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Decomposition dynamic of higher plant pigments by HPLC analysis

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Abstract: The fate of the litter of dominant vegetation(willows and reeds) is one of the aspects studied in the frame of the project "Onderzoek Milieu Effecten Sigmaplan". One of the questions to be considered is how long the litter stays within the estuary. In this paper, the time the leaf litter(*Salix triandra* and *Phragmites australis*) stayed in the Schelde estuary was studied by using plant pigment as biomarkers with HPLC application. After analyzing the original data from the incubation experiment described by Dubuison and Geers(1999), the decomposition dynamics patterns of pigments were analyzed and described, and these decomposition dynamics patterns were used as calibration patterns. By using Spearman Rank Order Correlation, the calibration patterns of the pigments which were significant ($p < 0.05$) were grouped. In this way, several groups of the calibration patterns of pigment decomposition were achieved. The presence or absence of these groups of pigments (whether they can be detected or not from HPLC) was shown to be useful in determining the time the litter has stayed in the water. Combining data of DW and POC, more precise timing can be obtained.

Keywords: decomposition dynamics pattern; *Salix triandra*; *Phragmites australis*; Spearman Rank Order Correlation; Schelde Estuary; HPLC

Introduction

High-performance liquid chromatographic (HPLC) systems were developed for higher plant pigments in the late 1970's and early 1980's. Subsequently, more complex HPLC systems were developed for chlorophylls and carotenoids from microalgae and natural phytoplankton populations(Gieskes, 1991; Hodgson, 1997; Bris, 1998). Both reversed phase and normal phase systems were used.

The HPLC technique was used more recently for plant pigment analysis, and it has led to a significant increase in separation and in the number of pigments that can be identified in water and sediment samples(Hodgson, 1997). None method was ideal to be used for analysis for the 50 or so chlorophylls, carotenoids and degradation products likely to be important in aquatic systems(Zhou, 2000; 2001a). Not only were selective HPLC methods needed for accurate quantitative analysis of chlorophylls free from degradation products but also pigments unique to certain algal classes, taxa or processes needed to be unequivocally separated. The value of such pigments in field oceanography was becoming apparent(Zhou, 2001b).

One of the branches of the OMES (Onderzoek Milieu Effecten Sigmaplan) Project which was approved by the Belgium Government was to study the pigments decomposition of dominant species of willow and reed leaf litter along the Schelde Estuary. The riparian trees could be an important energy input into the river systems. As a result the litter decomposition has long been studied in previous researches (Hill, 1996; Whiles, 1997; Latter, 1998; Tam, 1998; Kuehn, 1998). We then use the pigments decomposition dynamics pattern combining the POC (particulate organic carbon) and DW(dry weight) data to infer the time which the litter stayed in the water body. This can be acted as the biomarker to investigate the fate of the leaf litter in the aquatic ecosystem(Sun, 2001).

Previous studies (within OMES) have described the decomposition dynamics pattern of mass loss of vegetation along the Schelde Estuary for willow and reed when submerged in the water in terms of DW and POC(Dubuisson, 1999).

The double exponential model implies a quick decline of DW($\mu g/mm^2$) or POC($\mu g/mm^2$) during the first week and a very slow decline afterwards for both willow and reed (Dubuisson, 1999). Considering the speed in replicate measurements it would be difficult to deduct the time the leaves have been decomposed (in the water) from these curves. Thus we have to find other methods which could give us a better decomposition dynamics pattern. The possibility to look into pigment as "biomarkers" of the duration of decomposition, and possibly the time the leaves have been submerged in the water is investigated in this study. Pigments were used as biomarkers in this study, to determine the retention time of the leaf litter in the Schelde, to describe the

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pigments decomposition dynamics pattern.

1 Materials and methods

1.1 Materials

Higher plants species willow (*Salix triandra*) and reed (*Phragmites australis*) were selected.

1.2 Sampling

Leaves of standing shoots of willow and reed were collected in October, 1998 from the Schelde Estuary near Dendermonde. They were put into the nets whose mesh size is 500 μm and submerged into the water. During 3 months, leaves were collected from the nets at certain periods (Oct. 12, 19, 26; Nov. 5, 18; Dec. 8 in 1998 and Jan. 14 in 1999).

1.3 HPLC measurement

1.3.1 Pigment extraction

Pieces with an average surface of 24.52 mm^2 (± 10%) were cut out of the leaves with a perforator. For each sampling date, 3 leaves were analyzed, and 3 replicate pieces were taken out of each leaf. Each piece of leaf was brought into a test tube with 1.8 ml of 90% acetone. The leaf was crushed with a glass rod, and 0.2 ml of filtered (0.45 mm), double distilled water added. The sample was then further ground. The test tube was cooled during the procedure by putting it in ice. When completely homogenized, the sample was transferred to a centrifuge tube and centrifuged for 3 min. The supernatants were decanted and suck up in a syringe covered with a 0.5 mm filter (Millipore).

1.3.2 HPLC analysis

The samples were analysed by using a Waters HPLC, equipped with a Waters 600 Controller, an autosampler and an absorbance fluorometer detector. A reversed phase column (Spherisorb ODS2) was used. The following solvents were used in a gradient protocol: (1) methanol/0.5 mol/L ammonium acetate(80:20); (2) acetonitrile/water(90:10); (3) ethyl acetate.

2 Results and discussions

2.1 Pigments decomposition dynamics patterns

The height of the peak of pigments at their specific retention time was shown by HPLC data. The data covered the whole time range of the incubation experiment from 12 October 1998 till 14 January 1999.

From the original HPLC collected data over the whole time range of the incubation experiment, the concentration of different pigments versus the incubation time was considered. In this way, the dynamics pattern of pigment decomposition can be described. This can be used as calibration patterns of pigment decomposition. By using spearman rank non-parametric correlation statistics, the pigments which have a similar decomposition pattern (equal of which the concentration changes with the incubation time were significantly correlated, $p < 0.05$) were grouped, and several groups of decomposition calibration patterns of pigments for willow and reed leaves were described.

For group 7 (Fig. 1), we can see that before 57 d of incubation, the concentration of the pigments (RT 19.87 min, 22.12 min, 22.26 min) is fairly low and remained constantly (absorbance peak below 2000 AU/24.52 mm^2), even could not be detected when incubation time was from 24 d onward. After 57 d, the concentration increased sharply until at the end of the incubation experiment.

When we use the calibration pattern to infer the "age" of the leaf litter, since we only consider the presence("+") or absence ("−") of the pigment from HPLC detection, while, the calibration pattern for group 7(reed) did not give us the information of "−" within 57 d of incubation(Fig.1). However, if we use the concentration (in terms of AU/24.5 mm^2), the pattern still could be used, as variability for these data was limited.

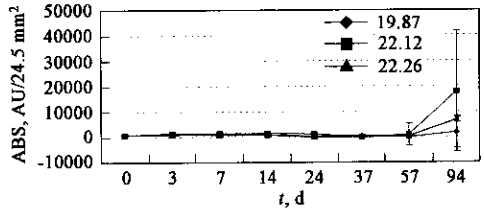

Fig.1 Pigments decomposition dynamics pattern of group 7(reed)

If combining calibration pattern of group 7 and chlorophyll-*a* decomposition pattern (Luo, 2002) we then have the ratio (%) of pigment concentration in group 7 to chlorophyll-*a* as shown in Fig. 2. The ratio is quite low within 57 d of incubation comparing with going up quickly from 57 d onwards until the end of the incubation. So, the ratio can also be used as an indicator of the "age" of the litter within 57 d or not.

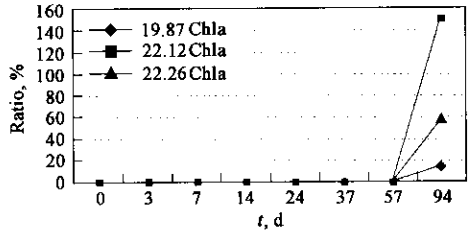

Fig.2 Ratio(%) of pigment concentration in group 7 to Chla(reed)

Leaf senescence is accompanied by the metabolism of chlorophyll to nonfluorescent catabolites (Hoertensteiner, 1999). The pathway of chlorophyll degradation comprises several reactions and includes the occurrence of intermediates. After removal of phytol and the central Mg atom from Chl by chlorophyllase and Mg dechelatase respectively, the porphyrin macrocycle of pheophorbide-*a* is cleaved(Hoertensteiner, 1999; Zhou, 2003).

Although no pigment identification was done in this study, we can still have some useful information from the calibration pattern of pigment decomposition. Combining the calibration pattern of chlorophyll-*a* decomposition for reed (Luo, 2002) and the calibration pattern for group 7 (reed; Fig.1), we found the sharp decomposition of chlorophyll-*a* from 57 d onward (until under the limit of detection at the end of the experiment) were accompanied by a sharp increase of the concentration of the pigments in group 7 from 57 d onward. This may imply that the pigments of group 7 may be the degradation products (chlorophyllides, pheophorbides, and pheophytins) of chlorophyll-*a*.

Combining the calibration pattern of chlorophyll-*a* for reed (Luo, 2002) with the POC data (Dubuison, 1999), the ratio of POC/Chla was obtained as shown in Fig.3. It shows clearly that within 57 d of incubation, the ratio is fairly low and remained constant (nearly zero), the ratio increases sharply from 57 d onwards until the end of the incubation experiment. This agrees with the sharp increase of pigment concentration of group 7 (probably the degradation products of chlorophyll-*a*), reflecting the converting of chlorophyll in the living tissues to non fluorescent catabolites in the detritus.

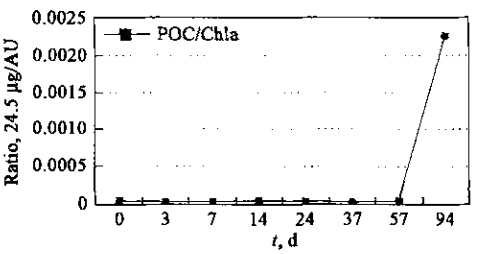

Fig.3 Ratio of POC/Chla for reed

Furthermore, combining the calibration pattern of chlorophyll-*a* with the DW data (Dubuison, 1999), the ratio of DW/Chla shows us the similar trend over the incubation time as the ratio of POC/Chla, as shown in Fig.4.

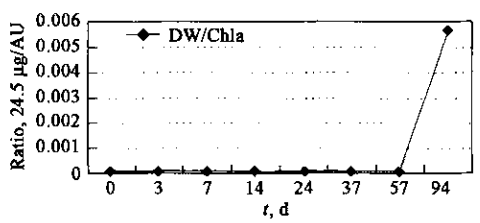

Fig.4 Ratio of DW/Chla for reed

2.2 Determination of the "age" of leaf litter combining pigment information and DW, POC decomposition dynamics

If the "age" of the litter is shown to be less than 24 d from the pigment information, then we can combine further with the DW or POC decomposition dynamics. If the DW we measured is higher than 85 $\mu g/mm^2$, or POC is above 39 $\mu g/mm^2$, we can infer that the "age" of leaf litter is less than one week. Otherwise, it is longer than one week. We can also make a table with combination with the pigment information, as shown in Table 1.

Table 1 The "age" of leaf litter by pigment information and DW, POC decomposition for willow

	DW, $\mu g/mm^2$		POC, $\mu g/mm^2$	
	> 85	< 85	> 39	< 39
Group 1 + Group 7 +	< 7 d	7—24 d	< 7 d	7—24 d
Group 4 − Group 7 +	< 7 d	7—24 d	< 7 d	7—24 d

Note: Group 1, 4, 7 are reviewed in Fig.1 (a, d, b) respectively (Luo, 2002)

In Table 1, for example, if calibration of group 1 and 7 are present, with DW > 85 $\mu g/mm^2$, the "age" of the litter is less than 7 d. If calibration pattern of group 1 and 7 are present, with DW < 85 $\mu g/mm^2$, the "age" of the litter is between 7 and 24 d. In this way, we can infer the "age" of the litter which is less than one week and 7—24 d. If the "age" of the litter is longer than 24 d, we can use the pigment information as we already described in the previous study (Luo, 2002).

Table 2 The "age" of the litter combining DW loss and calibration pattern of group 11 (RT 10.13) for reed

	Group 11	
	+	−
DW, $\mu g/mm^2$		
> 85	< 7 d	……
< 85	7—24 d	> 24 d

Note: Group 11 (RT 10.13) decomposition calibration pattern are reviewed in Fig.2 (Luo, 2002)

In Table 2, we can find that if group 11 (RT 10.13) (Luo, 2002) is present, and DW > 85 $\mu g/mm^2$, the "age" of the litter is less than 7 d. If group 11 is present, and DW < 85 $\mu g/mm^2$, the "age" of the litter is between 7 to 24 d. If group 11 is absent, and DW is < 85 $\mu g/mm^2$, the "age" of the litter is longer than 24 d, if combining the ratio of POC/Chla (or ratio of DW/Chla, ratio of pigment concentration in group 7 to Chla), we can get more precise time the litter stayed (in the water) between 24 and 57 d and longer than 57 d.

2.3 Advantages of the method and further suggestions

(1) The advantage of the use of pigment analysis by HPLC is that after sampling the leaves, we need not analyses them immediately in HPLC. The leaves can be kept in the deep freezer (−85℃).

(2) Identification of the pigments and calibration pattern of the pigments is not very necessary, since we can define the pigments in terms of their specific retention time.

(3) At the same time, the use of retention time only for characterizing a pigment has some disadvantages, since small shifts in retention times of a component between different runs of analysis can not be excluded. This may also create problems in comparison of patterns observed by various columns and various HPLC instruments. In our application, we tried to cover this aspect by using the shift in retention

time of a well-known and defined pigment(chlorophyll-*b*) as the accepted range of shift in retention time. The problem is also minimized because the method is not only based on the presence or absent of any one component, but also considered the groups of the components.

(4) When we infer the "age" of the litter from the calibration pattern, we only considered the qualitative data whether the pigments concentration can be detected from HPLC or not ("+" or "−"), instead of considering the quantitative data(the concentration of the pigments).

(5) Further identification of the pigments will be useful to study the chlorophyll degradation pathway and degradation products to help to investigate the fate of leaf litter in the Schelde Estuary.

3 Conclusions

Pigments degradation is well studied in phytoplankton, few studies on pigments dynamics in decaying of higher plants are reported. The reason may be due to more difficult on the identification of pigments of higher plants than phytoplankton. Without identification of pigments in this experiment, we still can get the pigments decomposition dynamics by defining each of their retention time from HPLC and get some valuable information on inferring the "age" of leaf litter(willow and reed) in the Schelde Estuary.

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