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A Voltammetric Study of the Reactivity of Folic Acid in Algal Cultures and in Natural Waters*

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Folic acid (FA) can be determined in natural waters by adsorptive cathodic stripping voltammetry. The measurement suffers from interference due to the presence of humic acid (HA) in natural waters. At 2 mg/L of HA, the peak of FA is fully suppressed. It was concluded from non-equilibrium adsorption isotherms that the adsorption of HA and FA is competitive, and that the folic acid is more strongly adsorbed than the HA. Measurements of the reactive folic acid concentration at low levels of HA found no evidence of complexation of folic acid by humic substances analogous with metal ions. The interfering effect of HA was eliminated by passing the sample through a C-18 cartridge. Water samples from marine and freshwater origin revealed the presence of folic acid. Algal culture experiments using marine algae illustrated that the folic acid is taken up by Phaeodactylum but not by Emiliania and Dunaliella; this finding is tentative due to the possibility that bacteria contributed to the uptake since the cultures were not axenic. The results of these experiments suggest that folic acid could be important to certain species of marine microorganisms.

* Dedicated to Marko Branica on the occasion of his 65th birthday.

INTRODUCTION

Folic acid is widespread in biological systems and is also found in natural waters as a result of activities by microorganisms.[1] Recently, a very sensitive method, based on adsorptive cathodic stripping voltammetry (CSV),[2] was developed to determine folic acid in natural waters. Preliminary measurements[2,3] in seawater have indicated that not all folic acid occurs in an electrochemically reactive form, suggesting that it may occur as a complex or otherwise associated with other organic matter. Humic acid, a widespread unidentified part of organic matter in natural waters, and known to interact with many trace substances of organic and inorganic nature, is used here to study its interaction with folic acid. Availability of folic acid to microorganisms was investigated by measuring its uptake by marine algae in cultures.

The AC-polarographic wave of folic acid is distorted and suppressed by the presence of certain synthetic surfactants, including polyethylene glycol methylcellulose[4] and gelatine.[5,6] The hypothesized interaction of folic acid with humic acid could be by complexation or adsorption and would in this case include adsorption on a well-defined and easily controlled model interface between the mercury electrode and an electrolyte solution (buffered seawater or buffered sodium chloride solution). The measured adsorption at the electrode is influenced by dissolved surface active substances, depending on their solution concentration, adsorption strength, kinetics of adsorption, and on the thickness of the adsorbed layer. In the case of adsorption of several adsorbing solutes, the capacitive behaviour of the Hg/water interface can be modelled as a parallel capacitor;[7] a simplified equation denoting the decrease of double layer capacitance caused by adsorption of two different organic molecules in a mixture is defined by:

$$C = C_0 (1-\theta_1-\theta_2) + C_1\theta_1 + C_2\theta_2$$

where

C = total differential capacitance ($\mu F\ cm^{-2}$),

C_0 = capacitance of the interface covered by water molecules

C_1 respectively C_2 = capacitance of the interface totally covered by organic molecules of individual components of the mixture,

θ_1, θ_2 = surface coverage for the components in the mixture.

Surface coverage is defined as $\theta = \dfrac{\Gamma}{\Gamma_{max}}$

Γ_{max} is the surface concentration at saturation ($\theta = 1$), and surface area $A = \dfrac{1}{\Gamma_{max}}$ is the area occupied by one adsorbed molecule. At low surface coverage, the measured capacitance decrease is additive with respect to indi-

vidual components of the mixture. At higher surface concentrations, the capacitance decrease is lower than the sum of individual effects because of competition for the limited surface area. If interactions between the adsorbed molecules take place, the capacitance decrease of the mixture could either exceed or could be below the value corresponding to the sum of individual components. Apparent adsorption isotherms can be constructed for different time domains of adsorption that are shifted with respect to the true adsorption isotherm in the direction of higher concentrations. Non-equilibrium adsorption at the mercury electrode has been studied before[8,9] in mixtures of surface active substances. Additional information on the adsorption behaviour of the mixture and the structure of the layer can be obtained by investigation of the adsorbed layer on the electrode processes of other ions and molecules which are used as a probe.[10,11]

In this work, the adsorption of folic acid on the mercury electrode was investigated alone and in the presence of HA. The influence of HA on the folic acid peak was minimized by passing the seawater through a SEP-PAK C-18 column which did not adsorb the folic acid at rapid sample flow. The presence of folic acid in water samples from varying origin (seawater, lake water and phytoplankton cultures) was verified. Its possible importance to microorganisms was evaluated from uptake experiments by cultured marine algae.

EXPERIMENTAL

Voltammetric instrumentation: a μ-Autolab (Eco Chemie, Netherlands) connected to a Metrohm hanging mercury drop electrode (HMDE) (model VA 663) was used for the measurement of folic acid in algal cultures; a Metrohm E-506 polarograph was used in the AC-mode (frequency 75 Hz, 10 mV amplitude, 90° phase angle) to determine the capacitance current in adsorption experiments; and a PAR 174A polarograph connected to a Metrohm HMDE was used to determine folic acid in lake and sea water samples.

Sodium chloride was purified by heating to 450 °C. Folic acid in natural water samples was determined after buffering at pH 8.3 with borate pH buffer (1 M boric acid, 0.4 M NaOH), final concentration 0.01 M. Folic acid solutions were diluted daily from a 0.02 M stock solution which was prepared weekly in 0.1 M NaOH and kept in the dark at 4 °C. Sample bottles were cleaned with chrome-sulphuric acid, and subsequently with 10% nitric acid, and Milli-Q water. All chemicals used were AR quality from Merck. Humic acid was from Aldrich. Its characteristics are described elsewhere.[12]

The determination of folic acid by CSV was similar to that of Le Gall and van den Berg:[2] culture media were filtered to remove the algae (1–2 mL through a GF/F glass fibre filter) and diluted to 10 mL with MQ water to minimize interference by organic surfactants occurring in the algal suspensions. The dilution factor was 10 for low folic acid concentrations (up to 50 nM) and 20 for higher folic acid concen-

trations. The sensitivity was calibrated for each sample individually with folic acid additions to correct for interference by natural surfactants.

Chlorophyll-a was determined spectrophotometrically according to the method of Parsons[13] using a Unicam SP8-1000 Spectrophotometer. Cell numbers were counted using an inverted microscope (Wild Heerbrugg) according to established methods.[14] Sample aliquots (5 mL) were thereto preserved with 20 μL Lugol solution. Volumes between 0.1 and 1 mL were used for counting.

Monocultures of *Emiliania huxleyi*, *Dunaliella minuta*, and *Phaeodactylum tricornutum* were grown in 250 mL F/2 medium in synthetic seawater (sterilized by heating), to which 1 mL of algal culture was added giving a starting concentration of ~ 3×10^3 cells mL^{-1}. The cultures were originally axenic and obtained from MBA, Plymouth, but microscopic inspection showed that the axenic condition had not been maintained by the time the cultures were used for these experiments; no attempt was made to restore the axenic condition. Incubation was carried out in temperature controlled incubator (Mercia Scientific) set to continuous (24 h) illumination and to 15 °C. Folic acid was spiked at the beginning of exponential growth (usually after 3 days), as established in prior experiments.

Seawater samples were collected from the Adriatic (44°59'N; 12°19'E) and from a beach near Selce (North Adriatic coast, Croatia) and the seasonally anoxic Rogoznica lake (Mid Adriatic coast, 43°N; 15°58'E). The samples (except for that from Selce) were filtered immediately upon sampling by vacuum filtration (using a mild vacuum of ~ 0.3 Bar below atmospheric pressure; through Whatman GFF filters, pore size 0.7 μm; pre-treated by heating to 450 °C) and stored at 4 °C until analysis.

RESULTS AND DISCUSSION

Interference of Humic Acid with the CSV Response for Folic Acid

Humic acid additions to folic acid solutions caused the peak height for folic acid to become suppressed at very low concentrations of HA (Figure 1). This suppression occurred both in the sodium chloride solution (0.55 M) and in seawater (both at pH = 8.3) but the suppression was weaker in the seawater. The ionic strength of the seawater is similar to that in the salt solution, so the difference in the double layer thickness was minimal; more specific interactions of the HA and the folic acid with the major cations in seawater are likely to play a role in this difference.

Humic acid is a large and complex molecule with charged as well uncharged sites similar to folic acid. Interactions similar to complexation reactions are therefore not impossible. The suppression of the FA response by HA could therefore be due to complexation of the folic acid with the HA or by competitive adsorption of the HA on the electrode. The voltammetric response of folic acid (Figure 2) in the presence of HA (0, 0.5 and 1.5 mg/L HA) was linear, in line with a mechanism consisting of competitive adsorption in which adsorbed HA is displaced by added folic acid, rather than

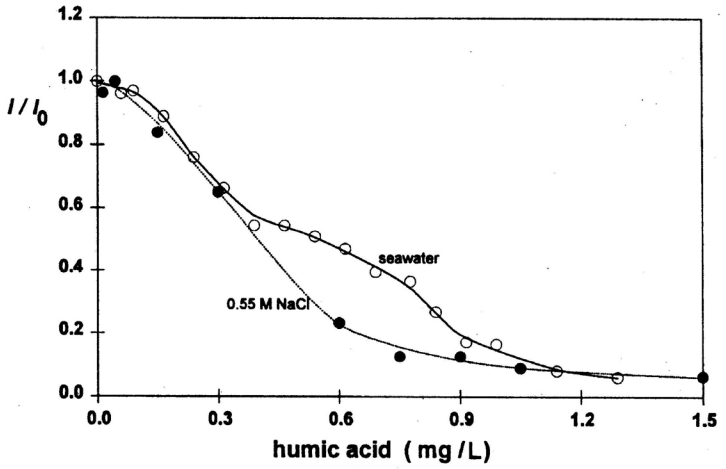

Figure 1. Decrease of the peak height of FA (40 nM L^{-1}) upon addition of HA from 0 to 1.5 mg L^{-1}; I/I_0 is the ratio of the peak height for folic acid in the presence over that in the absence of HA.

curved upwards, which would indicate suppression due to complexation reactions occurring in solution until saturation of the complexation sites on the HA with the added folic acid similar to metal complexation[11,15] reactions. The sensitivity for the folic acid determination was strongly decreased at increased HA concentration causing the plots at different HA concentrations to have greatly differing slopes.

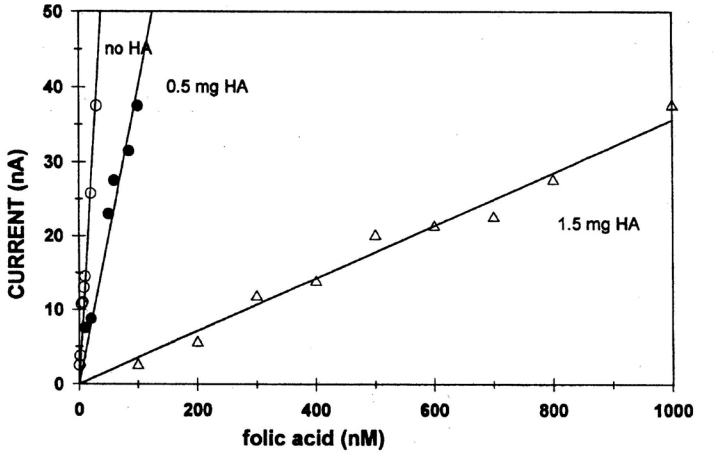

Figure 2. Calibration graphs for folic acid in 0.55 M sodium chloride in the absence and presence of added HA.

Adsorption isotherms were computed from the capacitance suppression caused by displacement of water molecules in the electrical double layer by adsorption of HA and folic acid on the mercury electrode; the capacitance suppression was determined using AC-voltammetry. The measurements represent steady state rather than equilibrium conditions: a constant adsorption time was used, limited to 60 s since the electrode would become practically fully covered if the adsorption were extended until equilibrium conditions were met due to the very strong adsorption of these organic molecules on the mercury surface. Comparison of the adsorption isotherms on a scale of mg L^{-1} organic matter (Figure 3) show that the folic acid is adsorbed more strongly than the HA as the electrode surface is fully covered at lower folic acid than HA concentrations. The slope of the adsorption isotherm for folic acid in the presence of 1.8 mg L^{-1} HA is steeper, and full electrode coverage is attained before that of humic acid alone, illustrating the stronger adsorption of folic acid and also indicating that the adsorption is additive, so both molecules are adsorbed simultaneously.

The presence of HA in natural waters clearly interferes with the folic acid determination, and UV-digestion, which is the usual method for removing the interference by natural surface active substances in natural waters, cannot be used in this case as it would also efficiently destroy the folic acid. Previous work has indicated that folic acid adsorbs poorly on Sep-Pak C18 cartridges at high flow rates but that it is adsorbed efficiently at very slow flow rates of 0.5 mL min^{-1}.[3] A high flow rate of 15 mL min^{-1} and a lowered pH (pH 2) were used here to test the removal of HA from seawater without

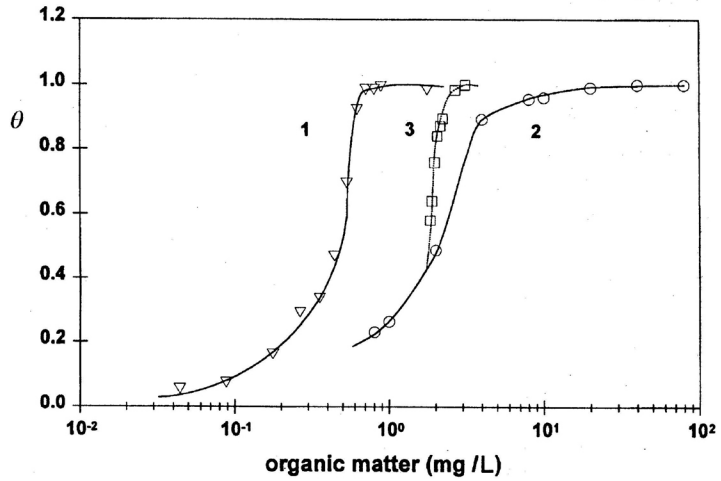

Figure 3. Adsorption isotherms of folic acid (1), humic acid (2), and their mixture (1.8 mg L^{-1} HA + additions of FA) (3); the adsorption time was 60 s at –0.2 V; θ = degree of surface coverage.

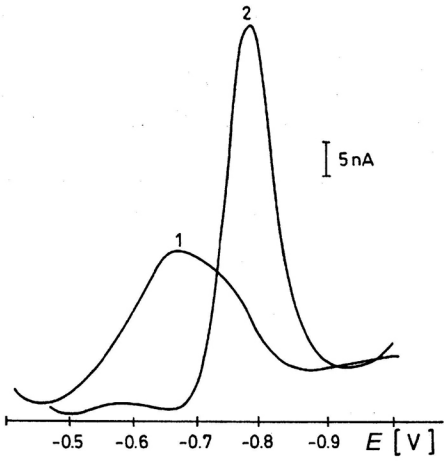

Figure 4. Voltammetric waves of 10 nM L^{-1} folic acid in the presence of 1.9 mg L^{-1} HA in 0.55 M NaCl also containing borate pH buffer; 1: before and 2: after Sep-Pak C18 treatment.

removing folic acid. The sample pH was adjusted again to pH 8.3 after passage through the column. Prior to this treatment, the folic acid peak height was fully depressed by the presence of 1.8 mg HA (Figure 4, curve 1) whilst the peak was fully recovered after the Sep-Pak treatment indicating that this is a convenient sample treatment for natural water samples containing interfering levels of humic acids. This method was used to treat samples from lake water and seawater for folic acid analyses.

Folic Acid in Seawater and Lake Water Samples

Voltammetric analyses of seawater from the North Adriatic revealed the presence of folic acid throughout the year (Table I). The highest folic acid concentration was found in a surface seawater sample in May, which may be the result of increased biological activity in spring. The surface folic acid concentration was less than the deeper water concentrations in July and September possibly because of the sensitivity of folic acid to photo-decomposition. However, the July data show that the folic acid concentration was rather variable over the 30 m sampled. Other data from the North Atlantic have shown the presence of folic acid throughout the oceanic water column, suggesting in-situ production and uptake of the folic acid by marine microorganisms.[3] Our data are in agreement with this previous work.

Lake water samples were also found to contain folic acid. Lake Rogoznica, near Šibenik (Croatia) is connected to the Adriatic and has a salinity varying between 31–38. The deeper waters, below 8m from an overall

TABLE I

The concentration of folic acid in lake water and seawater samples. The samples from the North Adriatic (station 101) and Rogoznica Lake were filtered, whereas those from shallow coastal waters in the North Adriatic near Selce were not filtered.

North Adriatic (station 101)	May 1994 nM L^{-1}	July 1994 nM L^{-1}	Sept. 1994 nM L^{-1}	Nov. 1994 nM L^{-1}	Feb. 1994 nM L^{-1}
0 m	4.3	0.73	0.6	0.94	0.03
5 m		1.7	1.0	0.06	0.9
10 m		0.3			
20 m		0.2			
30 m		2.0			
North Adriatic – Selce	Aug. 1995 nM L^{-1}				
0 m	0.03				
Rogoznica Lake	April 1994 nM L^{-1}	June 1994 nM L^{-1}	April 1995 nM L^{-1}	Oct. 1995 nM L^{-1}	
0.5 m	4.0	0.18		3.0	
2.0 m			3.8		
8.0 m			3.7		
11.0 m			36.0	3.5	

depth of ~ 12 m of the lake, are anoxic. The folic acid concentration in the surface waters of the lake in April 1994 was 3.4 nM L^{-1}, similar to that found in the Adriatic surface waters in May, whereas in June it dropped to 0.18 nM L^{-1} again, similar to the situation in the North Adriatic.

The deeper lake waters contained high sulfide concentrations, reaching 100 μM L^{-1} at an 11 m depth in April 1994.[16] The folic acid concentration in the lake increased greatly from levels of ~ 4 nM L^{-1} at 2 and 8 m depth, to 36 nM L^{-1} in the anoxic waters at an 11 m depth (Table I). The 11 m sample was measured after dilution (1:100) to minimize the interference by the sulfide (the combined concentration of sulfur and sulfide was 600 μM L^{-1} in this sample[17]), which produces a voltammetric peak at a more positive potential than that of folic acid. The folic acid maximum in the anoxic waters coincided with the chlorophyll maximum, and the maximum in the concentration of iodide.[17] The anoxic conditions were much reduced in the lake in October 1995 when the concentration of sulfur and sulfide was lower than 10 nM L^{-1} throughout the water column and the concentration of folic acid was not enhanced at the 11 m depth. The high folic acid concentration in the anoxic waters is presumably derived from sinking decomposing algae and is not broken down by oxidative bacterial activity in the anoxic conditions.

Uptake of Folic Acid by Microalgae

Variations in the folic acid concentrations in these waters and in the water column of the Atlantic ocean[3] suggest localized production as well as uptake by microorganisms. Uptake by marine phytoplankton could be of interest if it could be shown that they derive an advantage from this uptake. This uptake was therefore studied using algal cultures. Reproducibility of the algal cultures was established in preliminary cultures. The exponential growth phase started after three days of incubation, and this stage was maintained for three to four days whereafter it was followed by the stationary phase.

Relationships between the number of cells and the chlorophyll-a concentration were established by comparing the chlorophyll-a concentration with the number of cells. Linear relationships between the concentration of chlorophyll-a and cell number were obtained well into the exponential phase with ratios (μg chlorophyll-a/10^9 cells) of 1.17 (*Emiliania*), 1.19 (*Dunaliella*) and 0.34 (*Phaeodactylum*). The specific growth rate of Phaeodactylum was computed as 0.706 day^{-1}.

Preliminary experiments showed that folic acid added to the seawater/algal medium was broken down slowly during incubation in the absence of algae: an addition of 50 nM folic acid decreased by ~ 20 nM after 10 days (Figure 5). Any decreases as a result of the uptake by algae therefore had to be at least of similar magnitude to become clearly apparent. Experiments in the presence of algae showed that the decrease was sometimes significantly less than in their absence (if no uptake occurred) presumably due to shading by the algal cells.

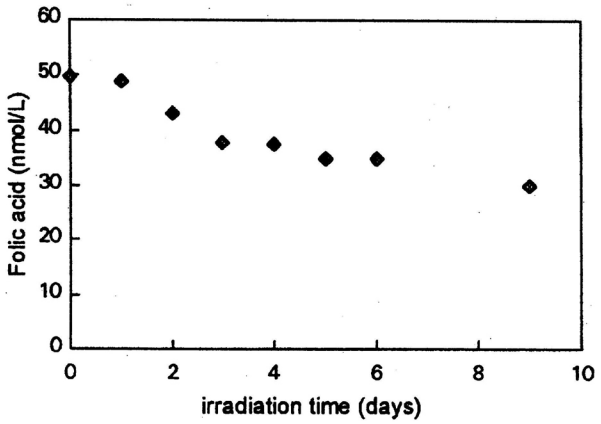

Figure 5. Breakdown of folic acid in algal medium in sea water during incubation in the absence of algae. The folic acid concentration (initially 50 nM L^{-1}) was monitored by CSV.

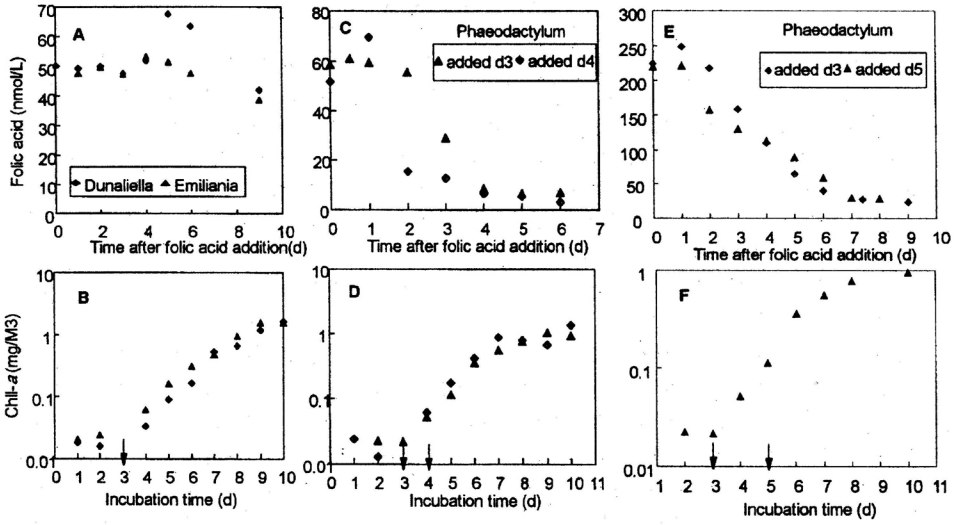

Figure 6. Folic acid uptake by *Dunaliella* (A), *Emiliania* (A), and *Phaeodactylum* (C and E); 50 nM L^{-1} folic acid was added in A and C, 200 nM L^{-1} was added in E. Growth curves are shown in B (*Dunaliella* and *Emiliania*), and D and F (*Phaeodactylum*).

The folic acid concentration in algal cultures of *Emiliania* and *Dunaliella* (50 nM folic acid added on day 3 of the cultures, the beginning of the exponential growth) remained stable for the next five days (Figures 6A and B): these species therefore did not consume folic acid throughout the exponential growth phase. Nine days after spiking the folic acid concentration decreased to about 40 nM L^{-1} in both cultures, which may have been caused by photochemical decomposition as a result of the irradiation rather than by uptake. The folic acid concentration appeared to increase (to ~ 65 nM 5–6 days after the folic acid addition) in the *Emiliania* culture: later measurements in media in which the cells had been damaged by acidification (see below) showed the presence of a peak close to that of folic acid but due to different organic matter which may have contributed to this increased peak height.

Contrary to the other two microorganisms, the *Phaeodactylum* culture clearly showed uptake of folic acid: folic acid at levels of 50 nM L^{-1} and 200 nM L^{-1} was consumed rapidly during the exponential growth phase. The uptake became most rapid after a short delay period of one to two days following the folate addition (Figures 6, C and E). The folic acid was consumed more rapidly when added later in the exponential growth phase, on day 4 (addition of 50 nM L^{-1}, Figure 6C) and on day 5 (addition of 200 nM L^{-1}, Figure 6E) presumably due to the greater number of algal cells. The shape

of the uptake curves of folic acid has an exponential appearance, mirroring the growth curves of the cultures, suggesting that the algal growth (rather than adsorption on the cells) is the dominant factor controlling the folic acid consumption.

The cultures were not axenic, so it is possible that bacterial uptake played a role in these experiments. However, the uptake was limited to the *Phaeodactylum* culture and the rate of uptake paralleled the rate of growth of this algal species, suggesting that if the uptake was bacterial rather than algal, the uptake was by bacteria specifically associated with the *Phaeodactylum* culture and with a parallel growth rate.

The removal of folic acid from the culture media could be due to adsorption onto the cell walls or to uptake followed by conversion to other material inside the cells. The possibility of adsorption was eliminated by measuring whether folic acid was released from the cells upon acidification of the culture to pH 2 with hydrochloric acid at the end of the uptake experiment and equilibration at this pH for an hour; this treatment caused the cells to become severely damaged. One hour later, the culture was filtered and the filtrate was analyzed by CSV in the usual conditions: a peak appeared at −0.76 V, approximately 50% higher than the original peak due to the folic acid but at a more positive potential than that of folic acid (at −0.83 V) which was not present in the filtrate. Previous experiments with humic acid showed that the folic acid could not have been masked by dissolved organic matter. Apparently, the consumed folic acid was not present as free molecules inside the microorganisms, presumably having been transformed into other organic compounds, possibly contributing to the large peak. The abundant folate uptake by *Phaeodactylum* (or the associated bacteria) could assist these microorganisms in periods of limitation by other essential compounds, which could be investigated in further experiments.

CONCLUSIONS

The reduction in the peak height for folic acid in the presence of humic acid is due to competitive adsorption on the mercury drop electrode rather than to complexation interactions in solution. Samples containing high concentrations of humic acids can be pre-treated with a Sep-Pak C18 column to remove the HA which is adsorbed at high flow rates when the folic acid is not adsorbed.

Seasonal and depth variations of folic acid were apparent in samples from marine and lake origin: surface waters contained relatively high folic acid concentrations in the spring with lower levels in the summer, possibly as a result of photochemical breakdown. Especially high levels occurred in anoxic waters, suggesting that folic acid released from sinking algal cells is

normally broken down in oxygenated waters but is accumulated in the reducing anoxic waters. Algal cultures indicated that the folic acid is available to some species of phytoplankton: uptake was clearly apparent by one (*Phaeodactylum*) out of three species tested (no uptake by *Emiliania* and *Dunaliella*), suggesting that the uptake of folic acid may be a luxury uptake rather than that of an essential nutrient. The finding of the uptake by *Phaeodactylum* is somewhat tentative as uptake by bacteria specifically associated with this species could not be eliminated.

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SAŽETAK

Voltametrijsko ispitivanje reaktivnosti folne kiseline u kulturama alga i prirodnoj vodi

Marta Plavšić, Xiankun Lu i Constant M. G. van den Berg

Folna kiselina (FA) se može odrediti u prirodnim vodama adsorptivnom voltametrijom katodnog otapanja. Na mjerenja količine folne kiseline (FA) utječe prisutnost humusne kiseline (HA) u prirodnim vodama. Kod 2 mg/L HA voltametrijski val FA je u potpunosti iščeznuo. Iz mjerenja neravnotežnih adsorpcijskih izotermi zaključeno je da je adsorpcija FA i HA kompetitivna i da se FA jače adsorbira od HA. Mjerenja koncentracije reaktivne folne kiseline pri niskim koncentracijama HA nisu uputila na moguće kompleksiranje folne kiseline s humusnom kiselinom analogno reakciji kompleksiranja metalnih iona. Utjecaj HA na mjerenje FA uklonjen je propuštanjem uzorka kroz kolonu Sep-Pak-C-18. Morski i slatkovodni uzorci iz jezera pokazali su prisutnost folne kiseline. Eksperimenti s kulturama morskih algi pokazali su da Phaeodactylum, za razliku od kultura *Emiliania* i *Dunaliella*, uzima dodanu folnu kiselinu, iako prisutne bakterije mogu doprinjeti uzimanju FA, jer kulture nisu bile aksenične. Rezultati eksperimenata upućuju na moguću važnost folne kiseline za neke vrste morskih mikroorganizama.