model substrate are appropriate for one and the same natural substrate; comparative studies, however, are very rare.⁹ A model substrate is usually composed of a fluorescent tracer molecule and an organic molecule which are linked by a specific binding. Despite their dimeric character, these model substrates are not only competitively inhibited by natural dimers but also by oligomers and polymers.³ The most commonly used model substrates, together with some of their specifications, are listed in Table 1. Model substrates are supplied by companies Serva, Sigma, Fluca, and others.

Preparation and Application of Substrate Solutions

In order to measure enzyme kinetics, model substrate concentrations should cover a sufficient range, depending on the trophic status of the investigated water. The number of concentrations used for a study depends on its specific aim. For instance, the application of a single, high concentration is recommended, only if measurements of relative activity are desired. The following description refers to 4-methylumbelliferyl (MUF)-substrates and 4-methylcoumarinyl-7-amide (MCA)-substrates, the model substrates which are most commonly used in water laboratories.

Model substrate solutions are prepared in sterile water, as long as they dissolve completely in water at the desired concentration. Water solubility differs with type of substrate. For dissolving higher concentrations of substrate, parts of the water may be substituted by an organic solvent, such as Methylcellosolve (ethylene glycol monomethyl ether). Low concentrations of Methylcellosolve do not influence the result of the measurement. Stirring supports the process of model substrate dissolution.

For our measurements in coastal waters of the Baltic Sea, we prepare a set of six concentrations whose molarities are 2, 20, 100, 200, 2500, and 5000 μM . Aliquots of these (50 μl) are pipetted into $\frac{1}{2}$ microcuvettes prior to the addition of 1 ml sample water, resulting in final concentrations of 0.1, 1, 5, 10, 125, and 250 μM . We have chosen relatively high volumes of model substrate in order to avoid mistakes due to inaccurate pipetting. $\frac{1}{2}$ microcuvettes give higher fluorescence readings than other types. Positioning of their small

side toward the emission collecting lens of the fluorometer is recommended. If incubation is not made in cuvettes but in greater or smaller volumes of water (e.g., in scintillation vials or microtiter plates), model substrate additions have to be adjusted to these volumes, or substrate solution concentrations have to be changed. Deep-frozen substrate solutions can be stored in sterile bottles for several weeks and have always to be kept in the dark.

Incubation

All incubations have to be made in the dark. Shaking of the water enhances enzyme activity in most cases, but in order to obtain representative results, it should be adjusted to natural water turbulence. Temperature of incubation depends on the aim of the study. The period of incubation depends on several preconditions: (1) the nature of water samples or sediment samples with respect to the EEA; (2) the mode of fluorescence reading — continuously or start-endpoint determination (see below); and (3) the sensitivity of the fluorometer. Incubation of 1 to 3 h is recommended for eutrophic limnetic waters and coastal

areas. In the open sea and clean inland waters 3 to 8 h are appropriate and up to 48 h for deep water samples. Continuous fluorescence reading, of course, reduces required incubation time considerably.¹⁰

Reading Fluorescence

Any filter fluorometer or spectral fluorometer may be used for fluorescence measurement. Excitation and emission characteristics of the fluorochrome have to be installed in the instrument (364 and 445 nm, respectively for MUF; and 380 and 440 nm for AMC, which is the fluorochrome of MCA substrates).

Start-Endpoint Measurement

Initial fluorescence is read shortly after mixing the model substrate with the sample. A second reading is made at the end of the incubation time. Time between the two fluorescence readings must be recorded for later calculations. Because the intensity of fluorescence is influenced by pH, a decision about pH adjustment has to be made before measurement. Maximum fluorescence of MUF is obtained at pH 10.3. Thus, 200 μ l of pH 10.3-Tris/HCl buffer are placed in the cuvette prior to the addition of 1 ml of the water to measure. When the cuvette itself serves as an incubation vessel, of course, measurements have to be made at the natural pH of the water. This can only be done when no pH changes are expected during incubation, e.g., with naturally buffered sea water. Depending on temperature and type of substrate, fluorescence increases are linear for several hours." Otherwise, time series experiments have to be made before starting routine work to check for the period of linear fluorescence increase.

The initial fluorescence reading detects some fluorescence, which is related to model substrate concentrations. This is due to impurities of the model substrates and does not influence the enzymatic reaction. This background fluorescence increases during prolonged storage of model substrate stocks. Heavy turbidity of the probe causes quenching. Centrifugation rather than filtration is recommended to avoid this effect. The endpoint measurement of fluorescence should be made with the same fluorometer-settings as used for the initial measurement, otherwise, factors for correction have to be considered.

Time Series, Continuous Fluorescence Reading

In the case of time-series measurements, subsamples are taken in short intervals from the incubation vessels for fluorescence detection. If incubation takes place in cuvettes, these are repeatedly checked for fluorescence development. Most suitable for this type of measurement, however, is an automatic microtiter plate fluorometer, e.g., the Fluoroskan II fluorometer supplied by Flow Laboratories Ltd. Incubations are made in the holes of the microtiter plates, which allows running many parallel samples. Microtiter plates are scanned automatically for fluorescence at any desired time interval. Temperature control of the microtiter plate chamber is an option of necessity. Microtiter plate fluorometers are not as sensitive as conventional types of fluorometers, and thus, their application is restricted to waters with high enzyme activity.

Calibration

Fluorochromes included in the model substrate are produced by the companies: Sigma, Serva, and Fluka. Molecular weights are 175.2 for AMC and 176.2 for MUF. Fluorochrome standard solutions in water are prepared according to the fluorescence readings of the enzyme

assay, because their concentrations should be in the same range as fluorochrome concentrations produced by model substrate hydrolysis. Suitable concentrations for fluorochrome standard solutions would be 0.1, 1, 5, 10, and 50 μM . Employing the method, standard addition is the best way of calibration. In this case, 20 μl aliquots of a suitable fluorochrome standard solution are carefully mixed with the contents of the cuvette after the last fluorescence reading of the enzyme assay and then measurement is repeated. By standard addition, all problems which may arise including matrix effects, water turbidity, aging of the lamp, etc., are avoided. Alternatively a 100% setting of an appropriate fluorochrome standard solution can also be used for calibration.

Blanks

It is not necessary to correct fluorescence readings for the autofluorescence of the water sample unless it has been proven that autofluorescence changes during incubation. Theoretically, measured fluorescence changes during incubation may also be attributed to reactions other than enzymatic hydrolysis. This can only be tested by boiling the sample water prior to incubation with model substrates. Boiled water samples, in most cases, do not show any changes in fluorescence after incubation, and thus, nonenzymatic hydrolysis appears to be negligible.

Calculations and Interpretation

Fluorescence readings of the replicates (four recommended) at any reading time are averaged. Corrections for autofluorescence or "boiled water" blank are made, if necessary. Fluorescence increases per hour are calculated by regression. Added standard fluorescence increases are also averaged and the velocity of enzymatic hydrolysis is calculated by the equation:

$$V = (A * C * D) / B \quad (1)$$

where V = velocity of hydrolysis ($\mu g C L^{-1} h^{-1}$), in terms of the C content of the organic component hydrolyzed from the model substrate; A = fluorescence increase per hour; B = added fluorochrome standard fluorescence; C = added fluorochrome standard concentration ($\mu M L^{-1}$); D = carbon content of the organic component of the model substrate which is equivalent to 1 μM of fluorochrome. D is 72 $\mu g C \mu M^{-1}$ in cases where the organic component is glucose or a 6-C amino acid.

Depending on the design of the study, this calculation is done for one, two, or more model substrate concentrations used in the enzyme assay. If a series of increasing model substrate concentrations has been applied, individual V -calculations may be used for enzyme kinetic calculations of the Lineweaver-Burk or Eadie-Hofstee type on the basis of the Michaelis-Menten equation.¹² This provides data of V_m (maximum velocity of hydrolysis) and H_t (hydrolysis time of the model substrate). In addition, the Michaelis constant (K_m) can be calculated from experiments where natural competitors of the model substrates were excluded.

The sensitivity of fluorescence detection of the applied fluorochromes is very high. Concentrations and increases of concentrations of these fluorochromes in the nanomolar range can be reliably measured by their fluorescence, if the power supply of the instrument is sufficiently stabilized. The standard error of the method depends on water quality and fluorescence intensity; in most cases it is <5% of the mean value.

Qualitative Determination of Extracellular Enzymatic Properties of Bacterial Isolates

Sample Preparation

The basis for this variation of the method are undefined bacteria colonies or pure culture colonies grown on agar plates. Tracing bacteria colonies for a variety of extracellular enzymatic properties with conventional "specific media" techniques is a laborious and time-consuming task. Using fluorogenic model substrates, a complete analysis can be made within minutes.¹³ Bacterial colonies may be grown on any kind of agar medium. Spread plate techniques are more suitable than pore plate techniques. Colonies should not be grown too big and they should not overlap.

We use this technique for the analysis of saprophytic bacteria colonies growing on conventional ZoBell-agar plates (prepared with fresh water or sea water). In the case of pure culture analysis, an agar plate is inoculated with 16 to 20 bacteria in a defined order, which are subsequently processed simultaneously. The analysis results in percentages of total saprophytes, which are able to hydrolyze given compounds, or in the detection of individual pure-culture enzymatic properties.

Model Substrates

Model substrates are again those which are listed in Table 1. For the quick identification of *E. coli*, there are some fluorogenic model substrates in use which refer to its specific metabolism. These are 4-methylumbelliferyl- β -D-galactoside and 4-methylumbelliferyl- β -D-glucuronide.^{14,15} Commercial media containing the latter substrate (e.g., Bnla-MUG-broth, Merck) are already extensively used for *E. coli* tests and they are on their way to becoming international standards.

Results obtained with model substrates should be comparable with those obtained by conventional agar techniques (e.g., growth characteristics on starch, protein, cellulose, and chitin). Comparative studies, however, are rare. It has been demonstrated, that chitin-agar and MUF-N-acetyl-glucosaminide medium give exactly the same results for all species tested.¹⁶ In the case of other enzyme identifications (e.g., peptidases and esterases), results obtained by the two techniques were the same with the majority of test species.¹³

Preparation and Application of Substrate Solutions

Model substrate stock solutions (5 mM L^{-1}) are prepared in Methylcellosolve (ethyl-ene glycol monomethylether, $\text{C}_3\text{H}_8\text{O}_2$) and stored at -25°C in the dark. Working solutions of 0.1 mM L^{-1} are made before the experiment by the dilution of stock solutions with specific buffers, which create the optimal pH for the desired enzyme reaction. Optimal buffer systems are: Tris/HCl, pH 7.4 for MUF-a-D-glycopyranoside, MUF- β -D-glucopyranoside, Leu-MCA, and MUF-butyrate and Tris/HCl, pH 8.3 for MUF-phosphate and Phosphate/Citrate, pH 4.95 for MUF-N-acetyl-glucosaminide.

Chromatography filter disks of the same size as the Petri dishes used are soaked with model substrate working solutions. The disks are then carefully laid on the bacteria colonies growing on the agar surface. Inclusion of air bubbles has to be avoided. After approximately 3 min of exposure at room temperature the disks are removed from the Petri dishes and placed in a clean and empty Petri dish. For the establishment of optimal pH conditions for fluorescence (pH 10.3) they may be exposed to NH_3 vapors for about 1 min.

Detection of Enzyme Activity

Pretreated filter disks are illuminated with excitation light (MUF-substrates, 364 nm; MCA-substrates, 380 nm) e.g., from an HBO epifluorescence microscope lamp. A conventional UV lamp cannot be used for this purpose; but it can be used for MPN (most probable number) *E. coli* fluorescence detection, e.g., in Brila-MUG-broth.

The whole filter shows a blue fluorescence, due to background fluorescence of model substrates. Sites of enzyme activity, however, can be clearly recognized by their bright fluorescence. Bright spots can easily be coordinated with colonies on the plates and thus, the percentage of total colony counts, which exhibits special extracellular activities, can be calculated. If the plate was inoculated with known species colonies, individual spectra of their enzymatic properties can be estimated. Enzyme spectra of total colonies show characteristic patterns in different habitats. They reflect the prevailing nutrient situation and other environmental conditions.

REFERENCES

1. Hollibaugh, J. T. and Azam, F., Microbial degradation of dissolved proteins in seawater, *Limnol. Oceanogr.*, 28, 1104, 1983.
2. Priest, F. G., *Extracellular Enzymes*, Van Nostrand Reinhold (UK), Wokingham, 1984, 79.
3. Chrst, R. J., Environmental control of the synthesis and activity of aquatic microbial ectoenzymes, in *Microbial Enzymes in Aquatic Environments*, Springer-Verlag, New York, 1991, 29.
4. Meyer-Reil, L.-A., Seasonal and spatial distribution of extracellular enzymatic activities and microbial incorporation of dissolved organic substrates in marine sediments, *Appl. Environ. Microbiol.*, 53, 1748, 1987.
5. Rego, J. V., Billen, G., Fontigny, A., and Somville, M., Free and attached proteolytic activity in water environments, *Mar. Ecol. Prog. Ser.*, 21, 245, 1985.
6. Goulder, R., Extracellular enzyme activity associated with epiphytic microbiota on submerged stems of the reed *Phragmites australis*, *FEMS Microbiol. Ecol.*, 73, 323, 1990.
7. Hoppe, H.-G., Microbial extracellular enzyme activity: a new key parameter in aquatic ecology, in *Microbial Enzymes in Aquatic Environments*, Chrst, R. J., Ed., Springer-Verlag, New York, 1991, 60.
8. Chappell, K. R. and Goulder, R., Epilithic extracellular enzyme activity in acid and calcareous headstreams, *Arch. Hydrobiol.*, 125, 129, 1992.
9. Pettersson, K. and Jansson, M., Determination of phosphatase activity in lake water — a study of methods, *Verh. Int. Verein. Theor. Angew. Limnol.*, 20, 1226, 1978.
10. Jacobsen, T. R. and Rai, H., Determination of aminopeptidase activity in lakewater by a short term kinetic assay and its application in two lakes of differing eutrophication, *Arch. Hydrobiol.*, 113, 359, 1988.
11. Hoppe, H.-G., Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methylumbelliferyl-substrates, *Mar. Biol. Prog. Ser.*, 11, 299, 1983.
12. Armstrong, F. B., *Biochemistry*, 2nd edition, Oxford University Press, New York, 1983, 653.
13. Kim, S. J. and Hoppe, H.-G., Microbial extracellular enzyme detection on agar plates by means of methylumbelliferyl-substrates, in GERBAM — Deuxieme Colloque International de Bactériologie Marine, Actes de Colloque, 3, IFREMER, Brest, 1986, 175.
14. Berg, J. D. and Fiksdal, L., Rapid detection of total and fecal coliforms in water by enzymatic hydrolysis of 4-methylumbelliferyl- β -D-galactoside, *Appl. Environ. Microbiol.*, 54, 2118, 1988.
15. Hofmann, G. and Schwien, U., Eds., Fluoreszenzmikroskopischer Nachweis von *E. coli*, *Grundlagen und Praxis*, GIT-Verlag, Darmstadt, 1989, 74.
16. O'Brien, M. and Colwell, R. R., A rapid test for chitinase activity that uses 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide, *Appl. Environ. Microbiol.*, 53, 1718, 1987.

17. Somville, M. and Billen, G., A method for determining exoproteolytic activity in natural water, *Limnol. Oceanogr.*, 28, 190, 1983.
18. Hoppe, H.-G., Kim, S. J., and Gocke, K., Microbial decomposition in aquatic environments: combined process of extracellular enzyme activity and substrate uptake, *Appl. Environ. Microbiol.*, 54, 784, 1988.
19. Hoppe, H.-G., Schramm, W., and Bacolod, P., Spatial and temporal distribution of pelagic microorganisms and their proteolytic activity over a partly destroyed coral reef, *Mar. Ecol. Prog. Ser.*, 44, 95, 1988.
20. Chróst, R. J. and Krambeck, H. J., Fluorescence correction for measurements of enzyme activity in natural waters using methylumbelliferyl-substrates, *Arch. Hydrobiol.*, 106, 79, 1986.
21. Somville, M., Measurement and study of substrate specificity of exoglucosidase activity in eutrophic water, *Appl. Environ. Microbiol.*, 48, 1181, 1984.
22. King, G. M., Characterization of β -glucosidase activity in intertidal marine sediments, *Appl. Environ. Microbiol.*, 51, 373, 1986.
23. Chróst, R. J., Characterization and significance of β -glucosidase activity in lake water, *Limnol. Oceanogr.*, 34, 660, 1989.
24. Chróst, R. J. and Overbeck, J., Kinetics of alkaline phosphatase and phosphorus availability for phytoplankton and bacterioplankton in lake Plußsee (north German eutrophic lake), *Microbiol. Ecol.*, 13, 229, 1987.
25. Perry, M. J., Alkaline phosphatase activity in subtropical Central North Pacific waters using a sensitive fluorometric method, *Mar. Biol.*, 15, 113, 1972.
26. Shadix, L. C. and Rice, E. W., Evaluation of β -glucuronidase assay for the detection of *Escherichia coli* from environmental waters, *Can. J. Microbiol.*, 37, 908, 1991.