ENVIRONMENTAL ANALYSIS

Determination of Carbon Dioxide Evolution Rates Using a Novel Noninstrumental Microrespirometer

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A novel noninstrumental microrespirometer was developed to determine carbon dioxide evolution rates of solid or liquid samples at μL/h levels accurately and rapidly. The respirometer is based on the simple principle of acid–base titration at a steady-state of carbon dioxide absorption/evolution. The structure and operation of the microrespirometer are simple and the cost is modest in comparison to instrumental methods. The microrespirometer is suitable for laboratory studies and field routine examinations of food, agricultural, and environmental samples.

Respiration is a common indicator of biological activity. Respirometry (measurement of respiration rates) has been applied to a broad spectrum of applied and environmental microbiology, such as toxicity–treatability, process control and prediction of biological oxygen demand (BOD_5) in wastewater treatments (1, 2), assessment of metal toxicity (3), living soil microbial biomass (4–6), and food quality (7, 8).

Respiration rates can be measured either by rates of oxygen consumption or CO_2 evolution. Rapid oxygen consumption rate can be measured by using an oxygen probe or a quantitative electrolytic cell (2). Most oxygen respirometers, however, are applicable only to liquid samples. Oxygen respirometers with an electrolytic cell can be used to determine respiration of solid or semi-solid samples, but their sensitivity is greatly compromised.

Sensitive and rapid CO_2 respirometers based on infrared (IR) detectors have been developed in the last 3 decades (9), and can handle solid samples with high speed and sensitivity. Instrumental respirometers are technically complicated and expensive if accuracy and sensitivity are demanded. Noninstrumental CO_2 respirometers operated by an alkaline trap and acid–base titration have been in existence for years (10). They are simple but relatively slow (measurement in days) and less sensitive (detection limit in mL CO_2/day). Sensitive and rapid determination of respiration rates is highly desirable in monitoring microbial activity in food and environmental samples.

We developed a novel non-instrumental microrespirometer capable of determining CO_2 evolution rates of solid or liquid samples rapidly (in 1 h) and sensitively (μL/h level). The operation is simple and the cost is very modest. The microrespirometer can be conveniently operated under laboratory or field conditions.

Experimental

Materials

(**a**) *Microrespirometer*.—The design of the microrespirometer is based on the simple principle of acid–base titration between an alkaline solution and CO_2, with phenolphthalein to indicate the end point. The microresprometer consists of a transparent reaction chamber and a sample vial (Figure 1). The reaction chamber is, in fact, a small alkaline trap (total headspace, 6–7 mL) with a septum hole. The size of a sample vial may vary depending on the need (vials of 25, 30, 40, and 75 mL are available; e.g., Fisherbrand [Suwanee, GA] EPA bottles). The reaction chamber and the sample vial are coupled through a standard threaded screw and septum liner to form a closed headspace. Alkaline solution can be injected into the reaction chamber with a syringe. The alkaline solution absorbs the CO_2 in the headspace. The indicator in the alkaline solution changes from a deep to a faint pink when the alkaline solution is consumed by CO_2. The microrespirometer is shaken at a fixed rate of ca 240 rpm on an orbital shaker to enhance the absorption of CO_2.

(**b**) *Alkaline solution*.—The alkaline NaOH–$BaCl_2$–indicator solution contains an equal molar ratio of NaOH and $BaCl_2$ and 0.5 mL indicator solution (0.5% phenolphthalein in 50% ethanol solution) per 50 mL alkaline solution. $BaCl_2$ in the alkaline solution precipitates the absorbed CO_2 that ensures the stoichiometry of 2 moles of alkaline spent per mole of CO_2 absorbed, i.e.,

$$CO_2 + 2NaOH + BaCl_2 = BaCO_3(s) + 2NaCl \quad (1)$$

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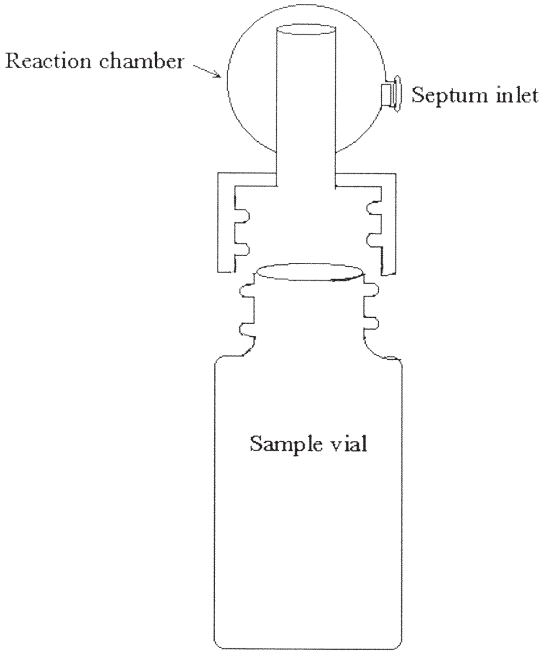

Figure 1. A graphic representation of the microrespirometer. The reaction chamber has a total headspace of 6–7 mL in which up to 1.2 mL can be used to hold the alkaline indicator solution. The size of the sample vial can be varied according to the test. Currently, 25, 40, and 75 mL sample vials are available commercially.

$BaCl_2$ also sharpens the change of color at the end point when a very low level of respiration is being determined. The alkaline solution is stored in a septum-capped vial to prevent absorption of CO_2 from the air. The alkaline solution is transferred through a syringe.

Procedures

The following experiments were performed to determine the optimal conditions for operating the microrespirometer:

(**a**) *Shaking rate experiment*.—The effect of shaking on the CO_2 absorption of the microrespirometer was investigated. Microrespirometers with empty 25 mL sample vials were coupled in a glove box of known CO_2 concentration (determined by an IR CO_2 analyzer). A 0.2 mL portion of 0.002M alkaline solution was injected into each reaction chamber. The microrespirometers were shaken at a fixed rate of 100, 150, 200, 250, or 300 rpm. The time required to consume the alkaline solution in each microrespirometer (as indicated by the color change) was recorded. Each treatment was performed in triplicate.

(**b**) *Alkaline concentration experiment*.—The effect of alkaline concentration on the absorption of CO_2 in a closed headspace was investigated. A 25 mL sample vial was connected to an IR analyzer as described by Hsieh and Hsieh (9) so that the vial and the IR detector formed a closed headspace in which air circulated continuously. The 25 mL vial was shaken at 240 rpm on an orbital shaker. A 1 mL portion of 0.2, 0.1, 0.01, or 0.001M alkaline solution was injected into the vial through a syringe needle port at the beginning of the experiment and the concentration of CO_2 in the vial was recorded periodically. The experiment was repeated twice.

(**c**) *CO_2 concentration experiment*.—The relationship between the CO_2 absorption rate and the CO_2 concentration in the headspace of the microrespirometer was investigated. Microrespirometers with a 75 mL sample vial were coupled in a glove box of known CO_2 concentration. An increment of 0.1 mL 0.002M alkaline solution was injected into the reaction chamber. The microrespirometers were shaken at 240 rpm and the time required to consume each increment of the alkaline solution was recorded. The consumption of each increment of the alkaline solution (i.e., 0.2 μmol alkaline, or 0.1 μmol CO_2) represents a 29.7 ppm (v/v) reduction of CO_2 concentration in the 82 mL microrespirometer at 25°C. Each treatment was performed in triplicate.

(**d**) *Microrespirometer procedure*.—A portion of solid or liquid sample was place in a sample vial and the vial was coupled to a reaction chamber. 0.8 mL alkaline solution of suitable concentration was injected into the reaction chamber using a syringe. The respirometer was shaken at a fixed rate of ca 240 rpm for 30 min (the pre-equilibration period), ensuring that the alkaline solution was not completely consumed during this time. If the alkaline solution wass about to be consumed, more alkaline solution was injected into the reaction chamber. After 30 min pre-equilibration, the shaker was stopped and the alkaline solution in the reaction chamber was

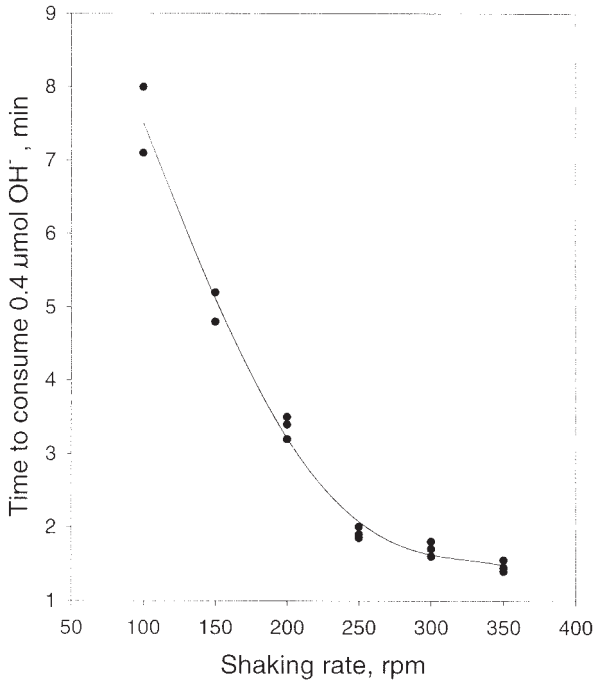

Figure 2. Relationship between CO_2 absorption and shaking rate of the microrespirometer at 25°C. Each dot represents a single measurement, not a mean.

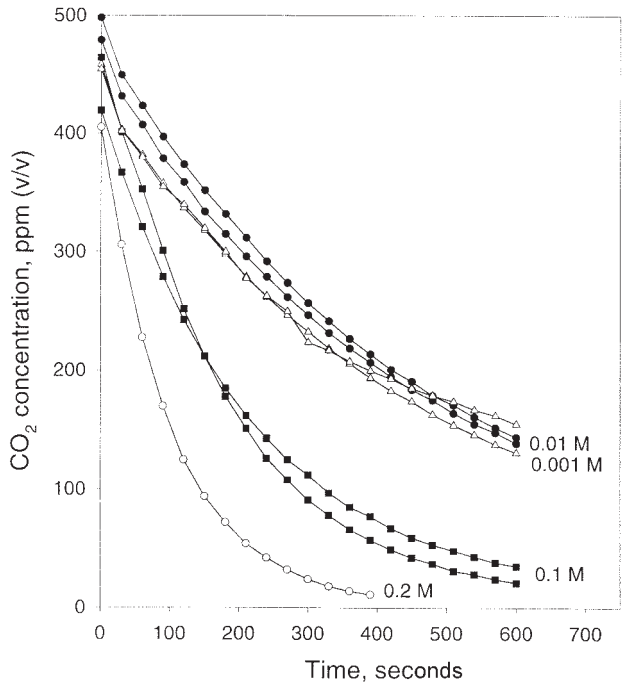

Figure 3. Relationship between CO_2 absorption and concentration of the alkaline solution in the microrespirometer. The experiment was performed at 25°C and a shaking rate of 240 rpm. Each dot represents a single measurement, not a mean.

withdrawn to ca 0.1–0.2 mL remaining. The respirometer continued to be shaken until the alkaline solution changed to a faint pink. The shaker was stopped immediately,and 0.1 mL alkaline solution was injected, shaking resumed, and the time required to consume the alkalinity was recorded. Increments of 0.1 mL alkaline solution were injected twice more and the time required to consume each increment was recorded. The average of the time required to consume the 0.1 mL increment alkaline solution was used to calculate the CO_2 evolution rate with the following formula:

$$CO_2 \text{ evolution rate, } \mu mol/h = \frac{0.1\times 10^3 \times M/2}{t/60} \quad (2)$$

where M is the concentration of the alkaline solution in mol/L and t is the time required to consume 0.1 mL alkaline solution in min. The CO_2 evolution rate can be expressed in μL/h by multiplying the molar volume of CO_2 at a specific temperature.

(**e**) *Validation experiment.*—The CO_2 evolution rates determined by the microrespirometer method were compared with those determined by an established IR analyzer method. Portions of soil samples of relatively low CO_2 evolution rates (2–5 μL/h/g), unfrozen processed meat samples of medium CO_2 evolution rates (10–100 μL/h/5 g), and room temperature milk samples of high CO_2 evolution rate (80–280 μL/h/20 mL) were placed in 25 mL sample vials. The CO_2 evolved by microorganisms associated with each sample was determined by the microrespirometer method. A duplicate sample in another 25 mL sample vial was also placed in a 250 mL flask and the CO_2 evolution rate was determined by the IR analyzer method described by Hsieh and Hsieh (9). The sample vials in the microrespirometers and those in the 250 mL flasks of the IR analyzer were exchanged and the CO_2 rates were determined again with the alternative methods.

Results and Discussion

The effect of shaking rates on CO_2 absorption of the microrespirometer is shown in Figure 2. The CO_2 absorption increased as the shaking rate increased from 100 to 250 rpm. The increase in CO_2 absorption leveled off when the shaking rate exceeded 250 rpm. Shaking at 200 rpm or higher improved reproducibility of CO_2 absorption. A fixed shaking rate between 200 and 250 rpm is recommended for the microrespirometer because the benefit of shaking is achieved while the difficulty of operation at higher rates is avoided.

The results of the alkaline concentration experiment are presented in Figure 3. As the concentration of alkaline solution decreased from 0.2 to 0.01M, the CO_2 absorption rate decreased as well. The CO_2 absorption rate did not further decrease as the alkaline concentration was reduced from 0.01 to 0.001M. It is not possible to have complete absorption of CO_2 in the headspace of a microrespirometer in a matter of hours when the concentration of the alkaline solution is less than 0.01M. The concentration of the alkaline solution has to be

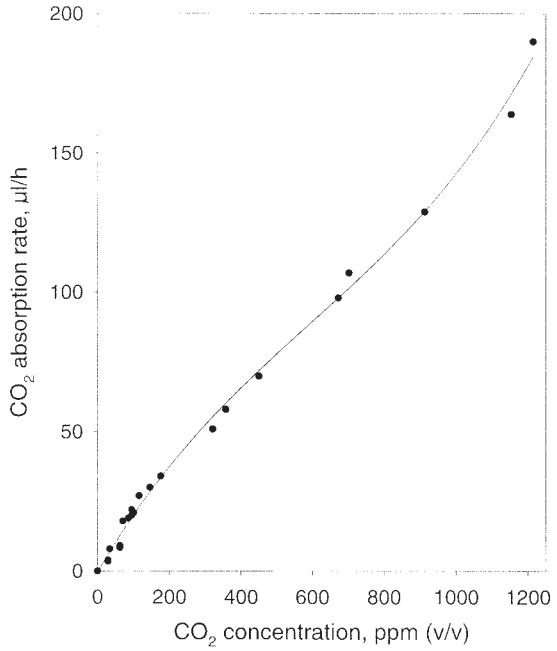

Figure 4. Relationship between CO_2 absorption and concentration of CO_2 in the headspace (82 mL) of the microrespirometer. The experiment was performed at 25°C and a shaking rate of 240 rpm. Each dot represents a single measurement, not a mean.

much less than 0.01M in order to determine CO_2 evolution rate at a μL/h level. The microrespirometer, therefore, does not work on the principle of a complete CO_2 absorption but on an absorption/evolution equilibrium principle that will be discussed in detail later. An alkaline solution of less than 0.0005M is not stable enough to be used in the microrespirometer because the possibility of contamination from the ambient CO_2 is too large for such low alkalinity. Phenolphthalein is not stable in alkaline concentrations exceeding 0.01M; the deep pink color will fade away by itself within 1 h. Therefore, a proper alkaline concentration range suitable for the microrespirometer is between 0.01 and 0.001M.

Figure 4 depicts the relationships between the CO_2 absorption rate of a 0.002M alkaline solution and the concentration of the CO_2 in the headspace of a microrespirometer. In general, the CO_2 absorption rate has a positive curve–linear relationship with the concentration of CO_2. The CO_2 absorption rate of the respirometer at a given temperature and shaking rate reflects the CO_2 concentration in the headspace of the microrespirometer which is, of course, not necessarily the CO_2 evolution rate of the sample. However, if a sample is equilibrated with the alkaline solution in the respirometer at a given temperature and a given shaking rate, then the concentration of CO_2 in the respirometer would eventually reach a constant value when the CO_2 absorption rate equals the CO_2 evolution rate. For example, if the beginning CO_2 concentration of the respirometer is 450 ppm and the CO_2 evolution rate of the sample is 100 μL/h, the CO_2 concentration of the respirometer will be increased to about 660 ppm (Figure 4) and stay there because an equilibrium of CO_2 absorption and evolution is established. If the CO_2 evolution rate of the sample is 20 μL/h, the CO_2 concentration of the respirometer will be decreased to about 150 ppm (Figure 4), where an absorption/evolution equilibrium is established. The CO_2 evolution rate of a sample, therefore, can be determined by the CO_2 absorption rate of the microrespirometer when an equilibrium or steady-state is established. That is, after a sample is equilibrated with an alkaline solution in a microrespirometer, the CO_2 evolution rate can be determined by the time required to consume a small increment of the alkaline solution (equation 2).

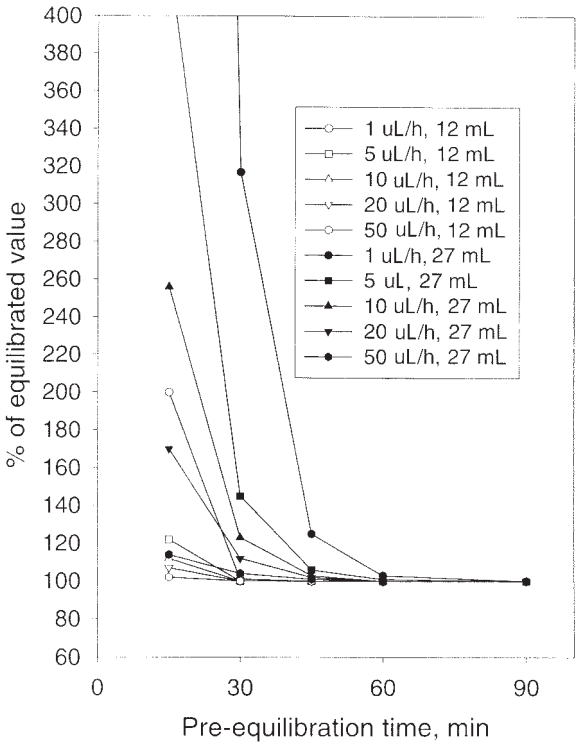

Figure 5. The relationship between the "pre-equilibration" time and the % of the equilibrated value (ratio of CO_2 absorption rate/equilibrated CO_2 absorption rate multiplied by 100) for a sample in a microrespirometer using 0.002M alkaline solution.

The minimum time required for a sample in the respirometer to reach an equilibrium was deduced from a computer simulation based on the relationship between the CO_2 absorption rate and the CO_2 concentration of the respirometer and the CO_2 evolution rate of the sample. That is, the concentration of CO_2 in the headspace of a respirometer after being shaken for a small increment of time Δt is,

$$C_{i+\Delta t} = C_i + \frac{(A_{Ci} - E)\Delta t}{V_{headspace}} \qquad (3)$$

where C_i and $C_{i+\Delta t}$ are the CO_2 concentrations of the respirometer at time i and time i + Δt, respectively. A_{Ci} is the CO_2 absorption rate of the respirometer at time i and a function of the CO_2 concentration C_i . E is the CO_2 evolution rate of the sample and $V_{headspace}$ is the volume of the headspace. The mathematical relationship of A_{Ci} and C_i was generated by a nonlinear regression curve fitting program (TableCurve, Jandel Scientific, San Rafael, CA) using the data of Figure 4. The regression enabled the calculation of A_{Ci} based on C_i. The values of A_{Ci}, C_i, and $C_{i+\Delta t}$ for each small time increment (0.5 min) of Δt were calculated and tabulated using a spreadsheet software (Excel, Microsoft, Redmond, WA) based on equation 3. An equilibrium is attained in the simulation when the CO_2 concentration in the respirometer approaches a constant, i.e., $(A_{Ci} - E)$ approaches 0 and $C_{i+\Delta t}$ approaches C_i. The minimum time required to attain an equilibrium is the sum of all small time increments, Δt, during which CO_2 concentration approaches a constant. The ratio of the CO_2 absorption rate to evolution rate (i.e., A_{Ci}/E) expressed as a percentage of the CO_2 evolution rate during the time course of reaching an equilibrium is presented in Figure 5. Two headspace volumes of the respirometer, i.e., 12 mL (5 mL remaining headspace in the sample vial plus 7 mL in the reaction chamber) and 27 mL (20 mL remaining headspace in the sample vial plus 7 mL in the reaction chamber) were simulated in Figure 5.

The results indicated that the smaller the headspace, the quicker an equilibrium is reached, and that the greater the CO_2

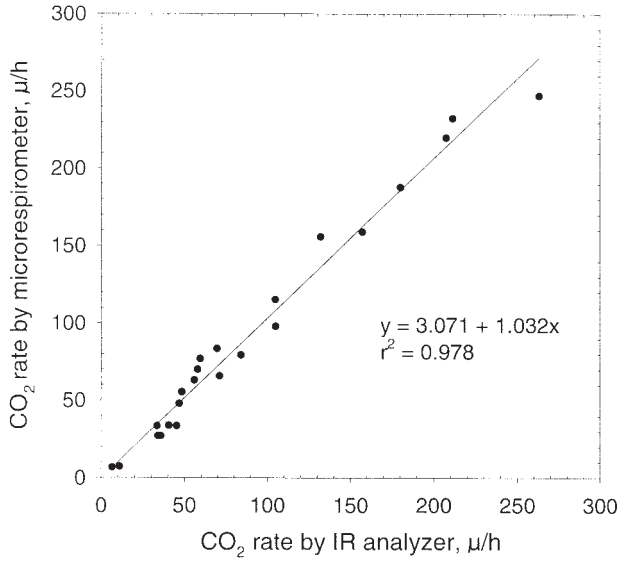

Figure 6. Comparison of the results of the CO_2 evolution rate determined by the microrespirometer with an established IR analyzer method. Each dot represents a single measurement, not a mean.

evolution rates, the quicker an equilibrium is reached. For example, in the 12 mL headspace case, 30 min pre-equilibration is sufficient for the measurement of all CO_2 evolution rates ≥ 1 μL/h. In the 27 mL headspace case, 100–107% of equilibrated value can be attained within 45 min for all CO_2 evolution rates, except the 1 μL/h case. The working range of the respirometer is designed to be 1–300 μL/h, which requires 30–45 min of pre-equilibration time, according to the condition of this study, to measure accurately the CO_2 evolution rate. If the CO_2 evolution rate is very low (≤ 5 μL/h), the headspace of the respirometer must be kept minimal to hasten the equilibration. The respirometer was designed so that the size of the reaction chamber stays the same while the size of the sample vial may vary according to the need of samples and the requirement of a minimal headspace.

The microrespiration was compared with an established IR respirometer on samples of various CO_2 evolution rates (Figure 6). The regression analysis indicates a very good 1:1 linear relationship (slope = 1.032 and r^2 = 0.978) between the 2 methods in a wide range (2–280 μL/h) of respiration rates.

One of the advantages of the microrespirometer is its ability to determine the CO_2 evolution rate accurately at the μL/h level in a short time. Determination of CO_2 evolution rates at a μL/h level is quite a challenge even for a sophisticated IR method. The IR analyzer must be able to detect less than 10 ppm (v/v) changes of CO_2 concentration with certainty during a period of hours. The accuracy of an IR analyzer method is further limited by the uncertainty of the volume occupied by a solid sample in most cases. Variation of headspace humidity, pressure, and temperature all affect the accuracy and precision of an IR respirometer. Because the microrespirometer method is based on the principle of CO_2 absorption–evolution equilibrium, its accuracy is not affected by headspace volume, humidity, pressure, or initial CO_2 concentration. The simplicity, noninstrumental nature, and very modest costs of the microrespirometer will make it available to many laboratory and field applications where accurate and rapid determination of respiration rate is required.

Conclusion

A novel noninstrumental microrespirometer was developed for the determination of CO_2 evolution rates. The microrespirometer is based on the simple principle of acid–base titration at a steady-state of CO_2 absorption/evolution. The microrespirometer is sensitive (detects μL CO_2/h), rapid (measurements made in 1 h), versatile (applicable to solid and liquid samples in a wide range of sizes), simple (in structure and operation), and economical (costs a fraction of an IR respirometer). The microrespirometer is suitable for monitoring real-time microbial activities in food, agricultural, and environmental samples, with regard to quality assurance and health safety concerns, under laboratory or field conditions.

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