USE OF A FLOW-THROUGH SYSTEM FOR ASSESSING MICROBIAL RESPIRATION RATES ASSOCIATED WITH DECOMPOSING LEAVES

M. Abelho & M. A. S. Graça
Departamento de Zoologia, Universidade de Coimbra, 3004-517 Coimbra, Portugal.

ABSTRACT

A flow-through system to assess microbial respiration rates associated with decomposing leaves in streams was tested under two different conditions. We wanted to determine (1) the sensitivity of the system to microbial respiration rates, and (2) the effect of antibiotics on microbial respiration rates during the incubation period.

The flow-through system was sensitive to the respiratory activity of microbial assemblages associated with decomposing leaves even during short incubation periods. Our results showed that microbial respiration rates did not change significantly during an incubation period of up to five hours. Due to the short incubation period and to the continuous stirring of the samples with 100% oxygenated water the method avoided much of the growth and death of cells that may be induced over longer incubation periods and changes in rates due to variations in oxygen concentration.

The results of tests estimating respiration rates during incubation with antibiotics showed that (1) at least some prokaryotes were not retained by 0.2 µm filters, and (2) oxygen uptake in the water column increased after the addition of antifungal antibiotics. This could be due to an inorganic effect of the antibiotics on oxygen chemistry, or to the enhancement of prokaryotic activity after the blocking of non-retained eukaryotes. However, the use of blanks allowed the removal of the expected and unexpected sources of variation.

Keywords: Microbial respiration rates, flow-through system, antibiotics, incubation period.

RESUMEN

Bajo diferentes condiciones experimentales se ha ensayado un sistema de flujo para medir las tasas de respiración microbiuna asociada a la descomposición de las hojus en ríos. Se quería determinur (1) la sensibilidad del sistema a las tasas de respiración microbiana, y *(2) el efecto de los untibidticos* ***en*** *las tasas* ***de*** *respiración microbiana durante el tiernpo de incubacidn.*

El sistema de flujo fue sensible a la uctividad respiratoria de la comunidad bacteriana asociada a la descomposición de las hojus incluso durante cortos periodos de incubacicin. Nuestros resultados han mostrado que las tasas de respiración microbianu no cambiaron significativamente durante un periodo de incubación superior a cinco horas. Debido a los periodos cortos de incubacicin y a la continua agitación de las muestras con agua al 100% de saturación de oxigeno el método limitó gran parte del crecimiento y muerte ***de*** *las células que podia haberse producido en periodos largos de incubacicin* y *cambios en las tasas como consecuencia de las variaciones* ***en*** *la concentración de osigeno.*

Los resultados de los ensayos para estirnar las tasas de respiración en incubaciones con antibióticos mostraron que (1) al menos algunos procariotas nofueros retenidos con filtros de 0.2 µm, y (2) *el consumo de oxígeno en la columna de aguu incrementó después de la adicicin de antibióticos antifúngicos. Esto puede ser debido a un efecto inorgánico* ***de*** *los antibidticos sobre la quirnicu del oxígeno, o a la estimulación de la uctividad procariótica después del bloqueo de los eucariotas* ***no*** *retenidos. No obstante, el uso de controles permitió eliminar las fuentes de variabilidad esperadas* y *no esperadas.*

Palabras clave: tasas de respiracidn microbiana, sistema de flujo, antibióticos, periodo ***de*** *incubación.*

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INTRODUCTION

One way to assess the contribution of organisms to a process is to measure their activity. Community respiration is a principal measure of biological activity, reflecting primarily the microbial utilization of resources (Fuss & Smock, 1996). The measurement of the respiration rates of microorganisms to asses their activity has been used in both sediments and soils (e.g. Fisher *et al.*, 1996; Griffiths *et al.*, 1997), and decomposing litter in forests (e.g. Dilly & Munch, 1996), marshes (e.g. Padgett, 1993; Hill & Perrotte, 1995), and streams (e.g. Triska *et al.*, 1975; Suberkropp, 1991; Fuss & Smock, 1996; Baldy & Gessner, 1997).

The methods used to assess the respiratory activity of microorganisms vary widely, from the specific staining of respiring microorganisms (e.g. Padgett, 1993; Fisher *et al.*, 1996) to the measurement of CO, or **O,** concentrations in chambers containing the samples. For instance, Triska *et al.* (1975) used a Gilson respirometer to assess respiration rates associated with decomposing leaves in streams, Griffiths *et al.* (1997) monitored the concentration of CO_2, and several authors monitored the variation in oxygen concentration over an incubation period in chambers containing the samples (e.g. Suberkropp, 1991; Hill & Perrotte, 1995; Fuss & Smock, 1996; Baldy & Gessner, 1997). The measurement of rates of processes, such as respiration, through changes in enclosed samples assumes that the rate measured in the chamber is the same as that in nature (Hobbie & Fletcher, 1979). Therefore, during the incubation there must not be any appreciable change in the concentration of the oxygen or the microbial populations (Hobbie & Fletcher, 1979).

The activity of microorganisms associated with decomposing leaves in streams is regulated by substrate quality, i.e., by internal factors, and by environmental, i.e., external factors, such as dissolved nutrients (Chamier, 1992; Suberkropp & Chauvet, 1995) or oxygen (Suberkropp, 1992). In most forested headwater streams, decomposing leaves are continuously stirred with fully oxygenated water. One way to avoid changes in the concentration of oxygen during the incubation period and thus to make the experimental conditions more similar to those in nature is to use a flow-through system where the samples are continuously supplied with 100% oxygenated water (e.g. Wrona & Davies, 1984; Graça, 1990).

Some authors have found that the use of antibiotics interferes to some extent with the inorganic chemistry of some compounds (Rosson *et al.*, 1984) and with rate measurements due to the presence of non-target organisms and to differential effects on target organisms (Taylor & Pace, 1987).

A laboratory experiment was carried out to assess if this flow-through system was adequate to measure microbial respiration rates associated with decomposing leaves in streams. The specific objectives of this experiment were: (1) to determine if the flow-through system was sensitive enough to measure differences in oxygen concentrations due to microbial respiration associated with decomposing leaf litter, and (2) to determine the incubation period necessary to detect differences in oxygen concentrations due to microbial respiration. Another objective of the experiment was to determine the potential interference of antibiotics with measurements of respiration rates. Thus, a second experiment was carried out to assess the effect of antibiotics on microbial respiration rates during the incubation period.

MATERIAL AND METHODS

Decomposing leaves and stream water were collected in stream S. João (Lousii Mountain, Central Portugal). In the laboratory, stream water was filtered (0.2 μm, Schleicher & Schuell), the leaves were carefully rinsed with tap water to remove attached debris and macroinvertebrates, and discs (14.5 mm diameter) were cut from each leaf and evenly assigned to each treatment.

Respiration measurements

The flow-through system consisted of a peristaltic pump with adjustable flow (Watson-

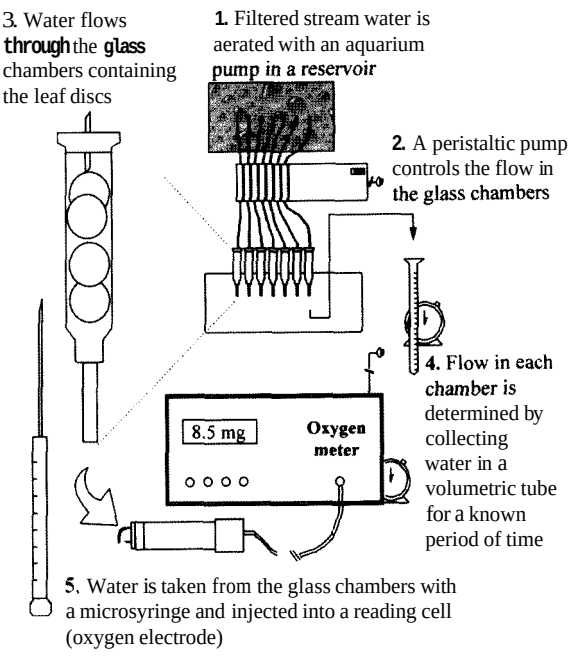

Figure 1. Flow-through system used to estimate microbial respiration rates associated with decomposing leaves (modified from Graça, 1990). *Sistema de flujo utilizado para estimar las tasas de respiración microbiuna asociada con la descomposición de las hojas (modificado de Graça, 1990)*

Marlow) provided with Watson-Marlow orange/green tubes (flow rate 0.10 cc minute-'). One end of the tubes was connected to the respiration chambers (8 mL glass syringes) and the other end entered a reservoir containing 100% oxygenated stream water (Fig. 1). The system was mounted in a room with temperature set at 15°C. On every measuring occasion, the chambers were filled with stream water plus the sample and flow was measured in each chamber by collecting the outflow water in a 5 mL calibrated glass vial during a period of 20 minutes. Final values were expressed as mL h^{-1}.

Measurements of oxygen concentrations were made only after the chambers' volume was totally replaced. The water flowing through the chambers was collected with a 1 mL syringe and injected into a 0.1 mL micro-chamber adapted to an oxygen electrode (Strathkelvin Instruments, model 781). Readings were made after two minutes, the time necessary for the stabilization of the electrode.

To determine if the system was sensitive to microbial respiration rates associated with decomposing leaves in streams, oxygen concentrations were determined in five chambers containing eight leaf discs and in five blanks. The peristaltic pump was set at position 40, corresponding to an approximate flow rate of 6 mL h^{-1}. Oxygen concentrations in the chambers were measured six times over a period of five hours.

Microbial respiration rates during incubation with antibiotics

To assess the effects of antibiotics on microbial respiration rates two treatments were prepared: (1) antibacterial: 50 mL L^{-1} of penicillin-streptomycin solution (Sigma N0906), and (2) antifungal: 50 mL L^{-1} nystatin suspension (Sigma N1638) plus 100 mg L^{-1} cycloheximide (Sigma C7698). The experimental design included six chambers for each treatment: five containing ten leaf discs and one containing only filtered stream water (blank). The peristaltic pump was set at position 20, corresponding to an approximate flow rate of 3.5 mL h^{-1}. Flow in each chamber was measured and the time necessary to replace all the volume calculated. After the volume of the chambers was completely replaced, three replicate measurements were made before the addition of the antibiotics.

The medium was changed to the treatments: six of the chambers were continuously supplied with 100% oxygenated stream water containing the antibacterial treatment and the other six chambers were continuously supplied with 100% oxygenated stream water containing the antifungal treatment. After the complete renewal of the volume in the chambers, seven measurements (in three replicates) were made at different times (5, 20, 24, 34, 46, 69 and 79 hours).

Leaf discs were oven-dried (60ºC, 3 days), weighed (± 0.01 mg), ashed (500ºC, 5 hours) and re-weighed to determine ash free dry mass (AFDM). To estimate respiration rates associated with decomposing leaves, the oxygen concentrations of the blanks were subtracted from the oxy-

gen concentrations of the samples and the values were corrected for the flow in each chamber and for the AFDM of the leaf discs. Respiration rates were expressed as mg O_2 h^{-1} g^{-1} AFDM.

Statistical analysis

Oxygen concentrations in the chambers (n=60: six measurements in each chamber, five with leaves and five blanks) were compared by a two-way ANOVA with replication (time vs. chamber content: leaves or blanks). Respiration rates before the addition of the antibiotics were compared by a one-way ANOVA (n=30: three measurements in each of the 10 chambers). Respiration rates after the addition of the antibiotics (n=210: three replicate measurements in each chamber, five with the antibacterial and five with the antifungal treatment, seven measurements over time) were compared by a two-way ANOVA (time vs. treatment). After rejection of the null hypothesis, multiple comparisons (Tukey test) were carried out to determine the points were differences occurred (ZAR, 1996). In all statistical analyses, the level of significance was set up at p*0.05.

RESULTS

Respiration measurements

The mean flow in the chambers was 6.2 mL h^{-1} (range 4.8-7.7 mL h^{-1}). Thus, the mean time for total renewal of the volume of one chamber was 79 minutes (range 62-100 minutes). Oxygen concentrations ranged from 6.6 to 8.4 mg O_2 L-' in the chambers containing leaf discs and from 10.0 to 10.4 mg O_2 L^{-1} in the blanks (Table 1). Oxygen concentrations in blanks and chambers containing leaf discs were significantly different, (F=908, P<0.0001, DF=1) but changes through time were not (F=0.89, P=0.05, DF=6; Table 1). There was no significant interaction between factors (F=0.43, P>0.05, DF=5).

Respiration rates during incubation with antibiotics

The mean flow in the chambers was 3.4 mL h^{-1} (range 2.7-4.5 mL h^{-1}), corresponding to a mean renovation time of 2.4 h (range 1.8-3.0 h). Before the addition of the antibiotics there were no significant differences in respiration rates between

Table 1. Variation in flow rate and in oxygen concentrations (mg O_2 L^{-1}) of the five chambers containing leaf discs (leaves) and of the five chambers containing only stream water (blanks). *Variación del flujo y de las concentraciones de oxígeno (mg O_2 L^{-1}) de las cinco cámaras conteniendo los discos de hojas y de las cinco cámaras conteniendo solo agua del río (blancos).*

Chamber	Flow	Time after starting						mg O_2 L^{-1}
content	(mL h^{-1})	1h55m	2h24m	3h30m	4h10m	4h35m	5h20m	(mean+ 1 SD)
Leaves								
1	4.8	7.3l	7.2 I	7.22	6.63	6.95	7.27	7.10 ± 0.26
2	6.0	7.23	7.40	7.68	7.42	7.92	8.02	7.61 ± 0.31
3	6.6	7.87	7.84	8.03	8.17	8.16	8.00	8.01 ± 0.14
4	4.8	7.95	7.73	7.95	8.37	8.18	8.21	8.07 ± 0.23
5	6.0	7.42	7.71	7.58	8.03	7.94	7.92	7.77 ± 0.24
Blanks								
l	7.7	10.12	10.03	10.28	10.18	10.00	10.05	10.11 20.11
2	6.0	10.10	10.02	10.26	10.26	10.16	10.16	10.1620.09
3	7.7	9.99	10.08	10.24	10.38	10.14	10.18	10.17 ± 0.13
4	6.6	10.11	10.18	10.31	10.41	10.14	10.27	10.24 ± 0.11
5	6.0	10.03	10.19	10.35	10.23	10.17	10.33	10.22 ± 0.12

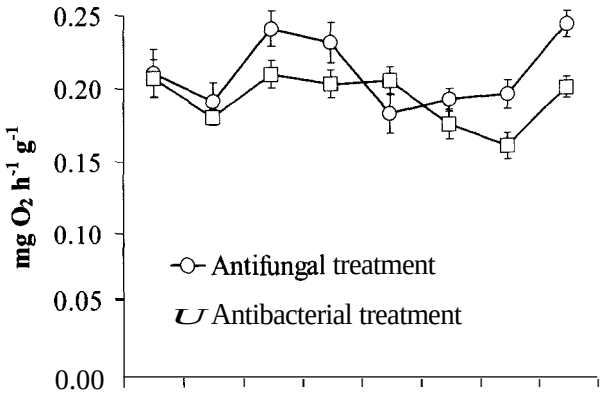

Figure 2. Respiration rates associated with decomposing leaves in both treatments before and after the addition of the antibiotics (mean ± 1 SE). *Tasas de respiración asociadas a la descomposición de las hojas en ambos tratumientos antes y después de la adicidn de antibióticos (media ± I SE).*

the treatments (F=0.027, P>0.05, DF=29). After the addition of the antibiotics respiration rates were significantly different in the two treatments (F=13.896, P<0.001, DF=1), being higher in the antifungal than in the antibacterial treatment. Respiration rates in both treatments experienced significant variations during the course of the experiment (F=7.741, P<0.001, DF=6). There was a significant interaction between time and treatment (F=2.33, P<0.05, DF=6).

Respiration rates in both treatments decreased during the first five hours after the addition of the antibiotics (Fig. *2)*. After that, respiration rates increased to a maximum at *20* hours, decreased to a minimum at either *34* hours (antifungal treatment) or *69* hours (antibacterial treatment) increasing afterwards until the end of the experiment (Fig. 2).

The oxygen uptake in the chambers containing no leaves (blanks) decreased in both treatments during the first five hours after the addition of the antibiotics (Figure *3)*. After that, oxygen uptake leveled at values close to zero in the antibacterial treatment maintained low values during the rest of the experiment. In the antifungal treatments oxygen uptake increased steadily during the first *34* hours of incubation, decreasing afterwards until the end of the experiment (Fig. *3)*.

DISCUSSION

Respiration measurements

Oxygen concentrations in the chambers containing the leaf discs were significantly lower than in blanks showing that the method was sensitive to the activity of the microbial assemblages associated with decomposing leaf litter. The flow rates used in the experiment allowed detection of oxygen consumption and at the same time provided incubation periods as short as *60* minutes, i.e., the time necessary for the renewal of the volume of chambers. However, when working with leaves in early stages of decomposition, it is probably advisable to use a slower flow. According to our results, a flow providing the replacement of the volume in five hours can be used without changing significantly the microbial respiration rates.

The flow-through system thus avoided much of the problems associated with measurements in

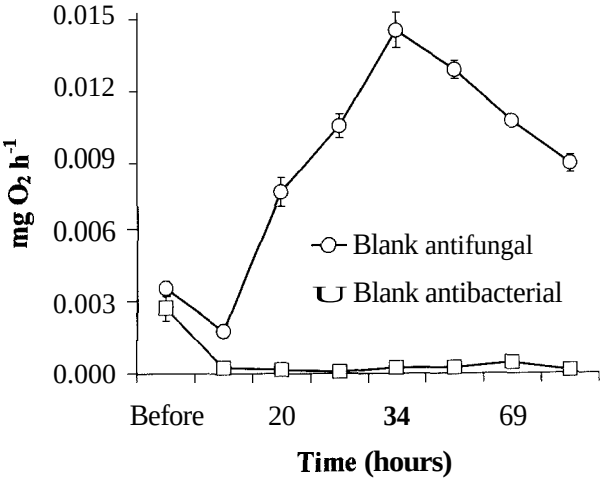

Figure 3. Oxygen uptake in the **blanks** of the two treatments before and after the addition of the antibiotics (mean ± 1 SE). *Consumo de oxígeno en los blancos de los dos tratamientos antes y después de la adición de antibióticos (media ± I SE).*

enclosed samples. First, the samples were continuously stirred with 100% oxygenated water during all the incubation period, avoiding changes in rates due to changes in oxygen concentrations (Hobbie & Fletcher, 1979). Second, the sensitivity of the method allowed short incubation periods, avoiding much of the growth and death of cells, and thus changes in rates that may be induced over longer incubation periods (Hobbie & Fletcher, 1979).

Respiration rates during incubation with antibiotics

To reproduce the natural conditions of decomposing leaves in streams and at the same time avoid oxygen consumption by microorganisms in the water column measurements of respiration rates were carried out in filtered (0.2 pm) stream water. Salonen (1974) showed that most bacteria from oligotrophic lake water were not retained by 0.2 μm filters. Moreover, in oligotrophic waters, bacteria are smaller than previously thought, increasing the percentage not retained by the filters (Salonen, 1979). In our experiment, there was some oxygen uptake in the water column before the addition of the antibiotics, suggesting that the filters did not retain at least some bacteria. In the antibacterial treatment, oxygen uptake in the water column decreased to values close to zero, showing that the bacteria not retained by the filters were blocked by the antibiotic. However, in the antifungal treatment, the initial decrease was followed by a steady increase in oxygen uptake until 34 hours after the addition of the antibiotic. The initial decrease may have been due to the blocking of eukaryotes not retained by the filters. In fact, several authors reported inefficient retention of filters, either due to pore defects, too high vacuum pressure or a combination of both (Salonen, 1979; Stockner *et al.*, 1990). Stockner *et al.* (1990), showed that the surface of some 0.2 μm filters may contain large holes or pores, some 5 times larger than the nominal pore size stated by the manufacturer, allowing passage of organisms as big as 12.8 pm. If some eukaryotes were not retained, the subsequent increase in oxygen uptake may have been due to increased prokaryotic activity after the blocking of the eukaryotes (Stolp, 1988). However, the eukaryotes not retained by the filters must have been present in very low numbers, since their activity was almost negligible in the water column treated with the antibacterial antibiotic. Another possible explanation for the oxygen uptake in the water treated with the antifungal antibiotic is that the compounds interfered in some way with the inorganic chemistry of oxygen, removing oxygen from the water column. In fact, it has been shown previously that antibiotics may interfere with the inorganic chemistry of other compounds (Rosson *et al.*, 1984).

Whatever the cause of the oxygen uptake in the water column treated with the antifungal antibiotic, its effect was removed from the calculations of microbial respiration rates associated with the leaves, because the oxygen uptake in the samples was subtracted from the oxygen uptake in the blanks. If we had instead used the water from the reservoir, the respiration rates associated with leaves treated with the antifungal antibiotic would have been greatly overestimated. It is thus advisable to correct respiration rates by the use of blanks where all expected and unexpected sources of variation may be removed from calculations.

The addition of the antibiotics resulted in a decrease of the respiration rates associated with decomposing leaves, suggesting that the compounds blocked at least some of the target organisms. After the initial decrease, respiration rates oscillated in both treatments, probably as a result of the factors discussed for the water column. In conclusion, several factors seem to influence the measurement of respiration rates associated with decomposing leaves treated with antibiotics. The use of blanks to correct estimations of respiration rate for whatever happens in the water column is advisable, since it can remove the influence of some of these factors.

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