USING THE RESPIRATORY QUOTIENT AS A MICROBIAL INDICATOR TO MONITOR SOIL BIODEGRADATION

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ABSTRACT

The effects of nutrient amendments on the variation in time of the respiratory quotient (RQ) were investigated in soil. Microbial activity measured by CO$_2$ production, biomass growth determined by plate counts technique and residual contaminants quantified by gas chromatography analysis were monitored in order to check their relation to RQ fluctuations. RQ values in all treatments displayed significant fluctuations over time which were closely related to the phases of the respiratory response as well as to microbial growth. After pollutant addition, an increase of RQ occurred in all microcosms. RQ values decreased when high degradation activity and microbial growth took place. RQ values slightly increased in all microcosms at the end of the incubation. These results show that the respiratory quotient is closely related to the physiological state of microorganisms and may be a determinable indicator for the efficiency of bioremediation.

Keywords: respiratory quotient, soil, biodegradation.

1. INTRODUCTION

Bioremediation is a well-recognized method for the treatment of contaminated soil [1]. However, bioprocesses are often operated under sub-optimal conditions due to the difficulty of identifying on-line the limiting parameters to biodegradation. Respiratory quotient, which is the molar ratio of carbon dioxide production to oxygen consumption, can display variations depending on composition of the examined microbial community as well as their available growth substrates [2]. Therefore, respiratory quotient could provide a valuable tool for a qualitative evaluation of microbial activity during bioremediation processes. For an on-line determination of the respiratory quotient, simultaneous and accurate measurements of oxygen and carbon dioxide evolutions are needed. Oxygen consumption in microcosms is usually determined by monitoring the air pressure after trapping the produced carbon dioxide into a strong base solution. CO$_2$ production is determined by acid/base titration of this solution. The objective of this work was to determine the relationship between the time-course biodegradation profile of a contaminant and RQ evolution and to investigate the effect of nutrient amendments
on RQ measurements. Hexadecane (C\textsubscript{16}H\textsubscript{34}) was used as a model contaminant for aliphatic hydrocarbons. RQ evolution curves as a function of time were compared to those of microbial growth and residual concentration of the contaminant.

### 2. MATERIALS AND METHODS

#### 2.1. Soil characterization

Soil samples (15.3 % of clay, 12.6 % of silt and 72.1 % of sand), collected from a natural field of Hanoi suburb (February 2017), were sieved at 2 mm and stored in the dark at 4 °C before use. Initial soil parameters were determined: soil water content of 15 % calculated from weight loss on drying at 105 °C for 24h; total organic carbon (TOC = 20.5 g/kg soil); total nitrogen (NTK = 1.69 g/kg soil); nitrate (NO\textsubscript{3}–N = 32 g/kg soil); nitrite (NO\textsubscript{2}–N<1 mg/kg soil) and orthophosphate (P\textsubscript{2}O\textsubscript{5}–P = 0.10 g/kg soil).

#### 2.2. Experimental and microcosms set-up

The biodegradation tests were performed in laboratory microcosms, consisting of Schott Duran bottles 500 mL, containing 50 g of soil at three (03) treatment options of nutrient-amendment: (1) soil contaminated by adding hexadecane at concentration 5.8 mg/g of dry soil without nitrogen (N) and phosphorus (P) sources (named thereafter as sample “H”); (2) adding hexadecane with the same concentration and (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} to reach C: N ratio of 100:10 (named thereafter as sample “HN”) and; (3) hexadecane and (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and KH\textsubscript{2}PO\textsubscript{4} to reach C:N:P ratio of 100:10:1 (named thereafter as sample “HNP”).

The soil microcosms were used for gas measurements (CO\textsubscript{2} production and O\textsubscript{2} uptake) and for chemical and biological analysis (microbial viable counts and residual hexadecane concentrations) during the 14-days bioremediation experiment. A tube filled with10 mL of 0.5M KOH solution, placed into each bottle, was used as alkaline trap to fix CO\textsubscript{2}. KOH solution was removed from the tubes and renewed daily in all microcosms. CO\textsubscript{2} production was determined by acid/base titration of this solution using hydrochloric acid 0.1 M HCl and some drops of phenolphthalein solution as indicator. O\textsubscript{2} uptake was determined daily by manometric measurement using OxiTop system (OxyTop-C controlled by the OxiTop OC110 system, WTW, Weilheim, Germany).

Identical microcosms were sacrificed at day 0, 2, 5, 8, 10 and 14 to monitor residual hexadecane concentration and hexadecane-degrading bacteria counts. Non-contaminated soil and abiotic microcosms (containing 0.02 % w/w of sodium azide (NaN\textsubscript{3})) were used as controls. All microcosms were incubated at 20 °C for all experiments. At each sacrifice time, two microcosms (duplicates) were sacrificed: 10 g of soil were collected from each microcosm to monitor specific hexadecane degraders and 5 g of soil were used to monitor the hexadecane concentration. Residual hexadecane was extracted from the contaminated soil by the Soxtec extraction unit then quantified using GC-FID gas chromatography analysis (Perkin Elmer model 8600).Hexadecane-degrading bacterial population was determined by spreading on minimal agar made from mineral medium solution, solidified with 17 g/L of Noble agar (Merck), enriched with 50µl of hexadecane and supplemented with 50 mg/L of cycloheximide.
3. RESULTS AND DISCUSSION

3.1. Hexadecane degradation and microbial activity

Hydrocarbon degradation in all microcosm experiments followed a 3-step pattern (Fig. 1a). The first phase (from 0 to 2 days) may correspond to the lag phase when the indigenous microbial population adapts and responds to the source of hexadecane. The hexadecane depletion in this phase was minimal. Microbial growth during this phase (Fig. 1b) corresponded to the hexadecane depletion. During the second “exponential” phase (from 2nd to 5th day), higher hexadecane depletion was observed. It corresponds to the maximum growth of the biomass. During the last phase (after the 5th day), hexadecane degradation slowed down to reach a plateau after 8 days for all three samples “HNP,” “HN” and “H”. The cumulative hexadecane mineralization during 14 days, calculated as a percentage of hexadecane initial concentration, ranged from 40 to 69% for three samples. However, after the 8th day from the beginning of the experiments, the hexadecane concentration remained stable or was completely degraded for the sample “HNP” (Fig. 1a). This means that the CO₂ produced after 8 days was not associated with the hexadecane biodegradation, but was likely due to other biotic processes such as microbial mortality or soil organic matter mineralization (Fig. 1b). Hexadecane degradation was correlated to microbial activity, expressed as CO₂ production, for all three samples (Fig. 1a and 1c). Figure 1 showed the important role of simultaneous presence of N and P on hexadecane biodegradation in soil. The highest hexadecane depletion, in agreement with the microbial growth and CO₂ production or microbial activity, was obtained at the sample “HNP” (corresponding to the C:N:P ratio of 100:10:1) and the lowest at the sample “H” (without N and P sources). Microbial activity and hexadecane degradation rate decrease for the samples “HN” and “H”, probably due to substrate supply restriction.

Figure 1. Residual hexadecane concentration (a), hexadecane degraders (b) and cumulative CO₂ production (c) in soil microcosms during bioremediation experiment.
The literature includes some works that indicated that the addition of nitrogen (N) and phosphorus (P) is particularly effective in stimulating hydrocarbon biodegradation rates [1, 3, 4]. Nutrients are essential for the growth and development of microbial cells. Through the results of experiments we can realize that the addition of inorganic nutrients, especially nitrogenous compounds, to the soil contaminated with hexadecane can significantly stimulate biodegradation compared with non-supplemented soil.

3.2. Respiratory quotient (RQ) evolution in soil microcosm experiments

The respiratory quotient (RQ), defined as the ratio of molar CO$_2$ production to molar O$_2$ uptake, is an integrative parameter that characterizes the respiration activity. Its temporal evolution is presented at Fig. 2 for all treatment options of nutrient amendments tested. For the non-contaminated soil microcosms, RQ was close to 1. For all the contaminated microcosms, a similar RQ profile was observed (Fig. 2). Initials RQ values ranged from 0.86 to 1.96. They increased from 1.1 to 2.2 during the first day of incubation. A decrease of RQ values was then observed between day 2 and day 4. Finally, RQ values increased until the day 5, before decreasing slowly until the end of the experiment. The theoretical value of RQ during hexadecane degradation can be obtained by the stoichiometric equation (Eq. 1) [1].

\[
C_{16}H_{34} + 24.5O_2 \rightarrow 17H_2O + 16CO_2
\]  

The above equation does not include biomass generation and therefore, does not involve nitrogen. On the basis of this equation, assuming that hexadecane is completely mineralized and that there is no biomass generation, the RQ of 0.65 mol CO$_2$ mol$^{-1}$ O$_2$ corresponds to the maximal value that can be obtained during hexadecane biodegradation. A horizontal line in Fig. 2 represents this maximal RQ value. The RQ profile curves followed a three-phase pattern as previously identified for both gas activities and contaminant degradation. The 0-2-day phase corresponding to RQ values greater than 0.65, the 2-5-day phase with RQs smaller than 0.65, and the 5-14-day phase when RQs become higher than 0.65. These three phases correspond to the lag, the degradation and the decay phase, classically observed in batch cell cultures.

RQs greater than 0.65 indicate that either not all hexadecane is degraded or the stoichiometric equation (Eq. (1)) is over simplified. If we include biomass (C$_5$H$_7$NO$_2$) generation and potential nitrification of nitrogen the Eq. (1) becomes [1]:

\[
C_{16}H_{34} + O_2 + NH_4^+ \rightarrow C_5H_7NO_2 + CO_2 + NO_3^- + H_2O + H^+
\]  

In this equation, when hexadecane carbon is consumed for cell production, the CO$_2$ production in the right hand side decreases compared to CO$_2$ produced in the Eq. (1). This would lead to a decrease of the RQ (RQ < 0.65). Meanwhile, additional O$_2$ consumption compared to the amount provided by Eq. (1) may be accounted for biomass respiration and nitrogen requirement. In addition, some researches stated that other biotic reactions such as oxidation of mineral soil constituents may consume O$_2$ [5, 6]. Thus, the RQ values for all the microcosms depend on relative contribution of biotic processes responsible of CO$_2$ production and O$_2$ consumption at each phase of degradation process.

During the 0-2-day lag phase, RQ values were greater than 0.65 for all microcosm experiments. These values are in the range reported by various studies [2, 3]. They suggested that environmental conditions may control the ratio of mole CO$_2$ evolution per mole O$_2$ depletion. RQ values greater than 1 appear when alternative electron acceptors, such as NO$_3^-$, SO$_4^{2-}$, are significantly involved in the current degradation of organic substances. The addition of external nitrogen (N) and phosphorus (P) sources, in the form of (NH$_4$)$_2$SO$_4$ and KH$_2$PO$_4$ for
the samples “HNP” and “HN”, to stimulate hydrocarbon biodegradation rates, may also influence RQ values greater than 1. The 2-5-day phase corresponds to the highest gas activities and hexadecane depletion phase (Fig. 1). Hexadecane carbon is converted not only into CO₂ but also into microbial cells, explaining RQ values below 0.65 (Eq.(2)). At the end of 2-5-day degradation phase (close to day 5), RQ values increased again and became higher than 0.65. After 5 days, a stationary “decay” phase was characterized by microbial mortality and a very slow hexadecane biodegradation phenomenon (Fig. 1). During this phase a slow decrease of RQ values was observed. This can be explained by a lower demand in oxygen as the substrate is less biodegraded and a higher CO₂ production due to the microorganism’s mortality.

![Figure 2. Evolution of the RQ values during hexadecane degradation for three treatment options of nutrient amendments. The horizontal line refers to theoretical hexadecane RQ value based on mineralization.](image)

3.3. Effect of nutrient amendments on respiratory quotient (RQ) profile during bioremediation

The nutrient amendments did not significantly affect the shape of the RQ evolution curves as a function of time (Fig. 2). It only modified the magnitude of RQ values. Indeed, the minimum value of the RQ can be identified during the 2-5 day degradation phase for all treatment options of nutrient amendments. The lowest RQ value was observed for the sample “HNP”, corresponding to the highest gas activities and to the highest hexadecane depletion rate.

These results tend to show that higher biodegradation rates are associated with lower RQ values indicating by the same way that higher biodegradation rates are associated with higher hexadecane carbon conversion into cell’s microorganisms. Various studies stated that the respiratory quotient is very sensitive to changes in the substrate availability and physiological adjustments of soil microbial communities [3, 6]. These results are very useful to better understand and describe contaminant biodegradation in soil batch processes. Indeed, strong variations in RQ values during batch contaminant biodegradation in soil prove that degradation processes cannot be described by only one biodegradation reaction. Others reactions corresponding to various phenomena (microbial adaptation in the lag phase and microorganism’s mortality in the decay phase) must be considered to better explain the experimental observations.

However, the RQ may be an indicator for easily biodegradable carbon sources in the soil and presents a relevant, quick and easy determinable indicator for the efficiency of
bioremediation. The commercial devices are capable of continuously and automatically measuring $O_2$ uptake and $CO_2$ production and have the advantage of giving a quick and ease information of the bioremediation over the course of time. In addition, RQ is highly influenced by environmental factors, such as the nutrient amendments as shown in this work. Thus, pre-conditioning and standardization of the soil before measuring RQ is necessary to minimize the effect of these variables.

4. CONCLUSIONS

A clear relationship was observed between RQ evolution, microbial activity and contaminant depletion. The lowest RQs were correlated to the highest hexadecane depletion rate and were obtained for the sample “HNP” (corresponding to the C:N:P ratio of 100:10:1). This results show that it could be possible to monitor the contaminant degradation and microbial activity indirectly by using RQ as a monitoring tool. The determination of RQ could also be useful as one of the on–line measured variables to better monitor and control soil batch contaminant biodegradation processes. Studies have been carried out under controlled laboratory experiments. Further studies are necessary to determine the applicability of these results to field conditions. In open field conditions, microbial degradation processes may be more complicate. Hydrocarbons contamination may not be homogeneously distributed in the contaminated soil. Heterogeneous soil induces a heterogeneous distribution of water content causing shifts in microbial communities and thus affecting contaminant degradation.

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