Technical communication

An improvement of the Zieman leaf marking technique for Zostera marina growth and production assessment

Silvia E. Ibarra-Obando*,a, Charles F. Boudouresqueb

*aCentro de Investigaci{on} Cientifica y Educaci{on} Superior de Ensenada (CICESE), Apdo. Postal 2732, Ensenada 22800, B.C., Mexico

bLBMEB, Facult{e} des Sciences de Luminy, 13288, Marseille Cedex 9, France

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Abstract

We present a modification of Zieman’s leaf marking technique that uses the meristem as reference level. This technique allows both a detailed description of eelgrass (Zostera marina L.) shoot dynamics by dissection and a rapid estimation of shoot growth from field data. The average number of leaves per shoot, and the average number of new leaves per shoot were compared between a traditional technique, analyzing only the visible portion of leaves, and the long version of the proposed technique (previous growth analytical technique); significantly higher values were found with the latter technique. Higher correlation coefficients with environmental variables such as light and temperature were also found, indicating an improvement in the data quality. The rapid approach had a 0.8 correlation coefficient with shoot growth and production, making it evident that the distance we call the previous growth can be used as a morphological index to assess these shoot variables.

Introduction

Because Zieman’s (1974) leaf marking technique is considered to give reliable productivity values for seagrasses (Zieman and Wetzel, 1980), it has been used and modified by several authors, with regard to both the marking device and the reference level. To establish a mark on Zostera marina L. leaf blades, workers have used felt pen, staples or hypodermic needles (Sand-Jensen, 1975; Jacobs, 1979; Mukai et al., 1979; Kentula and McIntire, 1986). The reference level has been either the leaf tip (Mukai et al., 1979; Roman and Able, 1988) or the top of the sheath (ligula) (Sand-Jensen, 1975; Hamburg and Homann, 1986; Kentula and McIntire, 1986; Kemp et al., 1987).

Zieman’s technique (or modified versions) has also been used with other

*Corresponding author.

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seagrass species, particularly *Thalassia testudinum* Banks ex König (Zieman, 1974), *Posidonia australis* Hook. (Kirkman and Reid, 1979), *Posidonia oceanica* (L.) Delile (Caye, 1979, 1980; Béđhomme et al., 1983; Bay, 1984) and *Zostera capricorni* Ascherson (Kirkman et al., 1982; Larkum et al., 1984). For *Z. marina*, we consider that these methods provide only a relative estimate of growth, because they overlook Tomlinson’s (1974) recommendation of considering the meristems as the original sources of biomass. This is what Tomlinson described as “meristem dependence”, i.e. the need for continually active shoot apical meristems to maintain populations.

We developed a new marking technique in order to assess *Z. marina* blade growth from its origin, the meristem. This paper describes the proposed technique, which has a long (detailed assessment) and a rapid version, and presents the results of a series of comparisons to test the two versions of the proposed technique. In order to test the long version (previous growth analytical technique) we compared the average number of leaves per shoot, the average number of new leaves per shoot, and growth and production data between this technique and a traditional one (analyzing only the visible portion of leaves). To represent the traditional technique, we used Kentula and McIntire’s (1986) modification of Zieman’s technique. In this last case, a shoot is marked by inserting a 22-gauge hypodermic needle through all the leaves just above the level of the youngest sheath. The leaf segment between the original location of the mark and the location of the mark at the time of harvest, is considered to represent the new growth. To test the rapid version (rapid previous growth technique), we correlated field and laboratory data obtained from the use of the meristem as reference level.

**Methods**

*Previous growth analytical technique*

In the field, the number of exposed leaves (above the sheath) is recorded for every shoot to be marked. Leaves are marked at the top of the sheath with a hypodermic needle. The distance from the needle mark to the first node, sometimes below the sediment, is recorded. This value represents what we called the ‘previous growth’ (Fig. 1A). Two weeks later, the shoots are cut for dissection and leaf counts and measurements in the laboratory. The cut is made at the level of the first pair of roots to ensure the recovery of the reference node (Fig. 1B).

In the laboratory, the number of leaves emerging above the top of the sheath is recorded (whether or not marked). All these leaves will be considered as being present at the time of marking. The sheath is peeled to allow counting and measurement of the tiny leaves, not yet visible. All leaves are arranged in order of position on the shoot and are measured for new growth (Fig. 2).
Fig. 1. Assessment of *Z. marina* blade production using the previous growth analytical technique. (A) At the time of marking the number of leaves present is recorded. Leaves are marked with a hypodermic needle using the top of the sheath as the reference level. The distance from the needle to the first node is registered (previous growth). (B) At harvest time, shoots are recovered, being cut at the level of the first pair of roots.

Three categories of leaves are recognized:
(1) 'Outer' (older) leaves with the limbs (blades) already fully developed. The distance from the top of the sheath to the needle mark represents new growth because no growth occurs above the top of the oldest sheath (Fig. 2A).
(2) 'Middle’ leaves which do not yet have a measurable sheath; only their upper portion is visible without dissection. The distance between the needle mark and the reference node represents growth that took place both before and after marking. The former (previous growth) needs to be subtracted in order to measure the new growth which is located in the basal portion of the leaves (Fig. 2B). The base of the middle leaves is actually located above the reference node, so the previous growth is slightly overestimated.
(3) 'Inner’ leaves which are visible only by dissection of the shoot. Owing to the life span of *Z. marina* leaves (Roman and Able, 1988), it is possible that their total length represents new growth (Fig. 2C).

For each leaf, the new growth portion is cut away, placed in a glass plate, frozen and lyophilized. Prior to dry weight determination, leaves are scraped to remove epiphytes.

*Rapid previous growth technique*

In the field, the distance from the needle mark (top of the sheath) to the reference node is measured (previous growth). Because only one value is ob-
Fig. 2. In the laboratory, after recording the number of exposed leaves (with and without needle mark), the sheath is opened. The total number of leaves per shoot and the number of new leaves are noted. The number of sloughed leaves is also calculated. Three categories of leaves are identified: (A) outer; (B) middle; (C) inner. The first category uses the ligula as a reference level, while the last two use the first node as a reference. In the case of middle leaves, the previous growth needs to be subtracted in order to assess the new growth.

...tained per shoot, the field work is considerably reduced, allowing for an increase in the number of replicates being measured. Laboratory work is eliminated as field data only need data processing.

**Field work**

Two transects, parallel to the shore, were installed on a seagrass bed located in San Quintín Bay (Pacific coast of Baja California, Mexico): Transect I (lower) at \(-0.10 \text{ m MLLW}\) (mean low low water) (MLLW = 0), representing the lower intertidal zone; Transect II (higher), at +0.20 m, representing the transition zone between MLLW and MLW (mean low water) (Ibarra-Obando and Huerta-Tamayo, 1987). From January to August 1987, each transect was sampled at every low tide (12–19 days). At each visit to the site, 21 apical shoots and a variable number of lateral shoots were marked randomly in each transect (their position was determined by a random number table). On the following visit, the marked shoots were harvested and the same number of new shoots was marked. Individual harvested shoots were placed in labeled plastic bags and refrigerated until processed in the laboratory.
In order to compare growth and production values, two independent field experiments were designed, one in summer (July 1987) and the other in winter (February 1988). These seasons were thought to represent extremes of the annual growth cycle. In this case, 42 apical shoots and a variable number of lateral shoots were marked randomly along each transect. Half of the shoots in each transect was marked using the previous growth analytical technique, and the other half was marked using Kentula and McIntire's technique. In the laboratory, leaf pieces representing growth were measured according to each technique. Differences were tested for significance with a Kruskal–Wallis test.

At each visit to the site, water temperature and light were measured. The former variable was measured during high tide at 1 m depth using a bucket thermometer (0.1 °C precision). Light was measured at noon (legal time) using a Li-Cor Integrating Quantum/Radiometer/Photometer (Li-Cor, Lincoln, NE, USA), with an integration time of 1000 s.

Laboratory work

For every shoot, information on the number of leaves present, as well as the number of new leaves, was first obtained with Kentula and McIntire's technique. The sheath was then opened and the same information obtained using the previous growth analytical technique. The average number of sloughed leaves was not compared, as this remains the same independent of technique. Because leaf dynamics data did not fit the normal distribution, non-parametric statistics were used. Results from both techniques were compared with a one-way analysis of variance (Kruskal–Wallis test) and correlated with light, water temperature and sampling date using Spearman's rank correlation coefficient.

The possibility of using previous growth as a morphological index of shoot growth and production was tested by linear regression analysis. All statistical tests had a significance level of 0.05 and were run using the Statgraphics Program (STSC Inc., Rockville, MD, USA).

Results and discussion

In both transects, the average number of leaves per shoot was significantly ($P<0.05$) higher with the previous growth analytical technique. Kentula and McIntire's (1986) technique yielded average values of 4.0 leaves in Transect I and 3.8 in Transect II, whereas the previous growth technique values were 5.5 for Transect I and 5.3 for Transect II. No significant difference between the two techniques was found for the average number of new leaves per shoot on any sampling date in either transect, average values in both transects being between 1.4 and 1.5 leaves. However, when the variances were compared (Fisher statistics) on a seasonal basis (January–April and May–October; Ibarra-Obando, 1992), significant differences were found between the tech-
niques. For both variables, the trends through time were more clearly seen with the previous growth analytical technique (Fig. 3).

When the average number of leaves per shoot and the average number of new leaves per shoot were correlated with light and temperature, the highest correlation coefficients always corresponded to the previous growth analytical technique. The same was true with respect to the sampling date, confirming that a better understanding of the trends through time can be achieved with this technique. In general, we consider that use of the previous growth analytical technique allows a considerable improvement in the quality of the information obtained (Table 1).

When Transect I shoot elongation and production values were compared, significant differences were obtained only during summer ($P < 0.05$). In this season, the difference between techniques was 9.2 mm day$^{-1}$ for elongation

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**Fig. 3.** Comparison of the average number of leaves per shoot and the average number of new leaves per shoot between the previous growth analytical technique and Kentula and McIntyre’s technique. Transects I and II are represented. Vertical bars represent ± 1 SE. The period over which the comparison is made is from 14 January to 21 August 1987.
Table I
Comparison of Kendall correlation coefficients for the average number of leaves per shoot and the average number of new leaves per shoot with light, temperature and sampling date, as a function of the leaf marking technique

<table>
<thead>
<tr>
<th>Transect</th>
<th>KM</th>
<th>PGAT</th>
<th>Light</th>
<th>Temperature</th>
<th>Sampling date</th>
<th>KM</th>
<th>PGAT</th>
<th>Light</th>
<th>Temperature</th>
<th>Sampling date</th>
</tr>
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<tr>
<td>Transect I</td>
<td>1</td>
<td>0</td>
<td>0.09</td>
<td>0.08</td>
<td>-0.17</td>
<td>1</td>
<td>0</td>
<td>-0.009</td>
<td>0.40*</td>
<td>0.29</td>
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<td></td>
<td>1</td>
<td>0.13</td>
<td>0.41*</td>
<td>0.38</td>
<td>1</td>
<td>1</td>
<td>0.44*</td>
<td>0.65***</td>
<td>0.85***</td>
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<tr>
<td>Temperature</td>
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<td>1</td>
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<td>Transect II</td>
<td>1</td>
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<td>-0.02</td>
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<td>0.35*</td>
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<td>1</td>
<td>0.51*</td>
<td>0.59**</td>
<td>0.72***</td>
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<td>Light</td>
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KM, Kentula and McIntire (1986) technique; PGAT, previous growth analytical technique.
* = significant; ** = very significant; *** = highly significant.

and $1.2 \times 10^{-3}$ g dry weight day$^{-1}$ for production. In Transect II, the differences between techniques were not significant ($P > 0.05$) (Table 2). In order to explain the lack of significant differences in three of four cases, the sources of error in each technique must be considered. The previous growth analytical technique does not take into account blade changes with age, specifically their increase in thickness. Bédhomme et al. (1983), working with P. oceanica, considered these thickness changes with leaf age. Similarly, because this technique slightly overestimates previous growth, it underestimates shoot growth and production. In contrast, Kentula and McIntire’s technique underestimates a portion of blade growth (the basal section of middle and inner leaves), and overestimates growth and production values. We suggest the possibility that these sources of opposing errors could have compensated each other, preventing more significant differences from being found. However, we consider that the previous growth analytical technique provides a better estimate of net production, because whole leaves, instead of leaf segments, are analyzed.

Finally, when we plotted the previous growth data, it became evident that they followed the general trends of the growth and production graphs, measured according to the previous growth analytical technique. Thus, the possibility of using previous growth as a morphological index of shoot growth and production was analyzed. In Transect I, the correlation coefficient between previous growth and shoot growth and production was 0.8, with an $R^2$ value of 70%. In Transect II, the correlation was only significant for shoot production, 0.7 with an $R^2$ value of 50% (Fig. 4).
Fig. 4. Linear regression between shoot real growth and previous growth in Transect I, and between shoot net production and previous growth in both transects.

Table 2
Mean (±1 SE; n=15–25) shoot elongation and production values obtained with Kentula and McIntire's (1986) technique (KM) and the previous growth analytical technique (PGAT). Results of both experiments (summer and winter) are presented.

<table>
<thead>
<tr>
<th>Transect</th>
<th>Shoot measurement</th>
<th>Summer (23 July 1987)</th>
<th>Winter (17 February 1988)</th>
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<tr>
<td></td>
<td></td>
<td>KM</td>
<td>PGAT</td>
</tr>
<tr>
<td>I</td>
<td>Elongation (mm day⁻¹)</td>
<td>30.5±2.3</td>
<td>21.3±2.7</td>
</tr>
<tr>
<td></td>
<td>Production (mg dry wt day⁻¹)</td>
<td>3.4±0.3</td>
<td>2.2±0.4</td>
</tr>
<tr>
<td>II</td>
<td>Elongation (mm day⁻¹)</td>
<td>20.3±1.9</td>
<td>22.1±3.3</td>
</tr>
<tr>
<td></td>
<td>Production (mg dry wt day⁻¹)</td>
<td>2.0±0.3</td>
<td>2.0±0.4</td>
</tr>
</tbody>
</table>
This study and that of Bédhomme et al. (1983) are the only comparisons between two variants of Zieman’s technique, using the same shoots or shoot population. This comparison showed that the inclusion of the first node as reference level not only resulted in a better understanding of shoot dynamics, but allowed us to find a morphological index of production.

Sand-Jensen (1975) is the only author to have considered *Z. marina* leaf segments within the sheath. This author recognized that new leaves have been visible for only part of the period of growth, so their total growth is equal to double their visible length. This method was later used by Larkum et al. (1984) with *Z. capricorni*. Even in recent analyses of the marking technique (e.g. Brouns, 1985), the leaves or leaf segments that remain within the sheath are not taken into account.

The technique selected must be appropriate to research goals. In our case, we needed a detailed description of the biological cycle of *Z. marina* on the Pacific coast of Mexico, as this was only partially known. This goal was achieved by the use of the previous growth analytical technique; however, we must recognize that the use of this technique resulted in double the amount of shoot processing in the laboratory compared with the traditional technique. This additional work was justified by the quality of the data obtained, and cannot be compensated for by simply increasing replication of the Zieman technique. With this background, we can now use a faster and reliable estimator of shoot production, the rapid previous growth technique.

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