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## **Micronutrient (PO<sub>4</sub>, NO<sub>3</sub>) measurements in oligotrophic and high salinity range environments by flow injection analysis**

with 6 figures

PATRICK RUCH<sup>1)\*</sup>, CARLOS BECK<sup>1)</sup> & FLORENCE GENDRE<sup>1)</sup>

### *Abstract*

The determination of nutrients in oligotrophic waters associated with high salinity range environments such as estuaries or coastal regions where flows of fresh water resurgences are high, is problematic because of the matrix effect. The matrix effect is due to the difference of density (refractive index) between the sample and the carrier streams which strongly affects the original absorbance of the sample and induces overestimates in nutrient content. The increasing absorbance is proportional to the difference in salinity between sample and carrier streams and inversely proportional to the nutrient content of samples. The matrix effect is noted because of its resulting sinusoidal shape (negative absorbance) and positive or negative shift of the time of maximal absorbance. For reasonable salinity differences between carrier stream and sample (0-7.5 g/l), concentration overestimates can be up to 20 µg/l for a PO<sub>4</sub> range between 0 and 25 µg/l. For salinity differences > 7.5 g/l, overestimates can be > 80 µg/l. The matrix effect range depends on the nutrient concentration too.

Impact studies or monitorings in coastal environment require numerous analyses to establish good statistics to determine variations caused by tides, seasons, fresh water flows, etc.). Since the adjustment of the carrier stream's salinity to the sample takes a long time, it is necessary to develop a method which allows an high analytical rate. This note proposes a quick and simple way to correct the matrix effect without changing the sampling rate (≈ 70/h). The variability of measurements is about ± 2.5 µg/l for PO<sub>4</sub> and ± 1.5 µg/l for NO<sub>3</sub>, which is acceptable considering the range of natural nutrient variability in sea water or in fresh water.

### *Résumé*

La détermination des sels nutritifs dans des milieux oligotrophes soumis à des régimes de fortes variations de la salinité comme les estuaires ou des régions où les résurgences d'eau douce sont importantes, présente des difficultés en raison de l'effet de matrice. L'effet de matrice est dû à la différence de salinité (indice de réfraction) entre l'échantillon et le liquide porteur qui affecte grandement l'absorbance originale de l'échantillon et provoque une surestimation de la concentration en nutriment. L'augmentation de l'absorbance est proportionnelle à la différence de salinité entre l'échantillon et le liquide porteur et inversement proportionnelle à la concentration de l'échantillon en nutriment. L'effet de matrice se manifeste par une courbe sinusoïdale (absorbance négative et positive par rapport à la ligne de base) et par un déplacement positif ou négatif du temps théorique auquel le maximum d'absorbance devrait être atteint. Pour des différences de salinité raisonnables entre le liquide porteur et l'échantillon (0-7.5 g/l), la surestimation de la concentration peut être supérieure à 20 µg/l pour des concentrations en PO<sub>4</sub> variant entre 0 et 25 µg/l. Pour des différences de salinité > 7.5 g/l, la surestimation peut atteindre 80 µg/l.

Les études d'impact ou les surveillances à plus ou moins long terme dans le milieu marin côtier

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exigent généralement de nombreuses analyses afin d'établir une bonne statistique pour déterminer les changements liés à la marée, aux saisons, ou aux débits des eaux douces se jetant en mer par exemple. Comme l'ajustement de la salinité du liquide porteur à celle de l'échantillon prend du temps, il est donc nécessaire de pouvoir bénéficier d'un instrument et d'une méthode qui puissent fournir un taux analytique élevé. Cette note propose une méthode simple et rapide pour corriger l'effet de matrice sans changer le taux d'analyses ( $\approx 70/h$ ); La variabilité des mesures est de  $2.5 \mu\text{g/l}$  pour les phosphates et de  $1.5 \mu\text{g/l}$  pour les nitrates, ce qui est acceptable en considérant la variation naturelle des concentrations en sels nutritifs dans les eaux marines et les eaux douces.

## 1. Introduction

The concentrations in nitrate and phosphate are regularly found to be less than 60 and  $100 \mu\text{g/l}$  respectively in surface sea water (RILEY & CHESTER, 1971) and oligotrophic environments (REISS & HOTTINGER, 1985). Concentrations in phosphate are typically less than  $50 \mu\text{g/l}$  in the euphotic zone (primary production) of the ocean (JOHNSON et al., 1985). The determination of nutrient concentrations are particularly important and delicate in coral reef environments (corals live in oligotrophic waters) where the integrity of the coral community depends on the level of nutrient concentrations (KINSEY & DAVIES 1979, WALKER & ORMOND 1982). Under such conditions, nutrient analyses need to be very sensitive.

Flow Injection Analysis has demonstrated to be a high sensitivity and precise method (relative standard deviation  $< 1\%$ ) in marine research. However, the study of oligotrophic lagoonal waters of Mauritius in the Indian Ocean (RUCH et al. in MULLER et al. 1991) has shown that, during nitrate and phosphate measurements, high interferences of the absorbance signals occurred when water samples were collected in estuaries and near fresh water springs. These interferences induced by changes in salinity between the carrier stream and sample create a "matrix effect" leading to an overestimate of the concentration.

Causes of the matrix effect have already been discussed by BETTERIDGE et al. (1979). Changes in salinity between samples and carrier stream create a parabolic index gradient which acts as a lens focusing light to the detector and refracting it away alternately. In sea water analysis, samples should have the same salinity as the carrier stream to avoid the matrix effect and to obtain a good detection limit. This is problematic for the sampling rate in a highly variable salinity environment (i.e., estuaries, fresh water resurgences in coastal waters, etc.). JOHNSON et al. (1985) recommend the addition of  $\text{NH}_4\text{Cl}$  to the carrier stream and the samples in order to suppress the refractive index signal. However, our measurements have demonstrated that this method is not sensitive enough for phosphate and nitrate concentrations lower than  $30 \mu\text{g/l}$  (i.e., lagoonal environments). Therefore, the aims of this note are: a) to evaluate the amplitude of the matrix effect; b) to establish relationships between nutrient concentrations and the amplitude of the matrix effect; c) to propose a quick and simple way to reduce the matrix effect produced by the mixing of different saline solutions keeping an high analysis rate in low nutrient content environments. Experiments with phosphates will illustrate the matrix effect and the method developed to eliminate interferences on the absorbance intensity generated by the matrix effect.

## 2. Instruments and methods

### 2.1 *Principle of flow injection analysis*

Flow - Injection - Analysis (FIA) is a technique for automating chemical analysis. The principle of FIA (RUZICKA & HANSEN, 1988) is based on the injection of a liquid sample into a moving, non-segmented continuous and laminar carrier stream. The carrier and the sample are then mixed with reagents in a chemifold of which configuration (number of reagents, time of reactions) depend on the elements to be analysed. The reaction zone is transported towards the detector that continuously records the absorbance of the stream where the baseline was determined previously by the mixing of the carrier with the reagent (fig.1).

The sensitivity of FIA using a photometric detector with 1-2 cm path-length cells approaches the sensitivity obtained by manual methods using 5-10 cells (JOHNSON et al., 1985).

### 2.2 *Instruments and method*

A Tecator FIAstar 5010 Analyser and a 5023 Spectrophotometer with a 1 cm path-cell coupled with an autosampler and a computer was used in this study. This configuration allows to sample automatically, to program injections and the time of pumping.

Calibrations and measurements have been performed from the maximal intensity of the absorbance. Calibrations are linear for phosphates and nitrates.

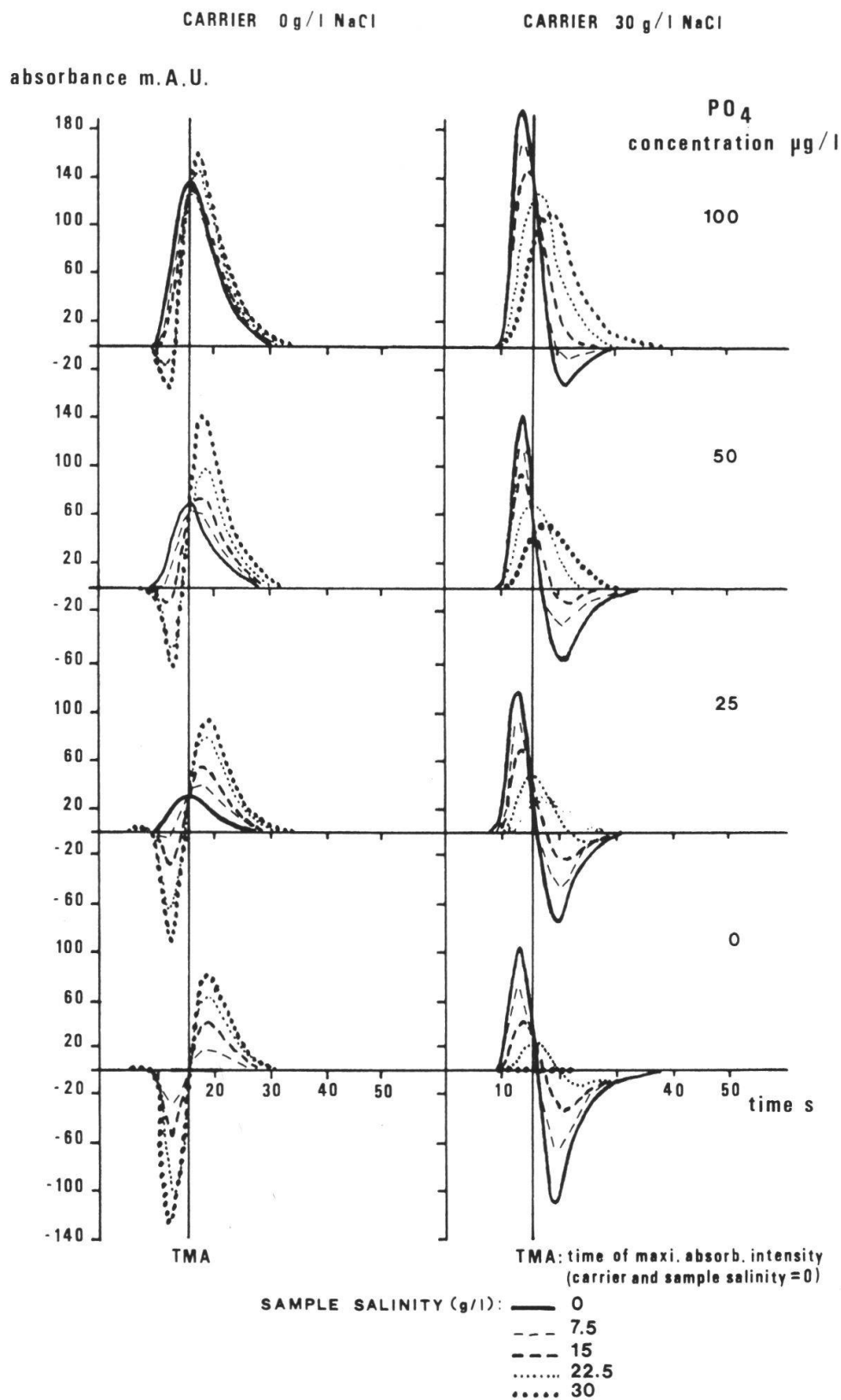
The method used for the phosphate determination is based on the reduction of the molybdophosphoric acid obtained by the reaction between ammonia heptamolybdate and orthophosphate and the subsequent reduction by stannous chloride. The use of stannous chloride as a reductant (JOHNSON et al, 1985), rather than ascorbic acid, allows to perform analyses at room temperature (30°C). The absorbance is measured at 690 nm.

The detection limit of nitrates and phosphates ranges from 0.5 to 1 µg/l in fresh waters. In sea water, the detection limit varies between 1 and 30 µg/l (depending on salinity).

### 2.3 *Matrix effect experiment*

In this study, the determination of the matrix effect is illustrated by the experiment performed with PO<sub>4</sub> standards. The matrix effect experiment with nitrate gives similar results.

The amplitude of the matrix effect was evaluated according to the following experiment: PO<sub>4</sub> standards of 0, 25, 50, 100 µg/l have been prepared in deionised water and 7.5, 15, 22.5 and 30 g/l NaCl artificial solutions. Absorbance of each standard was measured with carrier streams of deionised water and with waters of 7.5, 15, 22.5 and 30 g/l salinity. All analyses were performed four times with calibrations set up in deionised water and different salinity ranges. The experiment was repeated twice.



**Fig. 1:** Example of absorbance diagram (determination of PO<sub>4</sub> content) records. Influence of the salinity on the absorbance. A shift in time of the maximum absorbance peak position, a negative absorbance peak and a general increase in absorbance characterize the matrix effect. Note that the matrix effect depends on the carrier stream and sample salinity difference (carrier stream salinity < and > sample salinity) and PO<sub>4</sub> contents.

### 3. Results and discussion

#### 3.1 Determination and causes of the matrix effect

Results of the experiment are presented in figure 1. Comparisons of absorbance records between analyses realised under standard conditions (no salinity difference between sample and carrier) and analysis performed with different carrier and sample saline solutions show strong distortions of the standard signal (fig.1). The main changes consist of shifts of the time of the maximum absorbance intensity TMA (positive and negative shifts compared with the standard position TMA, fig.1) and the appearance of negative absorbance peaks before and after the maximum absorbance intensity, resulting in a sinusoidal shape of the absorbance signal.

Three characteristic types of absorbance signals have been recorded in relation to analytical conditions:

1/ - no negative absorbance recorded

- the time of maximal absorbance intensity (TMA) is at the right position

This situation corresponds to normal analytical conditions. Sample and carrier stream have the same salinity.

2/ - a negative peak is recorded before a positive peak

- the time of maximal absorbance intensity (TMA) is longer than the theoretical time for maximal absorbance (when salinities of carrier streams and samples are the same).

This situation occurs when the sample salinity is higher than the carrier stream salinity.

3/ - a negative peak follows a positive peak

- the time of maximum absorbance intensity (TMA) is shorter than the theoretical one.

This situation occurs when the sample salinity is lower than the carrier salinity.

BETTERIDGE et al. (1978) explain differences in absorbance by the parabolic shape of the reacting zone and by difference in refractive index between samples and streams.

In the case of a sample of higher refractive index than the stream, light travels from isohalines of high refractive index to those of low refractive index at the leading interface. The light is refracted and focused toward the detector leading to an increase in the amount of light reaching the photodiode. The absorbance decreases and a negative peak appears. At the trailing interface, light travels from a low to a high refractive index. Light is diverged from the path to the detector and a positive peak is observed. In the case of a sample of lower refractive index than the stream, the negative peak follows the positive peak.

The negative and positive shift with respect to the time of the maximum absorbance intensity measured under normal conditions (the carrier stream and



sample salinity are the same) is not only due to refractive index but to the viscosity of the solution as well.

### 3.2 *Influence of the matrix effect on concentration measurements*

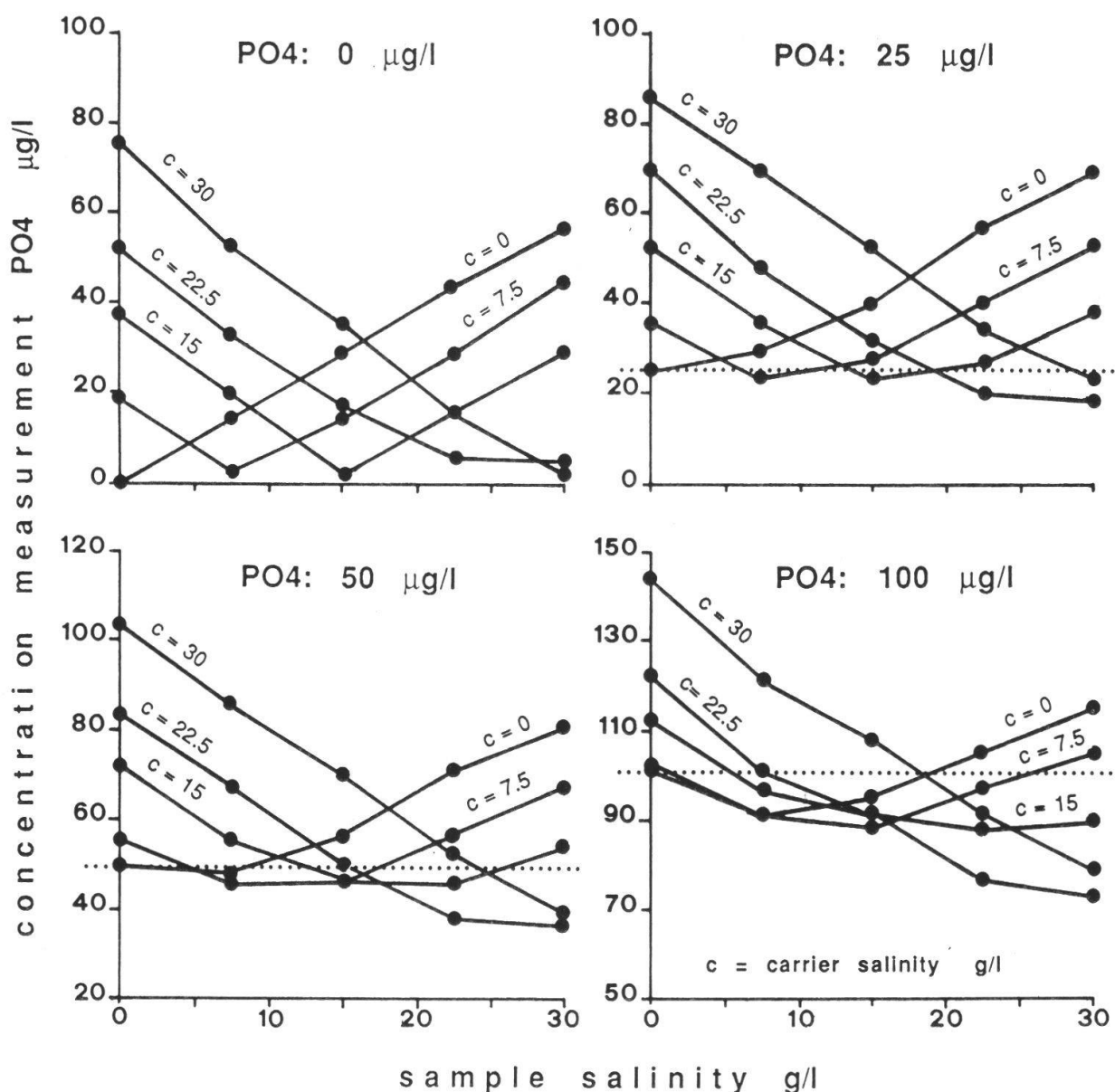
The relationship between sample salinity and concentration for definite phosphate concentrations and carrier salinities (fig.1 and 2) shows six main features:

- relationships are positive when the sample salinity is higher than the carrier stream salinity
- relationships are negative when the sample salinity is lower than the carrier stream salinity
- change in slope sign occurs when there is no salinity difference between the carrier stream and the sample
- overestimations and underestimations of concentration measurements
- the  $\text{PO}_4$  concentration influences the amplitude of the sinusoidal shape of the absorbance
- the increasing concentration of  $\text{PO}_4$  reduces the amplitude of the negative absorbance peak

The underestimation of results is greatly due to the automatic calculation of the baseline. Recalculated concentration values from recalibrations set up with carrier streams with same salinities as the samples (0, 7.5, 15, 22.5 and 30 mg/l NaCl) demonstrate that the baseline increases with increasing carrier salinity. The absorbance increasing of the baseline leads to an underestimation of results. Concentration values corrected from new calibrations show that results (except 3 samples) are in fact always overestimated (fig. 1).

Negative relationships between sample salinity and measured concentrations (fig. 2) occur when the sample has a lower refractive index than the carrier stream and positive relationships when the sample refractive index is higher than the carrier stream. The slopes of these functions are determined by the difference of salinity between the carrier stream and the sample. However, the difference of salinity between the carrier and the sample is not the only determinant factor for the matrix effect as shown in figures 3A and 3B. Excess in absorbance relative to standards (fig. 3A) and the negative absorbance intensity (fig.3B) depend on the salinity of both the carrier stream and the sample. When the carrier salinity is higher than sample salinity, the excess of absorbance is, as a general rule, higher and the intensity of the negative absorbance lower than when the sample salinity is higher than the carrier salinity for the same salinity difference. This means that the intensity of light reaching the detector is attenuated with increasing salinity of the carrier

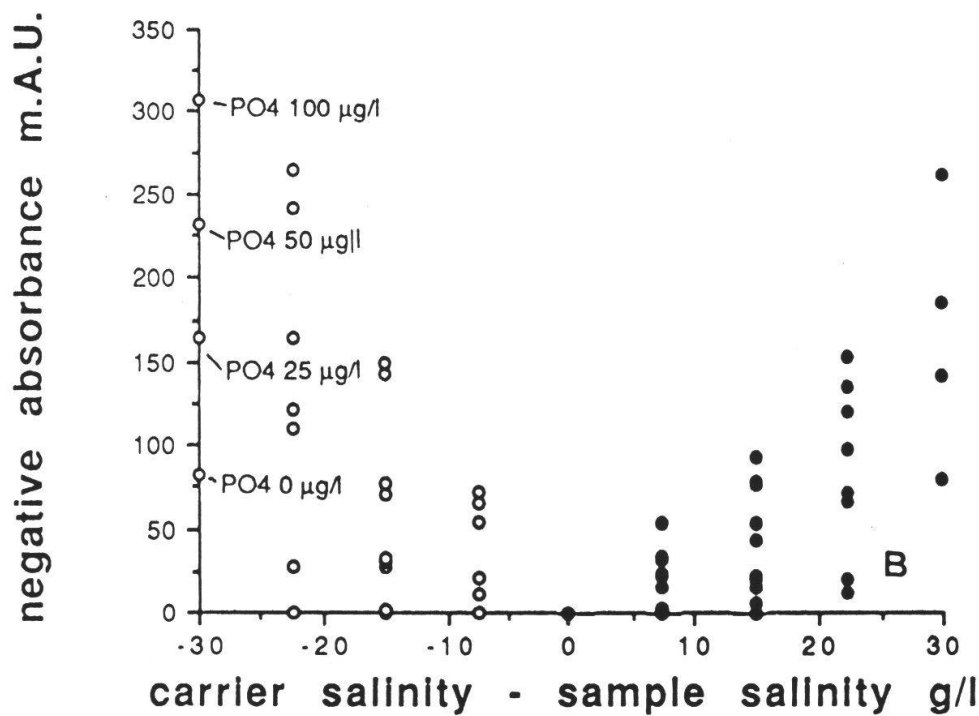
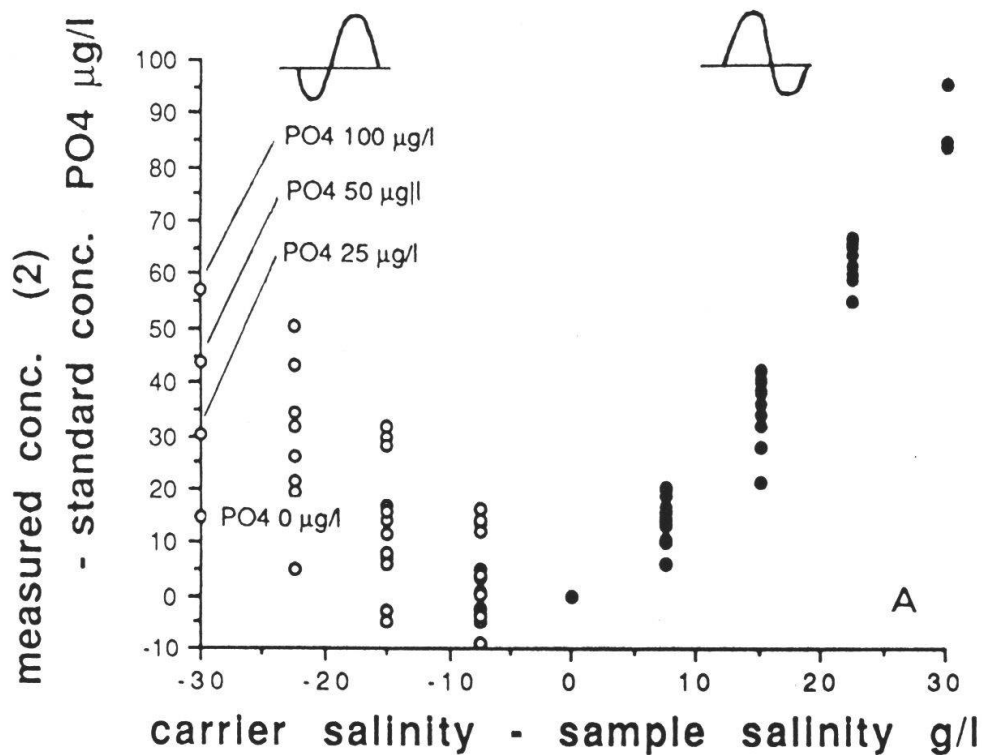
There are characteristic functions according to the relationship between the negative excess and excess in concentration (measured concentration -  $\text{PO}_4$  concentration on figure 4). The relationship is polynomial when the



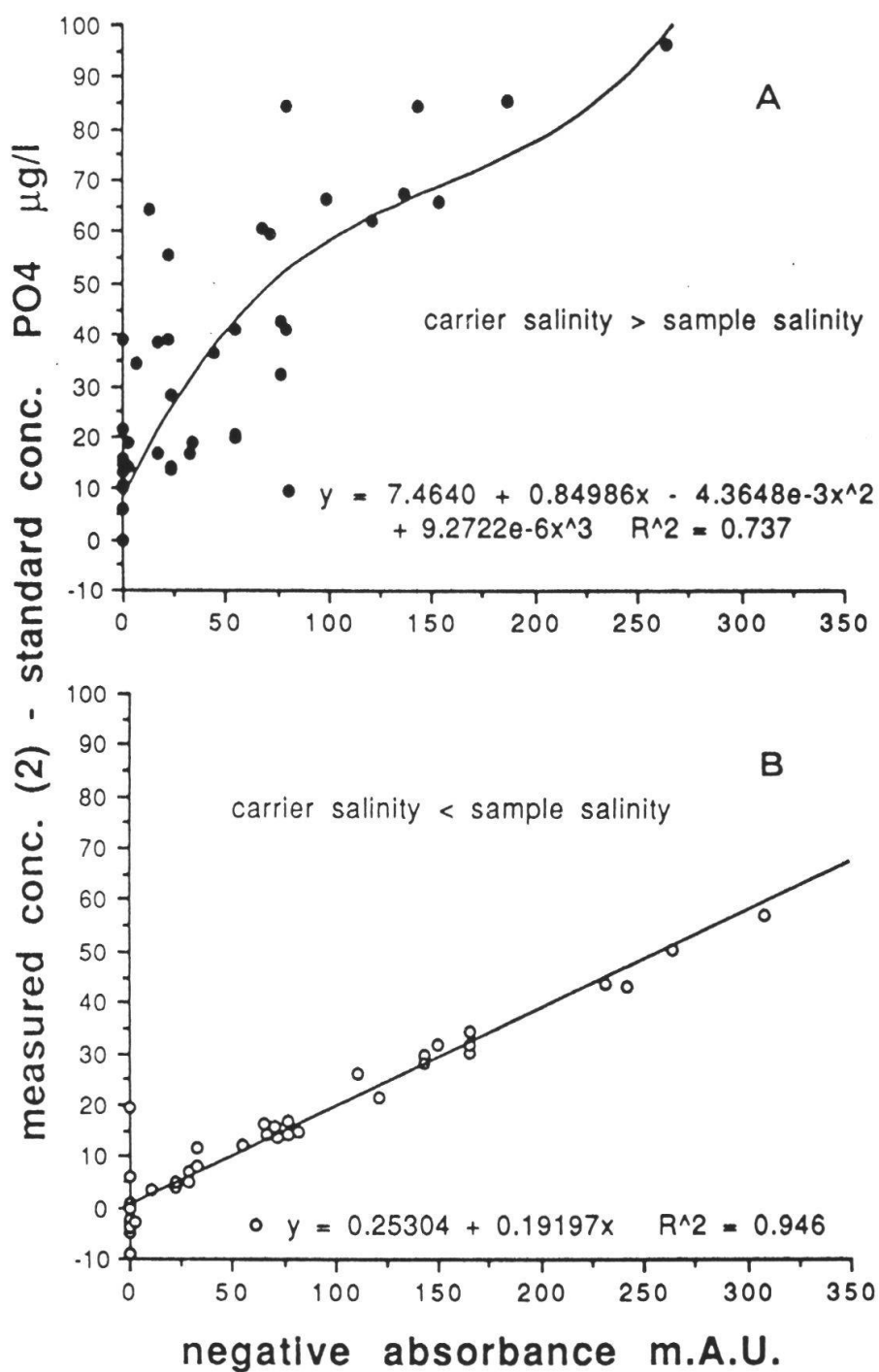
**Fig. 2:** Determination of 0, 25, 50 and 100  $\mu\text{g/l}$   $\text{PO}_4$  standards prepared in different saline solutions and analysed with 0 - 30  $\text{g/l}$  salinity range carrier streams.  $\text{PO}_4$  contents were calculated from a calibration set up in deionised water (salinity = 0  $\text{g/l}$ ). The change in slope sign occurs when carrier stream and sample salinity are equal. A positive relationship between carrier stream and sample salinity occurs when carrier stream < sample salinity and a negative one when carrier stream > sample salinity. Some sample concentrations are underestimated because of the automatic calculation of the baseline.

salinity of the carrier stream is higher than the salinity of the sample (positive difference, fig. 4A) and becomes linear when the sample salinity is higher (negative difference, fig. 4B). The absorbance is always higher when a carrier stream with a high refractive index is used.





**Fig. 3 A and 3 B:** Relationships between the salinity of the carrier stream and sample, the excess in  $\text{PO}_4$  concentrations and the negative absorbance (the number (2) on the Y-axis indicates that the  $\text{PO}_4$  contents were determined with calibrations performed in 0, 7.5, 15, 22.5 and 30 g/l NaCl). Note that the excess of the measured  $\text{PO}_4$  contents not only depends on the difference of salinity between carrier streams and samples but also on the specific salinity of carrier stream and sample. Concentrations are higher when the carrier stream salinity is higher than the sample salinity. The negative absorbance is higher when the salinity of the sample is higher than the carrier stream salinity.



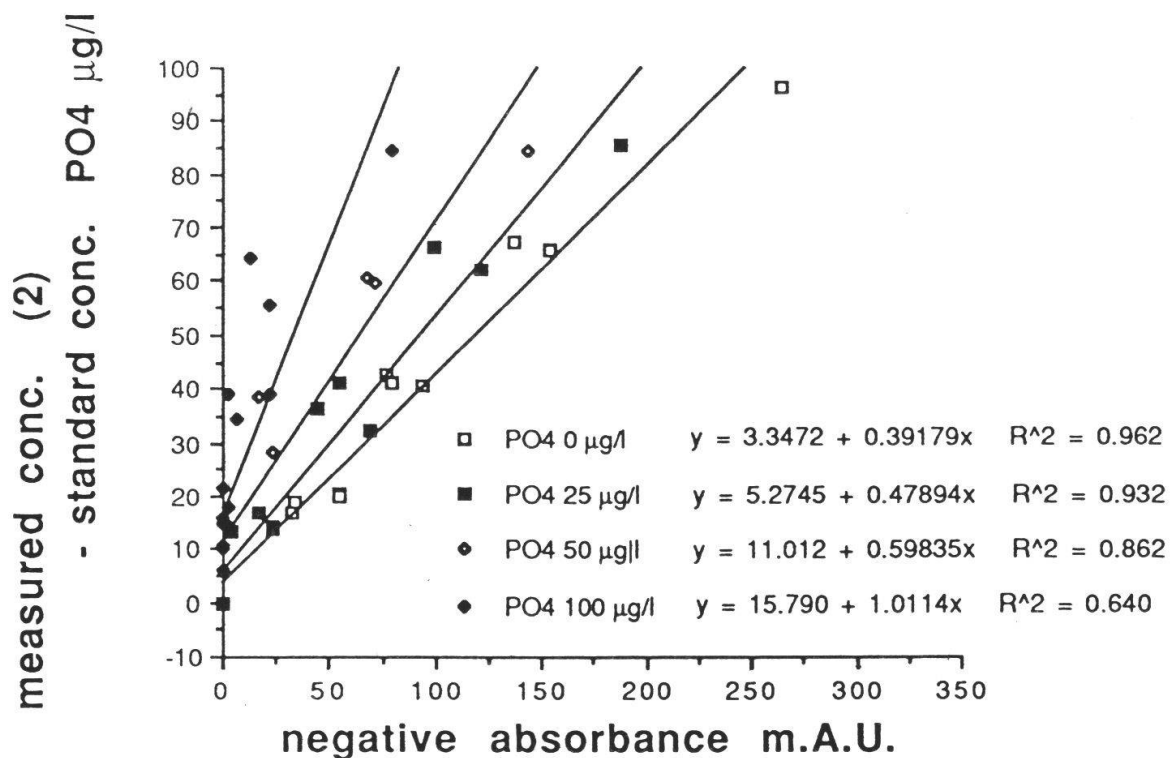
**Fig. 4 A and 4 B:** Relationship between the negative absorbance and the excess in PO<sub>4</sub> concentration. Note that the relationship is polynomial when carrier salinity > sample salinity and in particular linear when carrier salinity < sample salinity.

### 3.3 Influence of $PO_4$ concentrations

In the case of a carrier salinity < sample salinity, the linear correlation and the good correlation coefficient ( $r^2=0.946$ ) between the negative absorbance and the excess of concentration indicate that this relationship could be used for the correction of the matrix effect. At this time, it is evident that points (fig.4B) dispersed between -10 and 20  $\mu\text{g/l}$  for null absorbances cannot be used to correct the matrix effect.

However, these points correspond to  $PO_4$  concentrations  $\geq 50 \mu\text{g/l}$  and the highest errors (20%) occur when the  $PO_4$  concentrations reach 100  $\mu\text{g/l}$ . In the context of coral reef environments and coral mortality, the precision of measurements is not so important since such concentrations indicate a bad quality of water for the coral community integrity.

In the case of a carrier salinity > sample salinity (fig.5), the relationship between the negative absorbance and excess in  $PO_4$  concentration indicates that the correlation is decreasing with increasing  $PO_4$  concentration and high  $PO_4$  concentration reduce the amplitude of the negative peaks. Correlations are significant for  $PO_4$  concentrations  $\leq 25 \mu\text{g/l}$ .



**Fig. 5:** The  $PO_4$  concentrations influence the negative absorbance. Highest measured concentrations and negative absorbance are recorded for lower standard  $PO_4$  concentrations. The correlation decreases with increasing  $PO_4$  content.

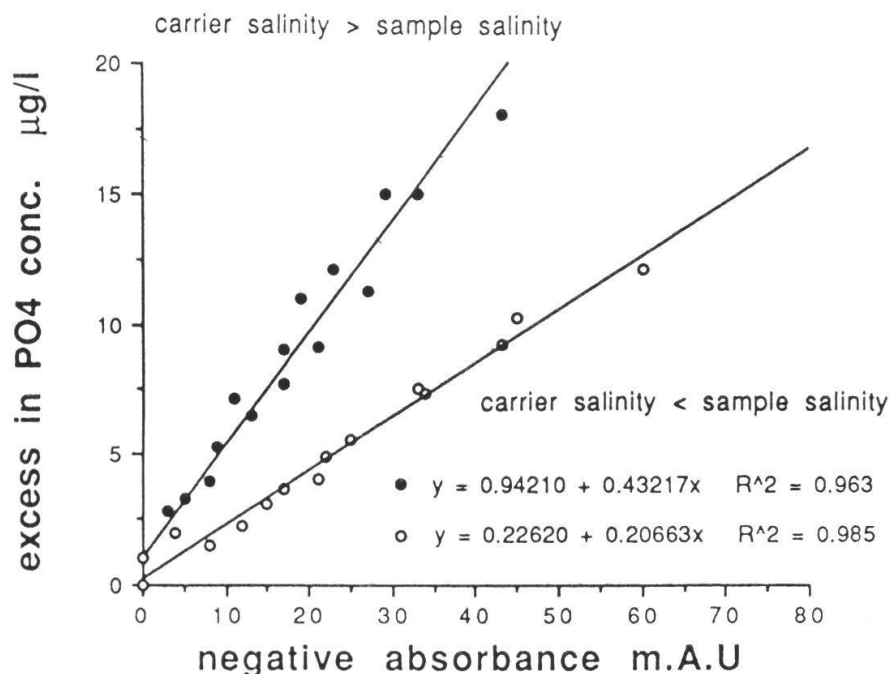
### 3.4 Correction of the matrix effect

Since the adjustment of the carrier to the sample salinity takes a long time, a correction of the measured concentration is necessary if studies need an high analytical sample rate.

The shift in time of the maximum absorbance intensity, caused by the refractive index of solutions, could be used to eliminate the matrix effect. However, the difference of the reaction time produced by the salinity difference is too small to establish a significant arithmetic relationship between the shift in time and concentration.

The matrix effect experiment has demonstrated that:

- the sinusoidal shape of the absorbance (negative intensity) is the best way to note the interferences due to the matrix effect
- the difference of salinity between the carrier stream and the sample must be  $\leq 7.5$  g/l to reasonably correct the measured absorbance
- the negative absorbance intensity is directly proportional to the excess in nutrient concentration and can be used to correct the interferences due to the matrix effect
- the  $\text{PO}_4$  concentrations  $\geq 50 \mu\text{g/l}$  do not need to be corrected considering the mean margin of error (10%) and the significance of such concentrations in the context of coral community integrity
- the  $\text{PO}_4$  concentrations  $\leq 25 \mu\text{g/l}$  need to be corrected because the measured concentrations can reach  $80 \mu\text{g/l}$  and therefore distort the interpretation of results
- the  $\text{PO}_4$  concentrations  $\leq 25 \mu\text{g/l}$  can be corrected from the negative absorbance.



**Fig.6:** Correlations between negative absorbance intensities and excess in  $\text{PO}_4$  concentrations elaborated for salinity difference  $\leq 7.5$  g/l NaCl and between standard  $\text{PO}_4$  concentrations ranging from 0 to  $25 \mu\text{g/l}$ . Note that the excess of concentrations are higher when the carrier stream is higher than the sample salinity. With these correlations the matrix effect may be corrected.

Following these observations, standard carrier solutions of 0, 7.5, 15, 22.5 and 30 g/l NaCl were prepared and calibrations set up with phosphate standards prepared in solutions with the same salinity as the carrier stream. Then, samples were selected according to their salinity determined before in sampling places with a CTD (conductivity, temperature, depth) probe (GENDRE 1992, RUCH et al. in prep.). In the case of lagoonal water sample analysis for example, the water salinity ranged from 30 to 35 g/l. Calibrations were set up with a carrier salinity of 30 g/l. Phosphate standard solutions of 0, 10 and 25  $\mu\text{g/l}$  were prepared in different saline solutions (30, 31, 32, 33, 34 and 35 g/l NaCl) and added to the sample series to be analysed. The negative absorbance intensity (in m.A.U. units) and excess of absorbance (in concentration units) of the different standards were determined and their relationship set up. Then, one needs only to replace the negative absorbance in the equation and remove the result from the measured concentration to obtain the right concentration. The variability of measurements with this method is about  $\pm 2.5 \mu\text{g/l}$  for  $\text{PO}_4$  and  $\pm 1.5 \mu\text{g/l}$  for  $\text{NO}_3$ .

#### 4. Variability of $\text{PO}_4$ and $\text{NO}_3$ in fresh and marine waters of Mauritius

In order to check the method, a certain number of analyses in fresh and marine waters of Mauritius were done to compare the natural variability of nutrient content with the detection limit.

In fresh water (where there is no matrix effect and therefore no correction of the absorbance intensity) the detection limit is 0.5 - 1  $\mu\text{g/l}$  for  $\text{PO}_4$  and  $\text{NO}_3$ . Natural variations of nutrients in oligotrophic waters of regions covered with forests were determined from 10 samples recolted in one hour. These variations are:

$\text{PO}_4$ ( $\mu\text{g/l}$ ):	min. = 0.9;	max. = 3.0;	mean = 1.8;	$\sigma$ = 0.85;	$\sigma\%$ = 54.0
$\text{NO}_3$ ( $\mu\text{g/l}$ ):	min. = 9.0,	max. = 12.0,	mean = 10.7;	$\sigma$ = 1.0;	$\sigma\%$ = 9.3

In marine waters, the detection limits for  $\text{PO}_4$  and  $\text{NO}_3$  are respectively  $\pm 2.5$  and  $\pm 1.5 \mu\text{g/l}$ . Variations of 10 samples recolted in one hour are:

$\text{PO}_4$ ( $\mu\text{g/l}$ ):	min. = 11.6;	max. = 77.0;	mean = 44.5;	$\sigma$ = 22.7;	$\sigma\%$ = 51.0
$\text{NO}_3$ ( $\mu\text{g/l}$ ):	min. = 7.4,	max. = 25.9,	mean = 15.2;	$\sigma$ = 5.4;	$\sigma\%$ = 35.5

The variability due to the tide is (for  $\text{NO}_3$  only):

$\text{NO}_3$ ( $\mu\text{g/l}$ ):	min. = 1.9;	max. = 12.8;	mean = 7.8;	$\sigma$ = 3.7;	$\sigma\%$ = 47.4
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The variability of  $\text{PO}_4$  and  $\text{NO}_3$  measurements with this method is therefore acceptable considering the natural variability of nutrients in fresh and sea waters. With this method, the monthly average (24 samples) for  $\text{PO}_4$  concentrations in oligotrophic lagoonal waters was determined between april 1990 and march 1991.

The  $\text{PO}_4$  content in waters ranges from 10.5 to 16.5  $\mu\text{g/l}$  (corrected) instead of values varying between 30 and 100  $\mu\text{g/l}$  (measurements). These differences between the corrected contents and raw measurements are very important since environments with  $\text{PO}_4$  concentrations  $\geq 50 \mu\text{g/l}$  are unfavourable to the coral reef community integrity.

By way of comparison with other methods, the detection limit obtained by JOHNSON et al. (1985) with rFIA method, a modified manifold and a 1 cm path length-cell, ranges from 5 to 8  $\mu\text{g/l}$  for phosphate in sea water.

## 5. Conclusion

The matrix effect is due to the difference of salinity (refractive index) between the sample and the carrier stream. The matrix effect is marked by a shift in time of the absorbance intensity position and by the presence of a negative absorbance peak in very low nutrient concentrations and for a strong salinity difference between the carrier stream and the sample. Density gradients cause an increase of the absorbance intensity and therefore an overestimate of the concentration. The overestimate can be up to 80  $\mu\text{g/l}$  for very low concentration (0-25  $\mu\text{g/l}$ ). The correction of the results is necessary to evaluate the significance of the results, especially in estuaries where salinity gradients could be the only cause of the increase in nutrient concentration. The variability of measurements is about  $\pm 2.5 \mu\text{g/l}$  for  $\text{PO}_4$  and 1.5  $\mu\text{g/l}$  for  $\text{NO}_3$  in this concentration range. For  $\text{PO}_4$  concentrations  $\geq 50 \mu\text{g/l}$ , the error of measurements (10%) is not so important considering that such high concentrations already imply bad environment conditions for the integrity of the coral reef community.

A method to correct the interferences caused by the matrix effect has been developed based on the negative absorbance intensity, because negative absorbances and excess in concentration show good linear relationships. The method is acceptable considering its rapidity, the range of nutrient natural variability in sea water, the high sampling rate and a comparison with the detection limit of other methods.

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