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The influence of selected water quality parameters on the decay rate and exoenzymatic activity of detritus of *Nymphaea alba* L. floating leaf blades in laboratory experiments

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Summary. A chemostat study was conducted to investigate the influence of water quality parameters related to acidification processes on the decomposition of floating leaves of *Nymphaea alba* L. HCO_3^- and total Al concentration and pH influenced the decay rate. The activity of four cell-wall decaying exoenzymes was measured in the detritus. The activity of two types of pectic enzyme and xylanase was low under acid conditions. Cellulase activity was little affected. The exoenzyme activity seemed to be influenced by the pH of the water within the detritus. Inhibition of pectic enzymes under acid conditions may be an important factor causing the slow decay of macrophyte remains in acid waters.

Key words: Decomposition – Acidification – Cell wall degrading enzymes – HCO_3^- – *Nymphaea alba*

Decomposition is a key process in the cycling of nutrients and carbon and it can be regarded as the counterpart of primary production. In aquatic ecosystems, most of the energy captured by the photosynthesis of macrophytes is made available to the rest of the ecosystem through degradation processes. Only a small part of the aquatic vascular plant material produced is consumed directly by herbivores (Pidgeon and Cairns 1981; Polunin 1982; Van der Velde et al. 1982; Van der Velde and Van der Heijden 1985). The role of decomposition in the nutrient cycles has been described by many authors (e.g. Brock 1984; Hemminga et al. 1988; O'Connel 1988; Kok et al. 1990b).

The factors leading to the mass loss of decaying macrophyte remains can be divided into physical factors like diffusion (leaching) of soluble components and fragmentation by wave action, and biological factors like fragmentation by detritivores and degradation by microorganisms. Saprophytic microbes cause about 60–70% of the mass loss of macrophyte detritus during decomposition (Harrison and Mann 1975; Brock 1984). Microbial degradation can therefore be regarded as the main process in decomposition.

The soluble compounds of dead plant material are very rapidly lost by leaching, so that the main substrate of saprophytic microorganisms consists of the insoluble parts of the material, mostly cell wall components like cellulose, hemicelluloses, pectines etc. (Chamier and Dixon 1982). Bacteria and fungi degrade their substrate by means of hydrolytic exoenzymes and the resulting soluble oligomers and monomers are taken up.

All the enzymes necessary for the degradation of the cell wall polysaccharides can be produced by the microbial community of decaying macrophyte remains (Chamier and Dixon 1982; Benner et al. 1986; Kok et al. in press). It is likely that the activity of exoenzymes in the decomposing material is a very important factor determining the rate of decay. There is, however, little information on the time course of the exoenzymatic activity during degradation of the plant detritus and the relation between decay rate (rate of mass loss from litter bags) and exoenzymatic activity. Information on these factors and their relation to water quality parameters would greatly enhance our understanding of the process of decomposition of macrophyte material in aquatic systems.

One factor greatly affecting the decomposition of plant remains in the aquatic environment is pH. In waters strongly influenced by acid and acidifying atmospherical deposition, the decomposition rates of plant material are generally much lower than in circumneutral or alkaline waters (Traaen 1980; Mckinley and Vestal 1982; Hoeniger 1985).

Besides a decline in pH, chemical changes in acidifying waters include a decrease of buffering capacity (HCO_3^- concentration) and a rise in Al and NH_4^+ concentration (Vangenechten 1980; Henriksen 1982; Leuven and Kersten 1988). All these water quality changes affect the decay rate of aquatic macrophytes (Rao and Dutka 1983; Brock et al. 1985; Palumbo et al. 1987; Leuven and Wolfs 1988). Therefore, it is likely that the combined

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changes in these chemical factors during acidification of aquatic systems are a major cause of slow decomposition in acid waters. The causal relation between the observed changes in water chemistry and the slow decay under acid circumstances has been proven in laboratory experiments (Brock et al. 1985; Leuven and Wolfs 1988; Kok et al. 1990a).

Brock et al. (1985) state that the chemical composition of the decaying plant material differs in alkaline and acid waters. In acid environments, these authors found an accumulation of sugar components belonging to the hemicellulose class. This indicates that certain processes in the exoenzymatic degradation of the cell wall are inhibited at low pH.

The ability of saprophytic microorganisms to produce certain types of exoenzymes in pure culture is clearly influenced by the chemical composition of the culture medium. For instance, the production of the enzyme pectin lyase by saprophytic fungi is inhibited by low pH (<6.5) and low calcium concentrations (Chamier 1985). Growth of selected aquatic fungi on pectin and isolated cell walls was also shown to be inhibited by low pH (Kok et al. in press). However, pH has not been found to influence mycelium growth on crystalline or amorphous cellulose. This indicates that, under acid circumstances, pectin and possibly hemicellulose degradation is slow.

This leads to the hypothesis that the activity of pectin and hemicellulose degrading eznymes is specifically inhibited under acid circumstances, and a laboratory study was conducted to test this hypothesis.

Materials and methods

The influence of $NaHCO_3$ concentration, pH, NH_4^+ and Al on the decay rate of *Nymphaea alba* floating leaf blade detritus, and the time course of (endo)cellulase (EC 3.2.1.4.), (endo)xylanase (EC 3.2.1.32), (endo)polygalacturonase (EC 3.2.1.15) and pectin and pectate lyase (EC 4.2.2.2 and 4.2.2.10), were studied experimentally.

Senescent floating leaf blades of *Nymphaea alba* were collected in the Voorste Choorven, (The Netherlands, 51° 34′ 54″ N, 5° 12′ 18″ E), a small, acidified moorland pool. The leaves were transported to the laboratory in polyethylene bags and stored in a freezer at −20° C until further use.

Leaf discs were obtained from the leaves using a cork borer (1.2 cm diameter). Litter bags (5 × 5 cm, mesh size 2 mm) were filled with 5 g (fresh weight) of leaf discs. Subsamples of the frozen leaf discs were lyophilized and the fresh weight/dry weight ratio was determined. Using this ratio, the initial dry weight in the litter bags was calculated. After the litter bags had been harvested, the material was lyophilized and reweighed. Dry weight of the detritus in the harvested litter bags was expressed as percentage of the initial dry weight. The lyophilized material was stored in a freezer at −20° C until determination of the exoenzymatic activity.

The experiments were carried out in 15-l glass aquaria placed in a water bath at 20° C. The aquaria were covered with black plastic to prevent algal growth. The water in the aquaria was continually refreshed from 120-l black polyethylene stock containers by means of peristaltic pumps (15 l · day^{-1}). The media were made by adding chemicals to twice demineralized water. The basic medium was the same as that used by Brock et al. (1985). Media differing in buffering capacity, pH and NH_4^+ and Al concentration were prepared by addition of sodium bicarbonate, NH_4Cl, $NH_4Al(SO_4)_2$ and/or hydrochloric acid to the basic medium. Since at pH 4.0 Al is present not only as Al^{3+} but also in hydroxide form (Dickinson Barrows 1977), Al concentrations mentioned here refer to total Al. The additions can be read from Table 1. Sampling was done weekly, and three litter bags were harvested for each treatment.

Table 1. Chemicals added to the basic medium to prepare the various solutions and the resulting pH of the media

Treatment	Additions	pH
1	5 mM $NaHCO_3$	8.0
2	0.5 mM $NaHCO_3$	7.2
3	0.3 mM $NaHCO_3$	6.5
4	–	7.2
5	HCl	4.0
6	HCl + 25 μM $NH_4Al(SO_4)_2$	4.0

Preparation of substrates for exoenzyme activity measurement

Cellulase and xylanase activity were estimated using the dye-release method of Biely et al. (1985). Substrates of the enzyme assays were carboxymethylcellulose (CMC) and xylan (both purchased from Sigma). Both polysaccharides were stained with Remazol Brilliant Blue (RBB) (Sigma). CMC or xylan (1 g) were dissolved in 30 ml twice distilled water. Subsequently the following compounds were added: 10 mg Na_2SO_4 in 10 ml water, 0.8 g NaOH in 10 ml water and 0.9 g RBB. The solution was stirred for 2 h. Afterwards, the reaction mixture was neutralized with HCl and 2 vols of 100% ethanol were added. The resulting suspension was filtered and the residue washed with ethanol-water (1 : 1), ethanol-water (1 : 4), ethanol 100% and acetone, until the filtrate was colourless. The dyed polysaccharide was dried at 60° C, 48 h and ball milled. The substrate was stored in a desiccator until use.

The dye content of the substrate was estimated using a spectrophotometer. Carefully weighed aliquots of dyed CMC (RBB–CMC) and dyed xylan (RBB-xylan) were dissolved in water and the absorption at 595 m was compared with the absorption of pure RBB dye solution. Since the absorption characteristics of the dye are not changed by the reaction with polysaccharides (Biely et al. 1985), it was possible to calculate the dye content of the RBB–CMC and the RBB-xylan. The dye content was between 7 and 9%, varying slightly between various batches.

Measurement of exoenzyme activity

Cellulase and xylanase assays were carried out using the same procedure, except for the different substrate. The dyed substrate (5 mg · ml^{-1}) was dissolved in 0.05 M acetate buffer (pH 5.2) containing 50 mM NaCl, 10 mM $CaCl_2$ and 3 mM NaN_3. The detritus was homogenized using an Ystral homogenizer in the same buffer (5 mg dry detritus · ml^{-1}), and 1 ml substrate solution and 1 ml detritus homogenate were mixed and kept at 30° C for 20 h. Afterwards, 6 ml 100% ethanol were added and the mixture was allowed to equilibrate for 30 min at room temperature. The samples were centrifuged at 5000 rpm for 10 min and the absorption of the supernatant was measured at 595 nm. The absorbency of the supernatant was compared to the absorbency of a dilution series of pure RBB in 75% (v/v) ethanol to calculate the amount of dye in the supernatant. From the amount of dye in the supernatant and the RBB content of the substrate, the amount of hydrolysed substrate could be computed. Enzymatic activity units were expressed as mg hydrolysed substrate · mg dry detritus^{-1} · h^{-1}. Reagent blanks were taken by stopping the reaction immediately after mixing the enzyme and substrate solutions.

Since the measurements of pectin-degrading enzyme activity took place in crude extracts and pectin methyl esterase activity was

present in the detritus damples, it was not possible to distinguish between pectin and pectate lyase activity with the method employed here. For this reason the activity of the lyases is referred to below as pectin/pectate lyase activity. Polygalacturonase activity and pectin//pectate lyase activity were measured turbidime/trically. Pectin (from citrus fruit, 10% methoxylated, Sigma) was dissolved (5 mg · ml^{-1}) in 0.05 M Tris buffer, pH 8.0, containing 1 mM $CaCl_2$ and 3 mM NaN_3 for pectin/pectate lyase measurements, and 0.05 M acetate buffer, pH 5.5, containing the same additions, for estimates of polygalacturonase activity. The detritus was homogenized using an Ystral homogenizer in the same buffers (5 mg detritus · ml^{-1}), and 1 ml substrate solution and 1 ml detritus homogenate were mixed and kept at 30° C for 20 h. Afterwards, 1 ml aqueous hexatin/pectate lyase activity were measured turbidime trically. Pectin and Anagnostakis 1975) was mixed with 1 ml enzyme-substrate mixture and the turbidity of the sample was measured at 600 nm in a spectrophotometer, after proper dilution. The samples were thoroughly vortexed to ensure a finely dispersed colloid. The enzyme activity was expressed as the decrease in absorbency per mg dry detritus^{-1} · h^{-1}.

Statistical procedures

Because the assumptions for ANOVA were not met, even after logarithmical or arcsine transformation, Wilcoxon's test was applied to see whether the treatments had any significant effects on the patterns of weight loss of the detritus and on the exoenzymatic activity.

Results

The decay rate of th *Nymphaea alba* detritus was clearly influenced by the media used in the experiment (Fig. 1). The highest decay rates occurred in media 1 and 2 (high $NaHCO_3$ concentration and high pH). Both a lower pH and a lower $NaHCO_3$ concentration inhibited the decomposition. There was no significant difference in the decomposition rate between medium 3 (0.3 mM $NaHCO_3$, pH 6,5, and medium 4 (0 mM $NaHCO_3$ pH 7.2). Low pH (medium 5, pH 4.0) inhibited the decomposition strongly and Al addition (medium 6) increased this effects. The statisticall analysis of the results is presented in Table 2.

The cellulase activityduring the experiment did not differ much between the various treatments. Only in medium 2 (0.5 mM $NaHCO_3$, pH 7.2) and medium 4 (0 mM $NaHCO_3$, pH 7.2) were lower activities found, and the activity in medium 1 (5 mM $NaHCO_3$, pH 8.0) was somewhat higher than in medium 5, 2 and 4 (Fig. 2 and Table 2).

The xylanase activity was much higher in medium 1 (5 mM $NaHCO_3$, pH 8.0) than in the other media. Detritus from media 3 and 5 showed a somewhat higher activity than the material from the media 2, 4 and 6 (Fig. 2 and Table 2).

The polygalacturonase activity was significantly lower in media 5 (pH 4.0) and 6 (pH 4, with Al). There was no significant difference between the enzyme activities in the detritus from the other media (Fig. 2 Table 2).

Pectin/pectate lyase activity was only clearly detectable in media 1 (5 mM $NaHCO_3$, pH 8.0) and 2 (0.5 mM $NaHCO_3$, pH 7.2). In the other media, very low activities were found (Fig. 2 and Table 2).

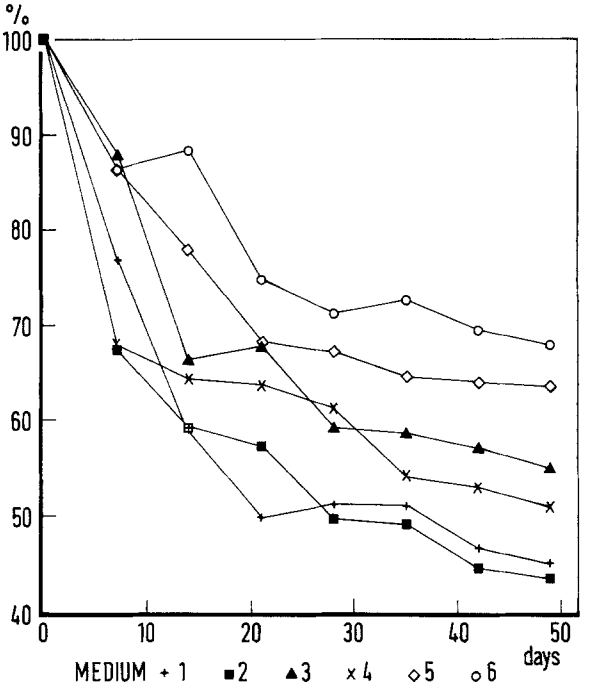

Fig. 1. Remaining dry weight of *Nymphaea alba* detritus during chemostat experiments. (Means of triplicates). Medium 1: pH 8.0, 5 mM $NaHCO_3$; Medium 2: pH 7.2, 0.5 mM $NaHCO_3$; Medium 3: pH 6.5 0.3 mM $NaHCO_3$; Medium 4: pH 7.2, 0 mM $NaHCO_3$; Medium 5: pH 4.0; Medium 6: pH 4.0, 25 μM Al

Table 2. Statistical analysis of the influence of the various treatments on the decay rate and the time course of the enzyme activities. Treatments are arranged from high to low rate of activity. Treatments followed by the same letter are not significantly different ($P > 0.05$)

Decay rate	Cellulase	Xylanase
2 a	1 a	1 a
1 a	3 ab	3 b
3 b	6 ab	5 bc
4 b	5 b	2 c
5 c	2 c	4 c
6 d	4 c	6 c

Polygalacturonase	Pectin/pectate lyase
2 a	1 a
1 a	2 a
4 a	4 b
3 a	5 b
5 b	3 b
6 b	6 b

Discussion

All the water quality parameters tested in this experiment influenced the decomposition rate of *Nymphaea alba* floating leaf blade detritus. Low buffering capacity (low $NaHCO_3$ concentration), low pH and elevated Al concentrations inhibited the decay. This is consistent with the results of other decomposition experiments (Brock et

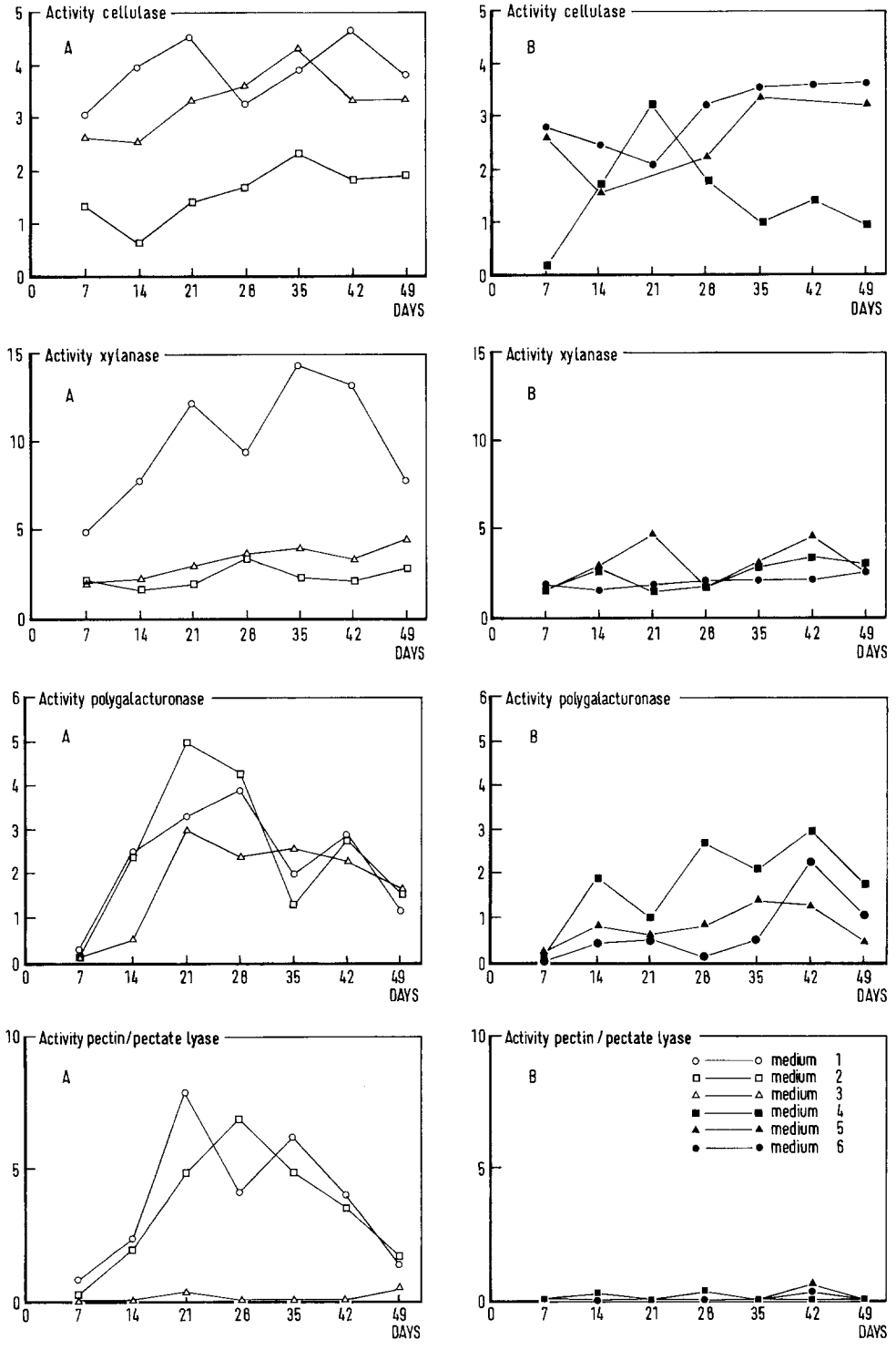

Fig. 2A, B. Activity of cell wall degrading enzymes in *Nymphaea alba* detritus during chemostat experiments. (Means of triplicates). *Medium 1:* pH 8.0, 5 mM $NaHCO_3$; *Medium 2:* pH 7.2, 0.5 mM $NaHCO_3$; *Medium 3:* pH 6.5, 0.3 mM $NaHCO_3$; *Medium 4:* pH 7.2, 0 mM $NaHCO_3$; *Medium 5:* pH 4.0; *Medium 6:* pH 4.0, 25 μM Al. For definition of activity units see Material and methods section

al. 1985; Leuven and Wolfs 1988; Kok and Van de Laar 1991; Kok et al. 1990a).

None of the enzymatic activities measured was significantly lower in medium 6 (elevated Al concentration). This means that the low decomposition rate in medium 6 cannot be ascribed to direct inhibition of exoenzymatic activity by the high Al concentration. It is not clear through which mechanism Al inhibits the decay rate. Palumbo et al. (1987) suggest that Al may be generally toxic to microorganisms. If this is true, the overall exoenzymatic activity in the detritus from medium 6 should have been low compared to those in medium 5 and in the other media. This was not the case. More research seems necessary to elucidate the underlying process of the influence of high Al concentrations on the decomposition process.

Differences in enzyme activity in detritus may be caused by differences in microbial densities. In the experiment presented here, this factor seems to have been of minor importance, since some enzymes differed strongly

Table 3. pH range from water extracted from detritus samples during chemostat experiments. (Data from Kok and Van de Laar 1991)

Medium	pH range
1	6.4–7.0
2	5.7–6.7
3	5.2–5.6
4	5.3–5.8
5	4.4–5.1
6	4.2–5.2

in their activities, whereas the differences for other enzymes were rather small. If the variation in enzyme activity had been due to differences in microbial biomass, than the effect would have been more or less the same for all the enzymes. It seems more likely that the activity of the enzymes was influenced by physico-chemical factors like pH. The enzymes under investigation in this study show rather large differences in pH optima. Pectate lyase has a pH optimum of 8.5–9, polygalacturonase of about 5.5 (Rexová-Benková and Marcovic 1976). Endocellulase has a low pH optimum (4.5–5) (Chamier 1985) and xylanases have a very broad optimum pH range, from 2.7 to 7.5 (Dekker and Richards 1974). So it is likely that differences in pH will have different effects on the activity of the various (exo)enzymes.

It has been shown that the pH of the water in *Nymphaea alba* floating leaf blade detritus is influenced by the pH and the buffering capacity of the surrounding water (Kok and Van de Laar 1991). Low pH and low buffering capacity of the medium caused a low pH of the water inside the detritus. It is plausible that the pH of the water inside the detritus is of greater importance for the exoenzyme activity in the material than the pH of the surrounding medium. Therefore, the results of the measurements of the 'internal pH' of the detritus are summarized in Table 3. (The estimates of 'internal pH' were done on the same detritus samples as the measurements of the enzyme activities presented here.)

Pectin/pectate lyase activity was only clearly detectable in detritus from media 1 (5 mM $NaHCO_3$, pH 8.0) and 2 (0.5 mM $NaHCO_3$, pH 7.2). The 'internal pH' of the material from these media was higher than that of the material from the other media, and showed roughly the same range as that required for the production of pectate lyase by aquatic saprophytic fungi (Chamier and Dixon 1982; Chamier 1985). It is likely that the pectin/pectate lyase activity in the other media was inhibited by low pH.

Polygalacturonase activity was low in the materials from media 5 and 6. The 'internal pH' in this material was clearly lower than the optimum pH of polygalacturonase. In the detritus from the other media, the 'internal pH' lay in the optimum range of the enzyme and within the pH range in which aquatic saprophytic fungi can degrade pectin (Chamier and Dixon 1982; Chamier 1985).

It is not clear why the xylanase activity of the detritus from medium 1 (5 mM $NaHCO_3$, pH 8.0) is so high compared to that in other media. All the ranges of the 'internal pH' in the detritus were within the pH range reported for this enzyme. There are indications that high pH is a stimulating factor in inducing xylanase activity in *Trichoderma viride* (Dean et al. 1989). It is possible that this applies to more fungal species. This xylanase-inducing effect of high pH would explain the high xylanase activity found in detritus from treatment 1. Further research will be necessary to clear up this issue.

(Endo)cellulase activities were not much influenced by the 'internal pH' ranges in the detritus, as was to be expected from the broad pH optimum of this enzyme. The fact that the differences in enzyme activity between the various media were small and not consistent with the overall decay rate probably means that inhibition of this enzyme is not an important cause of the slow decomposition rates under acid circumstances.

The activity of the pectic enzymes pectin/pectate lyase and polygalacturonase was inhibited at low pH. Inhibition of pectin/pectate lyase and of polygalacturonase will strongly inhibit the total decomposition of macrophyte material. These enzymes are very important in the first phase of establishment of fungi on plants and in the decomposition of the middle lamella of the macrophyte cell wall (Friend 1977 and references therein). If the middle lamella remains intact, fragmentation of the tissue will be very slow and this will reduce the available surface for microbial colonisation, resulting in a slow decomposition process (Hargrave 1972; Larsen 1982). Lack of fragmentation of decaying *Nymphaea alba* leaves in acidified water has been reported (Kok et al. 1990b) and the hypothesis proposed by these authors, viz. that this phenomenon is caused by inhibition of pectic enzymes, is supported by the findings presented here.

The results presented in this paper support the hypothesis that inhibition of exoenzymatic activity by the acidification process is a major cause of the low decomposition rates under acid conditions. Three of the four cell-wall degrading enzymes tested showed low activities under more or less acid conditions. The strongest inhibition by acid circumstances was found for xylanase and pectin/pectate lyase. These results are supported by the data of Brock et al. (1985), who found an accumulation of hemicellulose compounds in *Nymphaea alba* detritus from an acidified system. Furthermore, Kok et al. (in press) showed that low pH inhibited growth of saprophytic fungi on pectin and cell wall fraction, but not on native cellulose and carboxymethylcellulose.

It is clear that the inhibition of the cell wall degrading enzymes shown in this paper is not the only cause of the low decomposition rate in acidified aquatic systems. The effects of Al on decomposition cannot be explained by low exoenzymatic activity. Furthermore, changes in fauna and in nutrient cycles caused by acidification may also influence the decomposition rate (Mackay and Kersey 1986; Leuven and Kersten 1988).

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