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Proteolytic enzymes in coastal surface seawater: Significant activity of endopeptidases and exopeptidases

Abstract—We assayed proteolytic enzymes in coastal surface seawater using 16 types of fluorogenic substrates, including those for aminopeptidase, trypsin, elastase, and chymotrypsin. Hydrolysis rates were similar or higher for substrates of trypsin and chymotrypsin than for those of aminopeptidase. Substrates for elastase were hardly hydrolyzed. The results strongly suggest trypsin-type and chymotrypsin-type endopeptidases and aminopeptidases were present in the seawater. In most previous studies of proteolytic enzymes in aquatic environments, leucine-aminopeptidase activity measured using a fluorogenic substrate has been used as a model of proteolytic activity. From the results of this study using various peptide analog fluorogenic substrates, the significance of endopeptidases, which could play a key role in downsizing of dissolved proteins and polypeptides to oligopeptides prior to microbial respiration, was confirmed.

One of the largest reservoirs of organic matter on the Earth's surface is dissolved organic matter (DOM) in the sea. During the last several decades, the importance of the role of DOM in marine ecosystems has been recognized and its production, transformation, and removal processes have been investigated.

Microbial extracellular enzymes are of fundamental importance in the hydrolysis of polymeric DOM and particulate organic matter (POM). Enzymatic hydrolysis is thought to affect both the release of DOM from POM (Smith et al. 1992) and the biotic consumption of DOM (Hollibaugh and Azam 1983; Karl and Björkman 2002). Both result in the transformation between DOM and POM or mineralization of DOM in the microbial loop. The activities of hydrolytic enzymes, e.g., glucosidase, protease, lipase, phosphatase, in aquatic ecosystems have been investigated in order to describe the microbial activity and the dynamics of DOM.

Dissolved proteins and peptides may be important sources of energy and nitrogen for marine bacteria. Proteolytic enzyme (protease) should play an important role in the water column. Proteolytic activity has been assessed in various aquatic environments; however, in most of these studies, leucine-aminopeptidase activity measured using fluorogenic substrates has been used as a model of proteolytic enzyme activity (Hoppe 1983; Somville and Billen 1983). In this study, we investigated the types of proteases occurring in seawater using various peptide analog fluorogenic substrates and demonstrated the significant activity of endopeptidases in seawater.

Materials and methods—Surface seawater samples were collected from coastal waters of Matsuyama in the Ehime Prefecture, Japan, eight times between April and November 2003 using a plastic bucket. There was no river inflow near the sampling site. Unfiltered seawater samples were used for protease assays within 5 h after sampling.

Enzymatic activity was assayed using 16 types of peptide-analog methylcoumarylamide substrates (MCA-substrates; Peptide Institute) (Table 1). These peptide analogs included 2 substrates for aminopeptidase, 10 for trypsin, 2 for elastase, and 2 for chymotrypsin. Each substrate was dissolved in dimethyl sulfoxide (superior grade; Wako Chemical) prior to use.

Kinetics experiments using Leu-MCA (substrate for leucine-aminopeptidase) and Boc-Phe-Ser-Arg-MCA (substrate for trypsin) were carried out to determine the appropriate concentration of substrates. The saturation level for both substrates was about 100 μmol L^{-1} (Fig. 1). The apparent half-saturation concentration (K_m) for leucine-aminopeptidase was 16 μmol L^{-1} at 25°C and 21 μmol L^{-1} at 11.5°C, and K_m for trypsin-type enzymes determined using Boc-Phe-Ser-Arg-MCA was 13 μmol L^{-1} at 25°C. These K_m values were measured in samples obtained on 07 April (in situ temperature 11.5°C) for Leu-MCA and on 31 July 2003 for Boc-Phe-Ser-Arg-MCA.

Water samples were dispensed into disposable microtubes containing each substrate solution. The final volume of each mixture was 1 ml. The final substrate concentration was 100 μmol L^{-1} for all experiments except those on 30 September, which had a final concentration of 150 μmol L^{-1}. The mixtures of water sample and substrate solution were incubated at 25°C or at the in situ temperature for 1 h. After the incubation, the fluorescence of hydrolytic product, 7-amino-4-methylcoumarin (AMC), was measured with a spectrofluorometer (Hitachi F-2500) at an excitation/emission wavelength of 380/460 nm. The fluorescence was calibrated using the slope of a calibration curve obtained from several concentrations of AMC. Controls were prepared with autoclaved filtered seawater collected from the same sample. The controls were assayed using the same method in order to define the non–enzymatic-produced AMC. The hydrolysis rate of substrate was determined after subtracting both the blank fluorescence intensity of seawater and concentration of non–enzymatic-produced AMC from the concentration of AMC in the reactor. The hydrolyzed rate of substrate, namely the enzymatic activity, was calculated for nanomoles of substrate per hour per liter of sample seawater (nmol L^{-1} h^{-1}). This method identifies only the cleavage at the bond

Table 1. List of substrates used in this study.

Name	Substrate for	MW	Compound
Arg-MCA	Aminopeptidase	331.37	L-Arginine MCA
Leu-MCA	Aminopeptidase	288.35	L-Leucine MCA
Bz-Arg-MCA	Trypsin	435.48	Benzoyl-L-argine MCA
Z-Phe-Arg-MCA	Trypsin	612.69	Carbobenzoxy-L-phenylalanyl-L-arginine MCA
Glt-Gly-Arg-MCA	Trypsin	502.53	Glutaryl-glycyl-L-arginine MCA
Boc-Leu-Gly-Arg-MCA	Trypsin	601.70	*t*-Butyloxycarbonyl-L-leucyl-glycyl-L-arginine MCA
Boc-Leu-Thr-Arg-MCA	Trypsin	645.76	*t*-Butyloxycarbonyl-L-leucyl-L-threonyl-L-arginine MCA
Boc-Phe-Ser-Arg-MCA	Trypsin	665.75	*t*-Butyloxycarbonyl-L-phenylalanyl-L-seryl-L-arginine MCA
Boc-Val-Pro-Arg-MCA	Trypsin	627.74	*t*-Butyloxycarbonyl-L-valyl-L-prolyl-L-arginine MCA
Boc-Leu-Ser-Thr-Arg-MCA	Trypsin	732.83	*t*-Butyloxycarbonyl-L-leucyl-L-seryl-L-threonyl-L-arginine MCA
Boc-Val-Leu-Lys-MCA	Trypsin	615.77	*t*-Butyloxycarbonyl-L-valyl-L-leucyl-L-lysine MCA
Boc-Glu-Lys-Lys-MCA	Trypsin	660.77	*t*-Butyloxycarbonyl-L-glutamyl-L-lysyl-L-lysine MCA
Suc-Ala-Ala-Ala-MCA	Elastase	488.50	Succinyl-L-alanyl-L-alanyl-L-alanine MCA
Suc-Ala-Pro-Ala-MCA	Elastase	514.54	Succinyl-L-alanyl-L-prolyl-L-alanine MCA
Suc-Ala-Ala-Pro-Phe-MCA	Chymotrypsin	661.71	Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine MCA
Suc-Leu-Leu-Val-Tyr-MCA	Chymotrypsin	763.89	Succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine MCA

MW, Molecular weight; MCA, 4-methyl-coumalyl-7-amide.

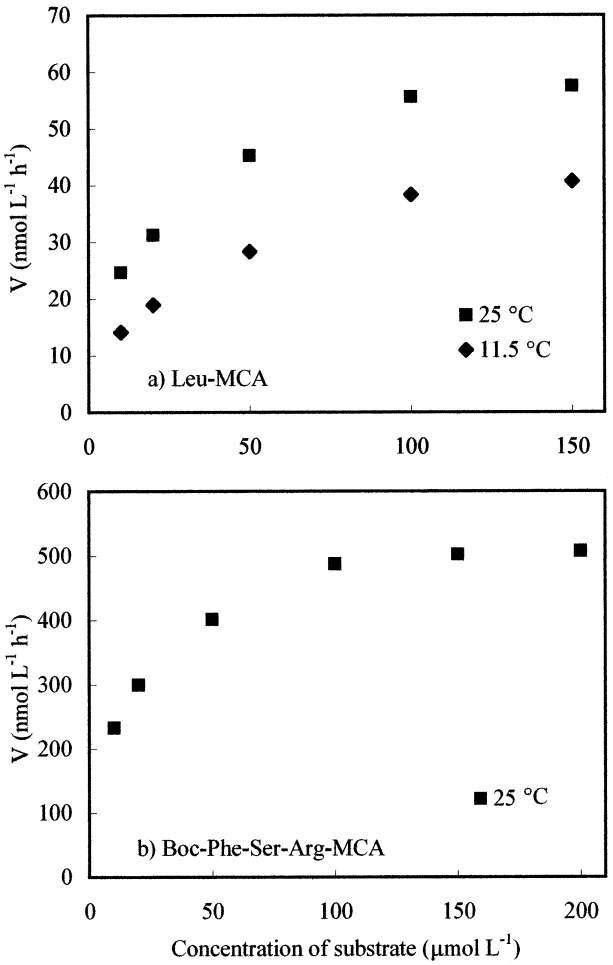


Fig. 1. Enzyme kinetics of (a) Leu-MCA at 25°C and 11.5°C for the sample obtained on 07 April 2003 and (b) Boc-Phe-Ser-Arg-MCA at 25°C for the sample obtained on 31 July 2003.

adjacent to the AMC moiety and cannot detect the hydrolysis of other peptide bonds. However, in this study, we defined the hydrolysis rate of each substrate as an enzymatic production rate of free AMC from the substrate.

Although it is better to use duplicates or triplicates to assess the variability associated with the hydrolysis measurements, the incubation tube was single for each substrate in this study because the number of substrates was too large to be measured in duplicate with the timeframe of the experiments. Coefficients of variation (CV) for hydrolysis rate of substrates estimated based on separate experiment were 2–7%.

As we know, we cannot assess the true in situ activity of enzymes in seawater. The activity estimated here is a potential activity, which was measured by addition of an artificial substrate. The in situ activity of enzymes may be regulated by the situation of both enzymes and substrates and the temperature in seawater. We intended to discuss the variation of factors related to the enzyme itself by comparing the potential activities at the same temperature (25°C). On the other hand, in terms of estimation of true in situ activity, measurement at in situ temperature is more appropriate than at a fixed temperature. In this study, hydrolysis rates were also estimated at the in situ temperature for selected samples, in addition to the measurement at 25°C for all samples.

Results and discussion—Among the peptide analog substrates added to natural seawater, substrates for aminopeptidase, trypsin, and chymotrypsin were hydrolyzed, while substrates for elastase were hardly hydrolyzed (Fig. 2). Most studies to date have assessed the proteolytic activity in natural seawater or freshwater by measuring leucine-aminopeptidase activity as a model enzyme using a fluorogenic substrate such as Leu-MCA. Carboxypeptidase activity in seawater has also been reported (Hashimoto et al. 1985). Figure 2 strongly suggests that aminopeptidases and trypsin-type and chymotrypsin-type enzymes were present in seawater. Aminopeptidase and carboxypeptidase are exopeptidases, which hydrolyze the peptide bond adjacent to the

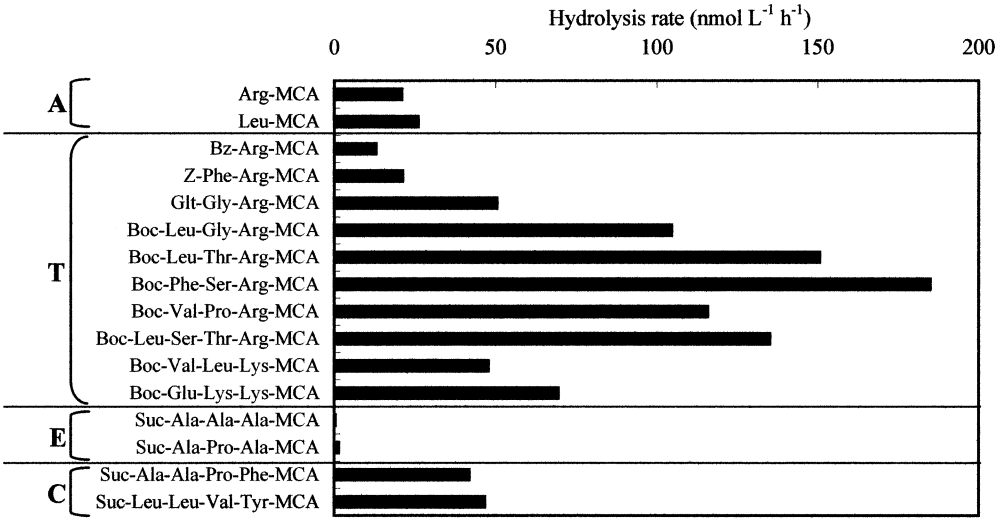
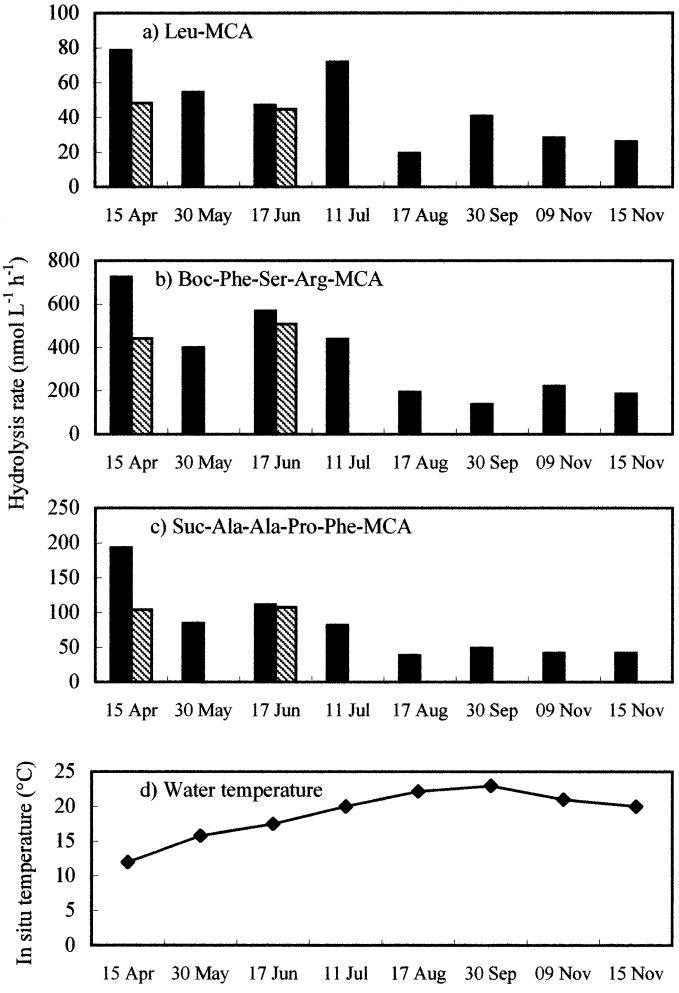


Fig. 2. Hydrolysis rate at 25°C of each substrate added to the seawater sample on 15 November 2003. A, Substrates for aminopeptidase; T, substrates for trypsin; E, substrates for elastase; C, substrates for chymotrypsin.



N-terminal and C-terminal amino acid, respectively. On the other hand, trypsin and chymotrypsin are endopeptidases, which are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain. The significant activities of trypsin-type and chymotrypsin-type endopeptidases in natural seawater probably play a key role in the downsizing of dissolved proteins and polypeptides to oligopeptides.

Figure 3 shows the hydrolysis rates of three substrates in seawater measured eight times between April and November. Leu-MCA, Boc-Phe-Ser-Arg-MCA, and Suc-Ala-Ala-Pro-Phe-MCA are substrates for leucine-aminopeptidase, trypsin, and chymotrypsin, respectively. The hydrolysis rates of the substrates for trypsin and those for chymotrypsin, namely the activities of trypsin-type and chymotrypsin-type enzymes, varied similarly during the observation. For all three types of enzymes, the potential activity measured at 25°C was higher between April and July than between August and November (Student t-test; $p < 0.05$). Variation of potential activities estimated at 25°C might directly reflect changes of the amount of enzymes in seawater.

The activities estimated at the in situ temperature (12.0°C) on 15 April 2003 were 54–61% of those estimated at 25°C and were still higher than the estimated activities in autumn (August–November). On 17 June 2003, the activities estimated at the in situ temperature (17.5°C) were 89–97% of those estimated at 25°C. From July to November, the activity

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Fig. 3. Hydrolysis rate of (a) Leu-MCA (substrate for leucine-aminopeptidase), (b) Boc-Phe-Ser-Arg-MCA (substrate for trypsin), and (c) Suc-Ala-Ala-Pro-Phe-MCA (substrate for chymotrypsin), measured eight times between April and November 2003. Solid and shaded bars show hydrolysis rate at 25°C and at in situ temperatures, respectively. Hydrolysis rates at in situ temperatures were measured on 15 April and 17 June 2003. (d) The in situ water temperature during sampling.

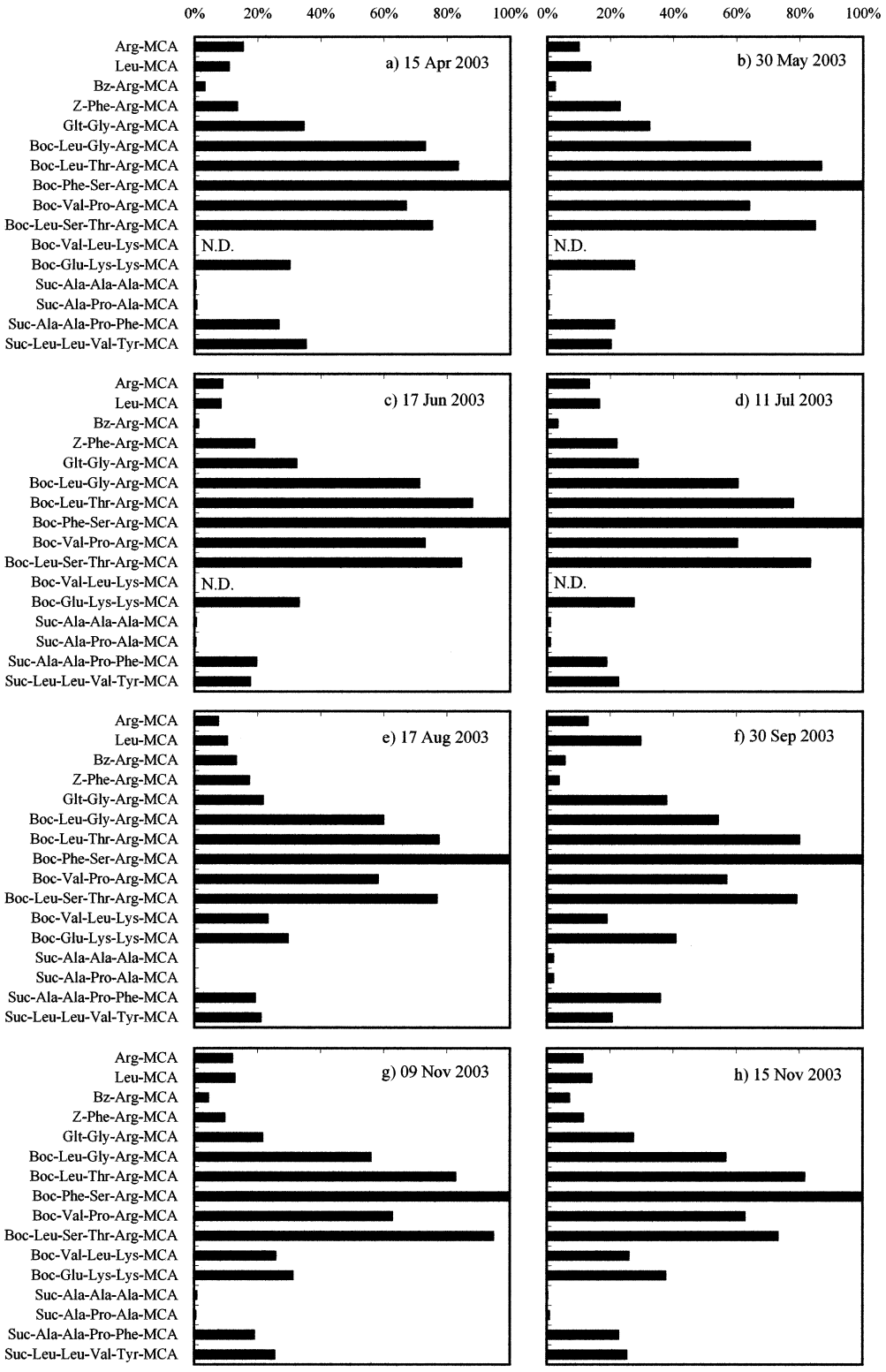

Fig. 4. Relative hydrolysis rate of each substrate expressed as percent of the substrate with maximum hydrolysis rate. Hydrolysis rate of Boc-Val-Leu-Lys-MCA on 15 April, 30 May, 17 June, and 11 July were not determined.

at the in situ temperature was not measured; however, because the water temperature was closer to 25°C (Fig. 3d) than that on 17 June, it is thought to be more than ~90% of the rates at 25°C. Taking account of the water temperature, a range of changes in activities at the in situ temperature during the observation period (from April to November) should be narrower than those of potential activity measured at 25°C.

During the eight observations between April and November, the activities of enzymes varied (Fig. 2); however, the features of substrate specificity for hydrolysis were common. At each observation, Boc-Phe-Ser-Arg-MCA (substrate for trypsin) showed the highest hydrolysis rates among the tested substrates. Relative hydrolysis rates of each substrate normalized by the hydrolysis rate of the substrate with the fastest hydrolysis rate are shown in Fig. 4. There are two aspects of the difference of hydrolysis rates among substrates: one is the type of enzyme and the other is the structure of the peptide. Pantoja et al. (1997) reported that hydrolysis of the longer peptides, LYA-ala$_4$, was faster than those of LYA-ala$_2$ and LYA-ala-leu. In Fig. 4, substrates for trypsin with more than three amino acids were hydrolyzed faster than substrates for aminopeptidase and the hydrolysis of substrates for chymotrypsin was usually about twice as fast as the activity of aminopeptidase. Among the 10 substrates for trypsin, peptides of similar lengths were also hydrolyzed at different rates, indicating that peptide length was not the sole variable driving these differences. It remains unclear whether this pattern reflects substrate specificity for a single trypsin-like enzyme or whether it is due to a mixture of trypsin-trypsin-like enzymes.

In aquatic ecosystems, contemporary DOM of high molecular weight has been shown to be efficiently utilized by bacteria (Amon and Benner 1994). The efficient utilization of high-molecular-weight DOM indicates the importance of bacterial extracellular enzymes because bacteria can take up molecules only smaller than ~600 Da. This enzyme pool should include both endo- and exohydrolases. The importance of endohydrolases has been pointed out (e.g., Hoppe et al. 2002); the cleave polymers into oligomelic compounds to suitable size for bacterial uptake or prior to generate monomers by exohydrolases. There are some studies focused on degradation of peptides or proteins in aquatic environment using LYA derivatives (Pantoja et al. 1997) and radiolabeled or fluorescent-labeled protein substrates (Hollibaugh and Azam 1983; Debroas 1999). The results of this study present evidence of trypsin-type and chymotrypsin-type endopeptidases in natural seawater, although the source organisms of these enzymes in the seawater remain unclear. The method using a series of substrates that are specific for endopeptidase provide a useful technique for assessing extracellular activity and for providing information about the degradation of DOM in the environment.

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