Hydrogen peroxide determination in estuarine and marine waters by flow injection with fluorescence detection

Hydrogen Peroxide (H_2O_2)
Flow injection
Fluorescence detection
Estuarine and marine waters

Peroxyde d'hydrogène (H_2O_2)
Injection à flux continu
Détection par fluorescence
Eaux marines et estuariennes

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ABSTRACT

The Scopoletin-Peroxidase fluorescence decay method has been developed using standard additions and a flow injection manifold for hydrogen peroxide (H_2O_2) determination in marine waters. The limit of detection of the method was optimized using stored milli-Q water with a minimum of 5 nM (3σ). The method was found to be linear from 5 to 700 nM, with an approximate relative standard deviation of 10 %. This accuracy was verified on both milli-Q and natural waters running systematically duplicate analysis.
The validity and the sensitivity of the technique was studied through the influence of pH, salinity and the dissolved organic matter (DOM) concentration. Standard additions avoided all matrix and experimental interferences, making it possible to use this method under any field conditions and on "humic-rich" waters. Additionally, the flow injection manifold improved the accuracy and the runtime of the analytical procedure and simplified the field manipulation

RÉSUMÉ

Détermination du peroxyde d'hydrogène dans les eaux marines et estuariennes par injection à flux continu et détection par fluorescence.

La méthode Scopoletine-Peroxydase d'extinction de fluorescence a été développée en utilisant des ajouts dosés et un système d'injection à flux continu pour la détermination du peroxyde d'hydrogène (H_2O_2) dans les eaux marines. La limite de détection de la méthode a été optimisée en utilisant de l'eau Milli-Q ultra pure stockée avec un minimum de 5 nM (3σ). La méthode est linéaire de 5 à 700 nM avec un écart-type relatif autour de 10 %. Cette précision a été vérifiée autant pour l'eau Milli-Q que pour les eaux naturelles en pratiquant systématiquement des analyses dupliquées. La validité et la sensibilité de la technique ont été étudiées au travers de l'influence du pH, de la salinité et de la concentration en Matière Organique Dissoute (MOD). Les ajouts dosés ont éliminé toutes les interférences de matrices ou expérimentales permettant l'utilisation de cette méthode dans des conditions de terrain variables et dans des eaux riches en matière humique. De plus, le système d'injection à flux continu a amélioré précision et durée de la procédure analytique et simplifié les manipulations sur le terrain.

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INTRODUCTION

The determination of hydrogen peroxide (H_2O_2) in environmental waters has received only little attention in marine sciences since the initial report of its occurrence in marine waters by Van Baalen and Marler (1966). However, H_2O_2 is ubiquitous and has been reported in a large variety of environmental samples, including marine (Zika *et al.*, 1985 *a, b*; Palenik and Morel, 1988; Johnson *et al.*, 1989; Weller and Schrems, 1993 ; Moore *et al.*, 1993) and continental waters (Cooper and Lean, 1989). It has also been detected in marine atmospheric liquid and gas phases (Cooper *et al.*, 1987; Weller and Schrems, 1993). Most studies relate the occurrence of H_2O_2 to photochemical processes taking place in the waters (Cooper and Zika, 1983; Cooper *et al.*, 1988; Cooper *et al.*, 1989; Zika, 1990). It is mainly generated as a secondary product resulting from the photo-reduction of dissolved oxygen (O_2) into the superoxide anion (O_2-).This reduction step is thought to be the consequence of an electron transfer from an excited photoreactive chromophore (triplet state) usually associated with the dissolved organic matter (DOM) pool. It also can be formed via photo-oxidation of reactive biogenic organic substances such as amino-acids and flavins (Draper and Crosby, 1983; Mopper and Zika, 1987). Finally, a few reports have also mentioned H_2O_2 as resulting from the development of biological activity (Stevens *et al.*, 1973; Palenik *et al.*, 1987; Zepp *et al.*, 1987; Palenik and Morel, 1988). Despite the limited number of studies performed to date, the understanding of the formation and fate of H_2O_2 is of considerable importance, since its occurrence in marine waters will indirectly control many chemical processes occurring *via* the induction of redox reactions. Redox reactions play a significant role in oceanic processes and specifically affect the cycling of organic and inorganic compounds in the ocean. Redox potentials involving H_2O_2 are high (O_2/H_2O_2 = 0.68 V, HO_2/H_2O_2 = 1.5 V, H_2O_2/H_2O = 1.78 V) and are therefore likely to play a significant role with redox speciation of trace elements in sea water (Zafiriou *et al.*, 1984; Millero and Sotolongo, 1989).

Different reports on H_2O_2 concentrations in sea water describe concentrations ranging between 0 and 400 nM, mostly in surface oceanic waters. H_2O_2 is a transient species due to reaction with other compounds in water, where its relative life time has been estimated to range from a few hours to three days. This period is mainly dependent on the concentration of organic matter present in the waters (Zika *et al.*, 1985*a*; Sikorski and Zika, 1993). H_2O_2 reactivity with organic matter has been fairly well described under light irradiation. Photochemically produced of "activated oxygen" species (OH, $^1O_2$, O_2-, H_2O_2) are reported to be an important sink of many organic substances (natural or pollutants) in surface sea waters (Draper and Crosby, 1981; Momzikoff *et al.*, 1983; Cooper *et al.*, 1989; Mopper and Zhou, 1990). Because its longer life time in comparison with other transient photochemical species, H_2O_2 is accumulated in natural waters under sunlight radiation. This allows it to be considered as a relative indicator of the photochemical reactivity in sea water (Zika, 1990). Such is particularly the case when dealing with photo oxidation processes (Zafiriou *et al.*, 1984).

In general, the determination of H_2O_2 concentrations in natural waters involves mainly either absorbance or fluorescence spectrometry. Previous papers on the subject (Kieber and Helz, 1986; Holm *et al.*, 1987; Miller and Kester, 1988) show that H_2O_2 measurements in natural waters must be carried out and interpreted carefully. These recent analytical developments for different techniques demonstrate that calibration and blank problems need to be solved. A peroxidase enzyme is used to catalyse the reaction of oxidation by H_2O_2 to quench or produce a measured absorbance or fluorescence signal from organic chromophores (phenols, anilines). The presence of organic peroxides, particularly light monoalkyl peroxides such as methyl peroxide (MeOOH), can generate interfering reactions with the peroxidase enzyme in competition with H_2O_2. Zika *et al.*, (1985 *a,b*) found the occurrence of organic peroxides in oceanic and coastal waters to be negligible in comparison with the concentrations found for H_2O_2. In comparison with methods based on absorbance detection, fluorometric methods are much more sensitive and selective. Limits of detection are in the few nanomoles range where the determination of sea water concentrations is concerned (Zika and Saltzman, 1982; Hwang and Dasgupta, 1986; Holm *et al.*, 1987; Miller and Kester, 1988). The analysis based on the following reactions:

$$\underset{\text{(fluorescent)}}{\text{Scopoletin} + H_2O_2} \xrightarrow{\text{Peroxidase}} \underset{\text{(non-fluorescent)}}{\text{"Scopoletin"}^{ox} + 2H_2O} \quad (1)$$

Scopoletin is a naturally occurring compound with a high fluorescence quantum yield. It is a coumarin derivative with a hydroxy group substituted on the phenyl ring which is then used as a specific hydrogen donor. Under the peroxidase activity, H_2O_2 reacts by oxidation with the phenolic group, inhibiting the fluorescent properties of scopoletin. Most techniques have been developed on open ocean water masses with low organic matter content. However, in the presence of high organic matter content, parts of the analytical scheme may be altered by competitive reactions yielding errors in the analytical results.

In this paper, we describe a method adapted from the fluorescence decay scopoletin-peroxidase approach developed by Zika and Saltzman (1982) or Holm *et al.* (1987) for the determination of H_2O_2 in various marine environments. This technique uses a flow injection manifold which permits faster and more reproducible analysis (Hwang and Dasgupta, 1986; Johnson *et al.*, 1987). We discuss the calibration and interference problems that may occur when the analytical method is used for very different matrices such as estuarine waters with high variation of DOM or open ocean waters with a very low organic matter load.

MATERIAL AND METHODS

Reagents

Millipore milli-Q water was used for all reagent preparations. It was also used for blank measurements under specific conditions. Careful cleaning of all glassware, polymer containers and bottles and polycarbonate filtration

apparatus is important. The cleaning procedure involves two steps. A detergent (RBS 50) is first used with hot tap water to remove bacteria and organic matter. Vessels are then flushed with tap water and milli-Q water. All vials and bottles are always controlled for organic matter impurities by molecular fluorescence emission measurements using different excitation wavelengths (250, 280, 313, 340 and 370 nm).

Synthetic sea water was made up for the interference experiments following the standard procedure of Kalle (see Annex In: Chemical Oceanography, J.P. Riley and Chester R. Ed., Vol. 1) for preparation. The major cations and anions were retained (Na^+, Cl^-, Mg^{2+}, SO_4^{2-}) and the solution was prepared with NaCl, $MgCl_2$, and $MgSO_4$ pure salts (Aldrich). Natural extracted reference fulvic acids (Contech, Canada) were also used for this study to simulate DOM interactions.

Analytical reagents for H_2O_2 determination

A scopoletin (7-hydroxy-6-methoxy-2H-1-benzopyran-2-one) stock solution of 2.5 mg.dm^{-3} is obtained by diluting crystallized scopoletin (Sigma Chimie) with milli-Q water. This solution remains stable for a few months if stored under dark refrigeration (4 °C). Crystallised Horse Radish Peroxidase (HRP Type II, Sigma Chimie) is used as a peroxidase enzyme. This chemical degrades rapidly and needs to be carefully kept frozen below -10 °C. Working solutions need to be prepared within less than 8 hours prior to use. The HRP solution is obtained by diluting 50 mg of HRP and 20 mg of sodium triazine (NaN_3, Aldrich) in 50 cm^3 of pH 7 phosphate buffer solution (K_2PO_3, Prolabo). The role of the buffer is to stabilize and optimize peroxidase efficiency during the determination procedure (Zika and Saltzman, 1982; Holm *et al.*, 1987). Finally, a small aliquot of NaN_3 is added to protect the HRP solution from bacterial degradation.

Standard additions of H_2O_2 are made with calibrated solutions. These solutions are prepared from initial commercial standard solutions of H_2O_2 3 % w.t. (Aldrich) diluted twenty times in milli-Q water to prepare a stock solution around 0.04 M. This concentrated solution is stable for a few months when stored under dark refrigeration. However, it needs to be regularly titrated prior use for accuracy in acidic medium (H_2SO_4) by a standard $KMnO_4$ solution. Later, these titrated H_2O_2 mother solutions need to be diluted ten thousand times every hour during field analysis to avoid potential inaccuracy of the results.

H_2O_2 blank determinations are always a problem. Fresh milli-Q water usually contents from nmoles up to µmoles of H_2O_2 and is therefore not directly suitable for blank determination at nmole levels in the environment. The catalase enzyme (Sigma Chimie) can then be used for blank study. When used under specific conditions, this enzyme can remove the H_2O_2 amount present in an sample in a few minutes (Lazrus *et al.*, 1985; Kok *et al.*, 1986). Blank determinations will be discussed later.

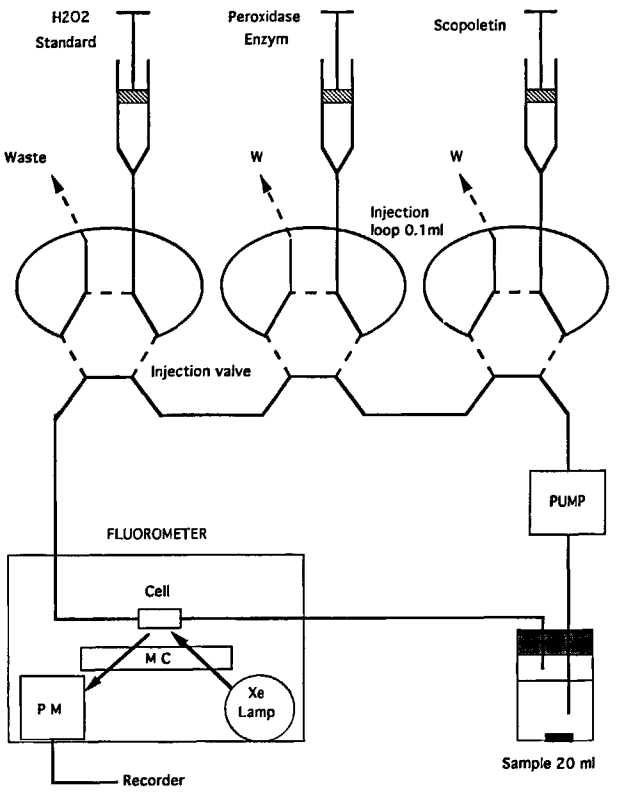

Figure 1

Flow injection manifold for H_2O_2 determination. The injection valves are on the loop-filling position (solid lines). When they are successively on the injection positions the injection loop is integrated in the system-loop (dashed lines). MC : excitation and emission monochromator, PM : Photomultiplier.

Apparatus

The small "ship-board" flow injection manifold is presented in detail on Figure 1. The principle of the apparatus involves operation on a closed reagent-injection flow system. The reagents are injected in a closed loop of constant volume, unlike most of the flow injection manifolds. Three 5 cm^3 PE syringes are used to fill the three different injection loops (100 mm^3) with the different reagents. These different injection loops are controlled by manual six-way valves (Rheodyne, Teflon FEP). All tubing and fitting are in Teflon FEP (0.8 mm I.D.) except for the pumping tube of the peristaltic pump which is made of Tygon (1.6 mm I.D.). Fluorescence spectroscopy detection for H_2O_2 determination was performed with a compact spectrofluorometer (LS 30 Perkin Elmer). Its high sensitivity is due to its miniaturized flowcell (7 mm^3) and powerful irradiation xenon discharge lamp (20 kW/ 8 µs), and to large fixed 10 nm spectral bandpass for both monochromators (excitation and emission). Fluorescence intensities were measured at fixed wavelengths optimized for scopoletin detection : 345 and 465 nm respectively for excitation and emission.

H_2O_2 determination procedure

Freshly prepared reagents are introduced in their respective 5 cm^3 syringes when starting the analytical scheme.

The remainder of the solutions is stored in a dark refrigerated place (cold box or refrigerator) in the meantime and only used when the syringes need to be refilled.

The analytical scheme is as follows. Sea water samples collected are first filtered on 0.45 µm pre-combusted GF/F filters with very gentle depression to avoid cell disruption using a Millipore filtration unit with manual vacuum pump. A volume of 20 cm^3 of the filtered sea water sample is introduced in the mixing reactor and the system loop is closed. The sample is then continuously circulated in the close loop system by a peristaltic pump delivering a continuous flow of 5 cm^3/min. In the reactor cell, the sample is mixed with a Teflon magnetic stirrer. This stirring action was found to enhance reaction rate and improve the reproducibility of the system.

After the sample introduction, the first injection valve introduces 100 mm^3 of the scopoletin solution which is rapidly mixed with the sample within less than one minute. The fluorescence signal is recorded after 1 to 2 min when constant. Then, 100 mm^3 of the HRP solution is added. In the presence of the enzyme, H_2O_2 in the sample reacts with the scopoletin and this reaction lead to a fluorescent intensity decay. After 1 to 2 min, this fluorescence quenching is stabilized and the intensity is recorded. The final step involves sequential introduction of calibrated H_2O_2 aliquots for standard addition. Two successive additions of 100 mm^3 H_2O_2 standard solutions and successive reduction of the fluorescence signal permit the elimination of matrix and experimental influence (2-4 min). This internal calibration procedure gives a standard addition slope which is then used to calculate the H_2O_2 concentration following the method described by Holm *et al.* (1987).

Between two analyses, the system loop is rinsed first with week-old milli-Q water of very low H_2O_2 content and with the next sample to analyse prior to its analysis.

All analyses are always performed in duplicate. A complete run including two determinations by standard addition takes approximately ten minutes. For each data set, we are then able to calculate a mean standard deviation based on the repeatability of the technique for a given period or experimental conditions. Such an approach avoids erratic determinations.

Sample collection and processing

Estuarine and marine waters were sampled along the estuarine gradient of the Gironde estuary (France), on the continental shelf in the Bay of Biscay and in the upper water column. During ECOFER 4 cruise (30/04-14/05/1991), on the R/V *Le Suroît* (IFREMER), water samples were collected with 8 dm^3 Niskin samplers (General Oceanic, USA) pre-rinsed with sea water flushing. During PhotoBiol cruises (23-25/09/1992, 15-16/12/1992, 16-17/05/1993), on the R/V *Côte d'Aquitaine* (CNRS), estuarine waters were collected with an acid-cleaned trace metal sampler (Nereides, France). Hydrological parameters were recorded with a multiparameter probe (Sea-Bird Electronics, USA) or a manual thermo-salinometer (Jenco). The samples were filtered under clean conditions on pyrolized 0.45 µm GF/F filters (Whatman). Filtered waters for H_2O_2 analysis were kept at room temperature (18-22 °C) in dark bottles (Nalgene PE 250 cm^3) for a maximum of 2 hours prior to ship-board determination. Dissolved organic carbon (DOC) samples were stored and analysed following the classical wet oxidation method (Menzel and Vaccaro, 1964; Cauwet, 1975).

RESULTS AND DISCUSSION

Analytical developments

Samples collected along the estuarine gradient and in the open ocean presented a large range of DOM content. These DOM concentrations could in all likelihood interfere during the H_2O_2 determination steps. Several authors have shown that these analyses must be performed with great care to avoid matrix interferences (Kieber and Helz, 1986; Holm *et al.*, 1987; Miller and Kester, 1988).

Non-stoichiometry, calibration and reproducibility

The standard addition method is usually applied to solve most of the problems related to unstable stoichiometry. Stoichiometry as understood here as relating to the number of moles of scopoletin reacting with one mole of H_2O_2. The H_2O_2 concentration in the sample is obtained after calculation of the fluorescence signal difference (FSD) between the fluorescence intensities (FI) prior to and after addition of HRP reagent:

$$FSD = FIscop - FIperox. \quad (2)$$

The H_2O_2 standard addition generates a curve that can be fitted with a linear regression. The slope is negative (-s) and is expressed in arbitrary fluorescence intensity units per mole of H_2O_2. The concentration of H_2O_2 in the sample is then determined by the ratio:

$$[H_2O_2] = FSD / s. \quad (3)$$

Standard additions have to be done with care because when the scopoletin signal extinction reaches an intensity amounting to half that of the initial signal, the reaction may become non-linear. Combination between scopoletin concentrations and the attenuation factor of the fluorometer allowed to obtain a linear concentration ranging between 5 and 700 nM. The use of a flow injection manifold resulted in simple and accurate successive additions of reagents. This modification directly translated into considerable improvement of the repeatability.

The reproducibility of the measurements has been estimated in waters of different DOM content (estuarine and marine samples). Figure 2 illustrates the variation in sensitivity recorded with different water matrix (duplicate measurements) performed under the same conditions within two days. The standard addition linear regression slopes for an estuarine sample (salinity 2 $g.dm^{-3}$, $[H_2O_2]$ = 148 ± 12 nM) and a marine sample (35 $g.dm^{-3}$,

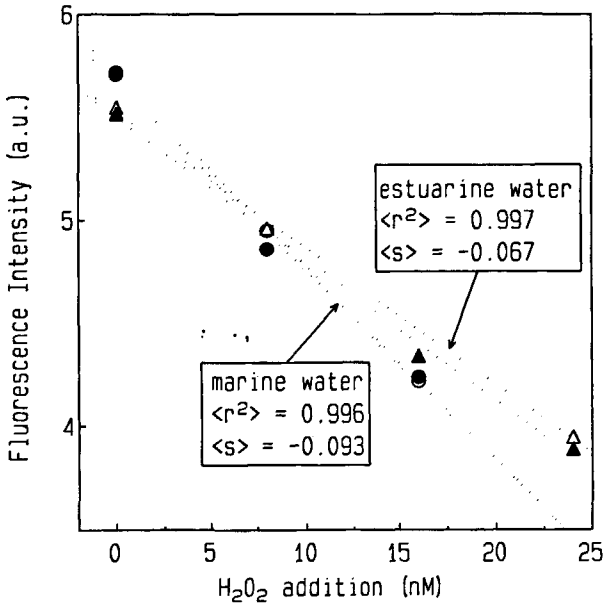

Figure 2

Comparison of linear regression standard addition slopes between 2 natural samples. Estuarine sample (3/5/91): depth 4 m, salinity 1.8 g.dm^{-3}, D.O.C. 1.5 mg.dm^{-3}, [H_2O_2] 148 nM. Marine sample (5/5/91): depth 120 m, salinity 35.6 g.dm^{-3}, D.O.C. 0.64 mg.dm^{-3}, [H2O2] 104 nM.

104 ± 6 nM) are compared. For both samples, the linearity is excellent ($r^2 > 0.99$) and the reproducibility high (5 %). The standard addition slopes are slightly different : –0.067 and –0.093 (fluorescence arbitrary unit/nM H_2O_2 added) respectively for the estuarine (DOC content = 1.5 mg dm^{-3}) and the marine water (DOC content = 0.64 mg dm^{-3}). Slope variations could be related to competition reactions during the determination process, due to the different content of DOC in the waters studied. H_2O_2 is a strong oxidant for many compounds in water, such as organic substances or metal ionic species (Zafiriou *et al.*, 1984; Zika, 1990). These interactions and their influence on the quality of the measurements will be discussed later.

Blank determination and limit of detection (LOD)

By definition, the LOD represents three times the blank standard deviation on a series of blank measurements. The standard deviation of the blank is directly dependent on the repeatability of the matrix and of the background concentration. The use of milli-Q water permits the removal of many interfering substances but this matrix contains significant concentrations of H_2O_2 reaching up to 100 nM. In general, the catalase enzyme is used for preparing H_2O_2 - free water (Lazrus *et al.*, 1985; Kok *et al.*, 1986) to yield low blank values. However, the use of catalase will generate interference reactions that modify the stoichiometry of the fluorescence quenching through competition with HRP. Therefore, the different detection limits were evaluated without the use of catalase. They were estimated in either fresh milli-Q water or milli-Q that had been stored for one week or more in a clean container (10 dm^3 PP, Nalgene). During the determination procedure, a first set of detection limits was evaluated after mixing the chemicals in fresh milli-Q water containing natural levels of H_2O_2 (experimental set A and B) or milli-Q water already presenting low levels of H_2O_2 after at least one week of storage (experimental set C). The results are presented in Table 1. The LOD obtained ranged between 5 and 18 nM. These values are in agreement with the LOD previously reported with the scopoletin-peroxidase method (Zika and Saltzman, 1982; Holm *et al.*, 1987). The highest blank values are obtained with the fresh milli-Q water. When the milli-Q water is stored for more than one week, the H_2O_2 concentrations decrease from 41 to 9 nM. The lowest LOD (5 nM) is obtained in the C type experiment, where the determination was made on aged milli-Q water and where the reagents have also been prepared from the same low H_2O_2 content water. Aged milli-Q water was finally retained as the best blank estimation due to its simple matrix and good reproducibility during the determinations.

Table 1

Blank determination and limit of detection (LOD). (n : number of replicates, Rel.Std.Dev. : Relative Standard Deviation for all replicates.)

Samples		Average [H_2O_2] (nM)	Rel.Std.Dev. (%)	LOD (nM)
A	fresh milli-Q water (n=4)	41	15	18
B	stored milli-Q water (n=8)	9	25	7
C	stored milli-Q water (n=6)	24	7	5

Interferences

The effect of the different parameters likely to influence the sensitivity and precision of the analysis were investigated. Relative sensitivity is defined here as the ratio of the sample standard addition slope to the "blank" standard addition slope (s/s_0). The "blank" standard addition slope is given with aged milli-Q water analysed in the same experimental conditions for a set of measurements. Three parameters were studied : pH; the salinity (ionic strength); and the DOM concentration (organic matter reactivity). The effect of temperature was omitted. Both laboratory experiments and field measurements have shown that no significant trend could be related to any temperature effect. All measurements were made in the 18 to 22 °C temperature range. The variations of the different factors ranged within the limits found in natural waters. All the measurements were done in duplicate. The mean relative standard deviation for all the measured sensitivities was less than 5 %.

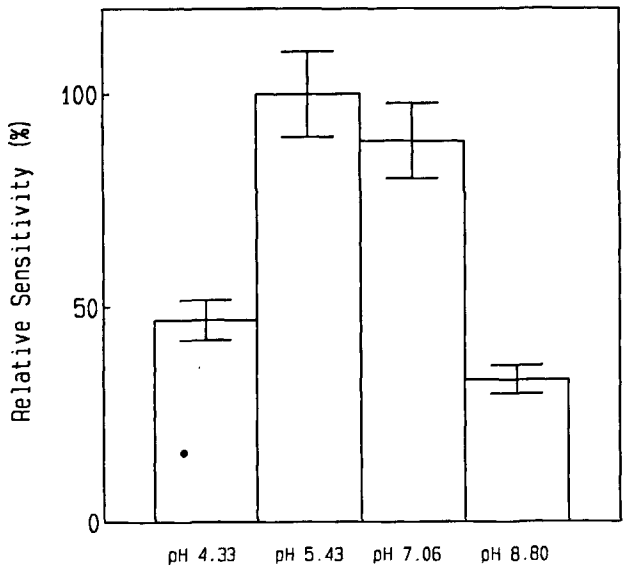

Figure 3

Relative sensitivity (s/s_0) versus *pH (error bars : estimated relative standard deviation 10 %).*

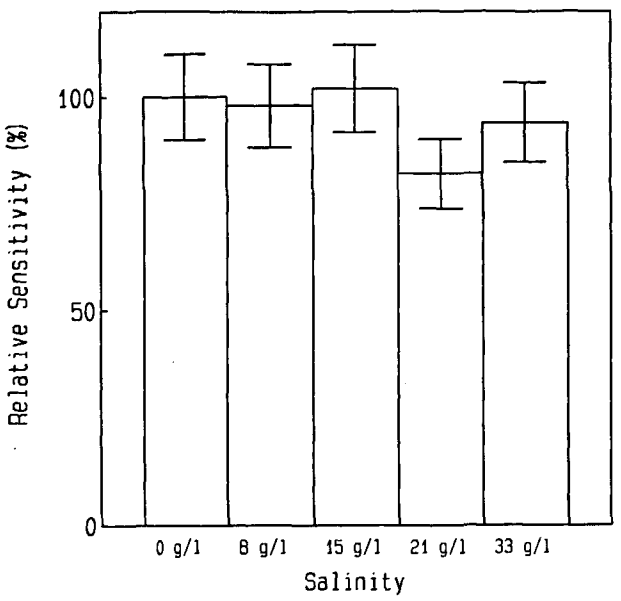

Figure 4

Relative sensitivity (s/s_0) versus *salinity (error bars : estimated relative standard deviation 10 %).*

This leads to determination of the relative sensitivities with a twice relative standard deviation of 10 %.

Influence of pH

The role of pH on H_2O_2 determination was studied with standard buffer solutions (100 mm^3, Prolabo) in Milli-Q to obtain the following values : 4.33, 7.06 and 8.80 (pH 5.43 was that of the pure milli-Q water). The results are expressed in relative sensitivity in term of H_2O_2 standard addition slope (s/s_0 : % normalized to the "blank" result estimated as 100 %). Results are presented in Figure 3. They illustrate that the scopoletin-peroxidase method is very sensitive to pH. The best results obtained for the relative sensitivity were procured by milli-Q water alone at pH 5.43. Both experiments performed under slightly acidic (4.33) or slightly alkaline (8.80) conditions give a lower yield of the analytical reaction (47 % and 33 % respectively). The different yields obtained for pH 5.43 or 7.06 are not significantly different. This is due to the fact that the milli-Q "blank" is buffered to pH 7 through the analytical procedure. The peroxidase enzyme activity is indeed highly sensitive to pH and its efficiency is best for neutral pH (Zika and Saltzman, 1982; Holm *et al.*, 1987). We therefore buffered to pH 7 the reaction medium of our analytical measurements, in order to obtain a compromise between sensitivity and reproducibility.

Influence of Salinity

The effect of variable ionic strength on the relative sensitivity was also studied. A solution of synthetic sea water was prepared from the major ions present in marine waters (Na^+, Cl^-, Mg^{2+}, SO_4^{2-}). Solutions of different ionic strenth were prepared with analytical grade salts dissolved in milli-Q water. Solutions with salinity of 0, 8, 15, 21 and 33 $g.dm^{-3}$ were prepared with aged milli-Q water stored in a clean tank. The fact that the pH increases slightly with salinity was corrected during the analytical procedure by the addition of the peroxidase-phosphate buffer solution. The results of the relative sensitivity response are presented in Figure 4. A salinity increase from 0 to 33 $g.dm^{-3}$ does not generate significant response with respect to the relative sensitivity. The approximate relative analytical yield of the reaction ranges from 82 % at 21 $g.dm^{-3}$ to 102 % at 15 $g.dm^{-3}$. These data have an analytical error (RSD) estimated at 10 %. The results suggest that in variable environments such as estuaries, fluctuation

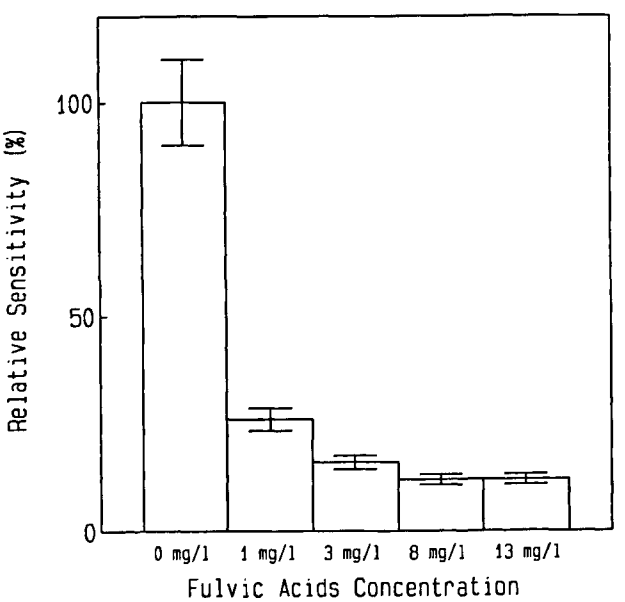

Figure 5

Relative sensitivity (s/s_0) versus *DOM concentration (fulvic acids) (error bars : estimated relative standard deviation 10 %).*

Table 2

Field results for different estuarine and marine waters during the ECOFER 4 and PhotoBiol cruises. (Time : local time = GMT + 2 hours; DOC : dissolved organic carbon; nd : not determined.)

Date Time	Site	Depth (m)	Salinity (g/dm^3)	DOC (mg/dm^3)	$[H_2O_2]$ (nM)	Std.Dev. (n=2, nM)
03/05/91	Gironde	0	1.64	1.50	169	8
15:00	Estuary	4	1.82	1.50	148	13
06/05/91		0	23.46	1.18	212	30
11.10		10	28.99	1.02	139	14
		18	30.54	1.09	110	11
06/05/91	Continental	0	34.95	0.72	138	11
05:00	Shelf	10	34.99	0.69	131	3
	(Bay of	20	35.12	0.77	147	5
	Biscay)	40	35.60	0.80	90	11
		65	35.60	0.61	69	10
05/05/91	Open	0	35.60	0.84	186	11
14:45	Ocean	5	35.60	0.76	175	8
	(Bay of	25	35.61	0.76	163	3
	Biscay)	80	35.61	0.75	125	nd
		120	35.59	0.64	104	11
		250	35.57	0.63	71	7
23/09/92	Gironde					
10:15	Estuary	3	13.00	nd	69	11
12:00		3	4.00	nd	27	1
14:00		3	2.00	nd	17	2
16:00		3	9.00	nd	32	4
19:00		5	28.00	nd	82	8
24/09/92						
11:30		5	31.00	nd	54	6
14:30		5	25.00	nd	75	6
17:45		5	30.00	nd	78	nd
15/12/92	Gironde					
13:00	Estuary	3	2.00	nd	48	7
17:00		3	1.00	nd	53	5
21:00		3	19.00	nd	22	4
16/12/92						
09:00		3	20.00	nd	22	1
11:00		5	33.00	nd	69	4
14:00		3	16.00	nd	21	1
16/05/93	Gironde					
08:45	Estuary	5	28.50	2.3	256	17
13:30		5	23.50	1.3	158	12
18:00		3	10:00	1.09	228	5
20:45		3	2.00	1.7	64	7
17/05/93						
07:30		3	1.50	2.1	24	3
10:30		3	0.50	6.4	40	3

of the ionic strength will not alter the quality of H_2O_2 determination.

Influence of DOM concentration

Influence of DOM on the relative sensitivity of H_2O_2 determination was also studied. In marine waters, humic substances (HS) represent 60-80 % of DOM and mostly as fulvic acids (FA). Reference FA were used to simulate the interactions generated by the occurrence of DOM in the sample. Molecular structures and reactivity of the HS are still not well understood (Harvey *et al.*, 1984). However, these substances present a large array of functional groups that may become involved in different physico-chemical interactions, such as redox processes, photochemistry and/or complexation. In our experiments, dry FA were diluted in stored milli-Q water at concentrations relevant to those found in natural waters (*e.g.*: 1, 3, 8, and 13 $mg.dm^{-3}$). The expected decreasing pH with increasing FA concentration was balanced by the buffering step involved during the Peroxidase addition. All solutions were analysed within the same day, a few hours after their preparation. Results expressed as relative sensitivity obtained in presence of increased DOM concentrations are displayed in Figure 5. A rapid decrease in sensitivity is observed directly in the presence of a very small amount of

DOM. This kind of natural compound appears to be a strong interferent for this technique, leading to a severe decrease of the chemical yield. For 1 $mg.dm^{-3}$ FA, which can be assumed to represent the mean concentration of DOM in oceanic waters, the relative sensitivity is four times lower than that obtained in simple milli-Q water matrices. The relative sensitivity decreases rapidly with DOM concentration to reach 12 % of the blank value at 8 $mg.dm^{-3}$ and 13 $mg.dm^{-3}$. This strong inhibitive behaviour could be explained by the fact that some aromatic structures, derived from the continental FA used, have the same reactivity as scopoletin under peroxidase catalysis. Holm *et al.* (1987) listed a number of possible interferents with this method. The principal interfering species are then: ascorbic acid, glutathione and manganous ions; as well as nitrite, ethanol, some amino acids, phenols, thiols, diamines and iodide. Some of these molecular groups can be included in the FA structure and compete with Scopoletin with respect to oxidation by H_2O_2. Also, Zafiriou and co-workers (1984) proposed different hypothetical structures of natural aquatic HS. One has a base structure belonging to coumarin compounds (like scopoletin) that may interfere with the analytical reaction by its occurrence in natural waters. These overall sets of conditions will lead to a decrease of the relative sensitivity measured. Direct non-catalysed DOM oxidation reactions by H_2O_2 (*i.e.* natural oxidation) are not expected to interfere with the analytical procedure. Processes of this type have very low reaction kinetics at this H_2O_2 concentration range in comparison with the peroxidase activated pathway. In general, we can state that DOM interactions are apparently the strongest interferents with the scopoletin-peroxidase method due to the many organic compounds present in natural waters that are able to behave as hydrogen donors, competing then with the scopoletin reagent.

As a result, the chemical yield of the analytical reaction will be higher in clear blue sea water that in coastal or continental brown waters. Hence, we can state that the differences of sensitivity observed in Figure 2 can now be explained by the DOM interactions with the H_2O_2-scopoletin reaction.

This analytical study of interference processes with the scopoletin-peroxidase method for H_2O_2 determination demonstrates that standard additions approach is best suited to remove all the analytical problems occurring with this technique and as a result of experimental conditions or chemical interferences.

Field results

H_2O_2 concentrations in estuarine and marine waters have been determined on board ship during ECOFER 4 (May 1991) and PhotoBiol cruises (Sept. 92, Dec. 92, May 93). Different waters were sampled on the Gironde estuarine gradient, on the continental shelf and in the open ocean of the Bay of Biscay and characteristic analytical results are presented in Table 2. All the measurements were performed in duplicate and analysis presented the same accuracy as in the laboratory (5 to 10 %).

The concentrations were not strictly dependent on salinity or DOC levels in the samples. However, in coastal and marine waters a systematic gradient pattern was found for H_2O_2 vertical profiles where concentrations decreased slightly with depth. These results are in accordance with the previous studies performed by Zika *et al.* (1985*a*, 1985*b*) and Johnson *et al.* (1989), where *in situ* photochemical production of H_2O_2 was described as the predominant source. Particularly high concentrations were, however, observed in the deeper waters of the surface layer below 100 m. Different processes may explain this specific pattern. First, the different vertical profiles in marine waters were determined during and after a stormy period (Force 6-9). Wind forcing may activate the turbulent mixing of the surface layer, involving important vertical transport of H_2O_2. Additionally, Johnson and co-workers (1987) observed consequent amount of H_2O_2 at 100 m depth in similar frontal region where vertical advection phenomena may be enhanced. They also considered that because the life time of H_2O_2 in marine waters is of several days, the vertical distribution of H_2O_2 relates the recent history of the water column. Taking into account all these potential hydrological factors permits an explanation of the profiles observed during the ECOFER 4 cruise.

The second part of Table 2 shows characteristic data obtained during seasonal cruises on the Gironde estuary. A first comparison between the data obtained in May 91 and May 93 presents similar H_2O_2 concentrations for the same season in two different years. In both cases, the H_2O_2 contents were found to be high, presumably because of the high incident light-inducing photochemical processes and the intense biological turnover. On the other hand, the seasonal variation is well marked with minimum concentrations in December followed by slightly higher amounts in September. The global incident light appears to be a main factor controlling H_2O_2 concentrations in estuarine waters. Lowest concentrations were determined generally in the most turbid part of the estuary, demonstrating that suspended matter may alter both light penetration and H_2O_2 life time.

The flow injection technique applied to the scopoletin-peroxidase method permitted the determination of H_2O_2 concentrations in estuarine and marine waters with a high accuracy. This technique has been successfully used for on-board field measurements avoiding any matrix or experimental conditions interferences.

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