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IT ALL STARTED WITH MARGALEF'S PAPER OF 1951

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ABSTRACT
As early as 1951, Margalef speculated on the production of soluble phosphatases by zooplankton; "entomostracans" were producing their own phosphatases and so contributing to phosphate regeneration. After analytical techniques had been considerably improved and introduced into limnology laboratories, it was verified, almost three decades later, that the eminent scientist was right. The role of soluble phosphatase in fresh waters can be investigated either in straight relationship to plankton phosphatase producers or simply in connection with other chemical entities abundant in lake water, with capacity to condition enzyme activity. Such is the case of humic substances which complex with soluble phosphatase; the enzyme activity will be inactivated when making part of the complex (linked to the humic molecule) and reactivated again once the complex is broken down, e.g. by the action of UV light from the sun.
Key words: *Daphnia*, phosphatase, hydrolysis, photolysis

Todo empezó con el trabajo de Margalef de 1951

RESUMEN
Desde 1951 Margalef especuló sobre la producción de fosfatasas solubles por zooplancton; "entomostracans" produjeron sus propias fosfatasas contribuyendo así a la regeneración de fosfato. Casi tres décadas después se ha confirmado que el eminente cientista tenía razón, después de que las técnicas analíticas hayan mejorado significativamente y se hayan introducido en los laboratorios de limnología. El papel de la fosfatasa soluble en aguas dulces puede ser investigado en estrecha relación con el plancton productor o sencillamente en relación con otras entidades químicas que abundan en el agua de los lagos, con capacidad para condicionar la actividad de enzimas. Es el caso de las sustancias húmicas que forman complejos con fosfatasa soluble; la actividad enzimática será desactivada cuando hace parte del complejo (ligado a la molécula húmica) y reactivada de nuevo cuando el complejo es roto, por ejemplo por la radiación UV solar.

Palabras clave: *Daphnia*, fosfatasa, hidrolisis, fotolisis

INTRODUCTORY NOTE

This is a brief article, intended to be a tribute paid to Ramon Margalef, five years after his death on the 23.rd of May, 2004. It will be written from the point of view of a person who, before meeting him, was inspired by his work to do her Master thesis, her Ph.D. dissertation, and about half of her research thereafter. After meeting him, and similarly to

what happened to practically everyone, she would be taken by his natural sympathy and enjoy his company very much.

THE FIRST SUSPITION

I was at the time living in the United States, at Kent, Ohio, teaching and studying at Kent State University. After one year of course work for the Master degree, I needed a topic for my thesis. It was the year of 1980, there was no internet and only a few computers. One night, at the Kent State main library, I was fascinated with a paper published by Margalef, respecting the production of phosphatase activity by *Daphnia* (Margalef, 1951).

Margalef had run experiments in which he was measuring the phosphatase activity of both algae and zooplankton. He attributed the production of phosphatase activity to zooplankton, showing that the levels of the enzyme detected in his experiments could not be accounted for by the low density of algae present in his experimental set up. With an amazing intuition, Margalef suspected that not only phytoplankton, but zooplankton as well, were contributing directly to the production of phosphatase, and therefore indirectly contributing to the regeneration of orthophosphate in lakes.

FROM MARGALEF'S SPECULATION...

The topic was found, and I set up to demonstrate, utilizing equipment and laboratory techniques of almost 30 years later, that zooplankters were really producing their own phosphatases and not only releasing those ingested with their algal food (Boavida and Heath, 1984). In my experiments I did use *Daphnia magna* fed *Chlamydomonas acidophila*; *Daphnia* were grown in small aquaria and *Chlamydomonas* cultures were kept in a light-and-dark cycle of 12 h each and fed to the zooplankton only in exponential phase of growth (more details in Boavida and Heath, 1986).

It was believed by the scientific community of the time that the phosphatase released by *Daphnia*, and other herbivorous zooplankton as well, was the phosphatase produced by their algal food, eventually released into lake water after algal cell disruption in the digestive tracts of the zooplankton. The main purpose of the work was to determine whether the enzyme released by *D. magna* originated from the *Daphnia* itself, from its algal prey, or from both.

By growing one single zooplankter (*D. magna*) on one single, controlled algal food (*C. acidophila*) and by analyzing the phosphatases in the culture medium of *Daphnia* and comparing the eluted enzymes by anion exchange chromatography with those eluting from *Chlamydomonas* treated with a membrane detergent, it was concluded that the phosphatases released from *D. magna* were those of their algal food plus other of their own production; the elution peaks were very well distinct (Boavida and Heath, 1984). The conclusion that *D. magna* were producing their own enzymes corroborated what Margalef had apprehended 30 years earlier, with no technical means to demonstrate it.

PHOSPHATASES IN THE ENVIRONMENT

It was also found that algae were producing several phosphatases each, alkaline and acid, adaptively and constitutively (e.g. Boavida and Heath, 1986). If so many organisms in the phyto- and zooplankton were producing the enzyme, then it would have to be of some relevance to the several communities. It was known that phosphatases would hydrolyze larger phosphorus molecules in lake water (mainly phosphomonoesters, according to Kuenzler and Perras, 1965) and release orthophosphate in the process (Heath and Cooke, 1975).

However, when the importance of phosphatase activity for the whole lake metabolism was inferred, the result was somewhat a deception. In conditions of P-limitation, it seemed that

the biological demand for phosphorus was much larger than the orthophosphate supplied for growth by hydrolysis. The main substrate for alkaline phosphatase in natural environments is phosphomonoesters, therefore these molecules will have to be present in lake water simultaneously with the enzyme. In eutrophic East Twin Lake (Ohio, U.S.A.) the phytoplankton was limited by available phosphorus all year around. The significance of hydrolysis by phosphatase was assessed by comparing the orthophosphate release rates with its uptake rates, on a seasonal basis. Although the correlation obtained between alkaline phosphatase and phosphomonoesters in lake water was negative and highly significant ($P < 0.01$ for the significance of r), meaning that this process was important in the regeneration of orthophosphate, the phosphate made available through hydrolysis was but *circa* one percent of the total orthophosphate required for growth (Boavida and Heath, 1988). It was concluded that, although potentially important in the regeneration of orthophosphate, hydrolysis by alkaline phosphatase was insufficient to explain phytoplankton growth in terms of P-limitation in East Twin Lake.

The role of enzymatic hydrolysis by alkaline phosphatase as a recycling mechanism remained unclear, since the fraction of phosphorus made available by this mechanism was apparently very small with respect to the total phosphate demand of the seston, in spite of what was indicated by the high phosphatase activity found in lake water. In a mesotrophic lake (Lake Maggiore, Italy) very high levels of alkaline phosphatase activity were found concomitant with incommensurable, below detection levels of phosphomonoesters (Boavida, 1990, 1991). This result suggests that all phosphomonoesters in lake water were being utilized as substrate for phosphatase, and even though the released orthophosphate was not enough to justify all the phytoplankton growth, as evidenced by the situation of P-limitation. There were other autochthonous sources of phosphorus in that lake. The need for more research respecting this topic was obvious.

After what was found up to here, one question arose: If phosphatases are unimportant, why is there so much of them and so many organisms spending energy to produce these enzymes?

So far all research had been done in natural lakes. Would the results be similar if the same kind of research was undertaken in artificial lakes? The rare opportunity to study these aspects of phosphorus regeneration on a 35-year old reservoir being emptied for repair came up. Phosphomonoesters seemed to be relatively few during the emptying phase, although they gradually increased to substantial concentrations with the refilling. Alkaline phosphatase, on the other hand, increased with emptying of the reservoir but decreased when refilling began. In this study Marques and Boavida (1993) found that, although the orthophosphate concentrations were relatively high, in some periods alkaline phosphatase seemed to have higher importance in phosphorus dynamics, especially towards the end of the refilling process.

Another study on eutrophic reservoirs produced distinct results (Boavida and Marques, 1995). To assess the potential importance of alkaline phosphatase in orthophosphate regeneration, the activity of the enzyme was determined concomitantly with the determination of phosphomonoester concentrations, among other chemical forms of phosphorus. Contrary to the expectations for such productive waters where algal blooms were frequent, during the study period this process of phosphorus regeneration was not significant, probably because the product of hydrolysis was always abundant. It was concluded that, in spite of what had been repeatedly observed in natural lakes with similar trophic characteristics, the readily available fraction of phosphorus in these reservoirs was large and for that reason alkaline phosphatase activity was low. What seemed intriguing was the low concentration of phosphomonoesters found in the water; with little phosphatase activity this phosphorus fraction should always be high, unless hydrolysis took place as

soon as phosphomonoesters were released into the water in the organic matter decomposition process.

CONTRADICTIONS IN THE SCIENTIFIC FINDINGS

As more research was done on the subject, inconsistencies and contradictions gradually appeared. It was sure that plankton organisms were producing phosphatases. The doubts resided on the potential significance of the enzymes to orthophosphate regeneration and on the role of the enzymes as indicators of lake trophic state as well.

On one hand, alkaline phosphatase was found to be produced in large quantities by planktonic algae in response to low orthophosphate concentration in the environment (e.g. Smith and Kalff, 1981). In addition, alkaline phosphatase was included among the good indicators of eutrophication (Istvanovics et al., 1992). On the other hand, when a set of four European lakes and reservoirs of several degrees of trophy were examined (Boavida et al., 1997), inverse correlations were expected between enzyme activity and the substrate and also between phosphatase activity and the product of enzymatic hydrolysis, since it was assumed that the phosphatases were being produced adaptively, i.e. in response to the low concentrations of orthophosphate in the water. Some kind of phosphatase variation was also expected, following the trophic gradient. No significant correlation was found, however, between alkaline phosphatase activity and the concentration of phosphomonoesters, and a highly significant, although positive, correlation between alkaline phosphatase and soluble reactive phosphorus (taken as a measure of orthophosphate) was found. According to the above cited literature alkaline phosphatase should be produced as a response to low orthophosphate in the environment – that did not seem to be the case. Other enzymes, such as 5'-nucleotidase (Cotner and Wetzel, 1991) could be more relevant to phosphorus regeneration in the oligotrophic lake where phytoplankton likely were P-limited and bacteria were limited by organic carbon availability as substrates.

Unlike the overwhelming number of references in the literature for the importance of potential orthophosphate release by alkaline phosphatase, evidence for the "non importance" of alkaline phosphatase in orthophosphate regeneration is hard to find.

ONE EXPLANATION IS OFFERED

After some investigation on phosphorus dynamics in bog lakes, it was thought that orthophosphate would be released from humic substance molecules upon irradiation of these with mild UV light (Francko and Heath, 1979). This would be the UV light from the sun, at the intensities it reaches the earth surface and penetrates lake water only a few centimeters.

Later on it was found that orthophosphate concentrations in lake water would increase, not as a direct consequence of the degradation of complex phosphorus by UV, but, instead, as a result of more complex mechanisms involving phosphatase enzymes (Wetzel, 1991). Humic substances resulting from organic matter decomposition are usually abundant in lake water. These are able to form large complexes with phosphatases, when the two kinds of molecules approach each other in the natural hydrodynamics in the lake. When the phosphatases form complexes with the humic substances, they are inactivated. But, once these large phosphatase/humic complexes are hit by the UV light of the sun, the two original molecules separate and the phosphatase, once free in lake water, becomes active again and capable of performing hydrolysis (Wetzel, 1993).

All this was later tested in laboratory experiments where humic extracts of specific macrophytes were irradiated with low intensity UV light, similar to that of the sun, and the results corroborate what was said in the last paragraph: Phosphatase was able to complex with the humics, and in that condition no phosphatase activity could be measured, showing

that the enzymes were inactivated. After irradiation with weak UV light, to break down the humic/phosphatase complexes, phosphatase activity would again be measured, showing that the phosphatase was free and reactivated (Boavida and Wetzel, 1998).
In some relatively recent work (Geraldes and Boavida, 2003) it was seen that none of the factors investigated in two different lakes (water chemistry, reservoir age, and external disturbance) made a difference in phosphatase activity. It appears that much is still to be investigated on the role of phosphatases in freshwater environments (Boavida 2000a). It is necessary to go back to this already old subject and search for the actual function of phosphatases in freshwater environments, utilizing the new techniques available and all the knowledge scientists have accumulated until now, which they didn't have a few decades ago (Boavida, 2000b).

CONCLUSION

The control of eutrophication may be effected through regulation of phosphatase activity if a means is found to prevent UV photolysis, thus kipping phosphatases complexed to humic substances and, therefore, inactive, unable to perform hydrolysis and make orthophosphate available. It is very important to understand every phenomenon going on in lakes. As Margalef himself said about two decades ago, "The epicontinental (freshwater or limnic) part of the biosphere is not only a section of the global water cycle, but a most important part of it" (Margalef, 1991).

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