IRA-International Journal of Applied Sciences
ISSN 2455-4499; Vol.03, Issue 03 (2016)
Institute of Research Advances
http://research-advances.org/index.php/IRAJAS

Methods for elucidating microbial biomass

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DOI: http://dx.doi.org/10.21013/jas.v3.n3.p8

How to cite this paper:
Saxena, N. (2016). Methods for elucidating microbial biomass. *IRA-International Journal of Applied Sciences (ISSN 2455-4499)*, 3(3).
doi:http://dx.doi.org/10.21013/jas.v3.n3.p8

The microbial biomass of soil is defined as the part of the organic matter in the soil that constitutes living organisms smaller than the 5-10 $\mu m^{3.}$ It is generally expressed in the milligrams of carbon per kilogram of soil or micrograms of carbons per gram of dry weight of soil. Typical biomass carbon ranges from 1 to 5% of soil organic matter. Biomass literally means "mass of living material" and can be expressed in units of weight (grams) or units of energy (calories or joules). Biomass is an important ecological parameter as it represents the quantity of energy being stored in a particular segment of biological community.

Measurement of biomass is used to determine standing crop of a population and transfer of energy between trophic levels within an ecosystem. A measure of the total microbial biomass in soil is often required when studying productivity or fertility of soils. Sometimes the biomass of specific parts of the microbiota is required-for example fungal biomass versus bacterial biomass. Soil microbial biomass is an agent of transformation for added and native organic matter and acts as a labile reservoir for plant available N, P, and S (Jenkinson, et. al., 1981). The activity of the microbial biomass is commonly used to characterize the microbiological status of a soil (Nanniperi, et. al., 1990), and to determine the effects of cultivation (Beyer, et. al., 1991; Anderson, et. al., 1993), field management (Perott, et. al., 1992), or contamination (Chander, et. al., 1993) on soil microorganisms. The degradation of organic compounds, such as industrial chemicals and pesticides, can be monitored by following changes in soil microbial biomass. Microbial biomass is important since it acts as a sink and source of nutrients (Marumoto, et. al., 1982; Singh, et. al., 1989) and it is a principle component of the decomposer subsystem regulating nutrient cycling, energy flow and ecosystem productivity (Wardle, et.al., 1998). Soil physiological characteristics influence the level of biomass and the activity of microorganisms. It is well known that water and its biological availability is an important factor influencing soil microbial diversity. It has frequently been shown that soil microbial biomass decline upon drying and increase upon rewetting (Orchard, et.al., 1993; West, et. al., 1988, 1992;Wardle., 1998), whereas drying and rewetting of soils caused a decrease in microbial biomass (Van Gestel, et. al., 1993).Nevertheless, positive relationships between soil moisture and microbial biomass are not universal, and negative relations between these variables, mostly in conditions of high soil moisture, have sometimes been detected (Wardle., 1998; Rinklebe., 2004).

Generally soil microbial biomass can be limited by soil moisture under both dry and wet conditions. Exposure of microbial communities to fluctuating moisture may lead to selection for organisms better adapted to these conditions. Although some researches studied the effect of dry/wet cycles on soil microbial communities under different farming systems (Lundquist, et. al., 1999a) or on available carbon sources in the laboratory (Lundquist, et. al., 1999b), there is a gap to quantify impacts of flooding duration and type of soil on microbial diversity in floodplain soils, both in laboratory and in the field. Seasonal changes in soil moisture, soil temperature and C input from crop roots, rhizosphere products (i. e. root exudates, mucilage, sloughed cells, etc.), and crop residues can have a large effect on soil microbial biomass and its activity (Ross., 1987), which in turn, affect the ability of soil to supply nutrients to plants through soil organic matter turn over. Microbial biomass has been reported to vary seasonally in European soils (Patra, et. al., 1990). Singh, et. al., 1989 have

also reported a seasonal variation in the microbial C, N, and P in forests and savanna. Generally, a negative relationship between soil moisture content and microbial biomass was reported by Ross., 1987 for New Zealand soils under tussock grassland and introduced pasture. Short-term fluctuations of moisture and temperature conditions have been shown to influence the amount of microbial biomass carbon (MacGill, et. al., 1986).

Soil organic carbon levels, too, have been reported to be governed by climatic conditions (Jenny., 1980). Limited data however, exists on the magnitude of seasonal changes in soil microbial biomass and enzyme activity in soils under cultivation were determined at May when crop root and residue additions were minimal and at September when crop residues were maximal. Plant growth pattern also influences the microbial biomass. Singh, et. al., 1989 and Yang, et.al., 1991, found out that microbial biomass and nutrient pools declined during the period of most rapid plant growth (wet season). Singh, et. al., 1989 concluded that the principle function of the microbial biomass is to accumulate nutrients during the dry period, and to release them rapidly upon onset of the wet period. Andrew, et. al., 1993, gave a relationship between microbial activity, biomass, and organo sulphur formation in Forest soil. Collectively, the data suggested that increasing inputs of sulphate, such as those encountered in acidic precipitation, stimulate aerobic respiration by microbial populations in the soil, generating ATP, which is then used to drive the organic S formation process, using the added sulphate as a source of S for incorporation into organic matter. Unfortunately, the direct measurement of microbial biomass, such as by filtration and direct dry weight or by centrifugation and packed cell volume measurements as practiced on pure cultures, are rarely applicable to environmental samples.

2.2 Methods for elucidating microbial biomass of soil

More recently newer methods have been developed that use measures of components of all cells to estimate biomass. These methods include:

a) Dilution plate counts and direct microscopical counting

Dilution plates usually only are able to culture between 1 and 10% of the viable organisms in soil samples. Direct microscopic observation methods (FITC, acridine orange staining, etc.) usually overestimate the number of cells because they include dead organism or other particles in their count.

b) Determination of cell wall components of bacteria

Bacteria contain specific cell wall components such as muramic acid that can be released by acid hydrolysis and analyzed by High Pressure Liquid Chromatography (HPLC). The amount of muramic acid in bacterial cell walls varies depending on whether the cells are gram positive or gram negative (12μg MA $mg^{-1}C$ average in Gram negative and 44 μg MA $mg^{-1}C$ in Gram positive cells). Thus, unless the proportion of Gram positive to Gram-negative cells is known, this technique has serious limitations. Bacterial spores also contain upto 4 times the normal levels of muramic acid. Phospholipid fatty acid (PLFA) analysis is also used to determine biomass. In the research of terrestrial soils (Frostegard, et. al., 1993a; Zelles., 1999; Pennanen., 2001), sediment microbiology (Rajendran, et al., 1992; Macalady, et al., 2000) or groundwater microbiology (Green, et.al., 2000) the use of lipid-based techniques is well established. Several studies

determined the soil microbial biomass (Gaunt., 1993; Witt., 1997), the metabolic diversity of microbial communities (Bossio, et.al., 1995) or PLFA (Bossio, et.al., 1998; Bai, et. al., 2000; Nakamura et al., 2003) in paddy rice fields. Rinklebe., 2006 used PLFA biomass, microbial carbon (C_{mic}), basal respiration (BR), metabolic quotient (qCO_2), and C_{mic}/C_{org} ratio to characterize and discriminate flood plain soils with microbial parameters. They concluded that biomass increased in the order of submerged soil< intermediate flooded soil< short time flooded soil. PLFA biomass in flood plain soil is higher in comparison to terrestrial soil, because flood plain soils are often characterized through high carbon contents due to high intensity of accumulation of organic material (Rinklebe., 2004).

c) Soil fumigation method

Microorganisms are primary agents of the digenesis of organic material (Deming, et.al., 1993). Thus, a strong link between carbon flux and microbial activity and biomass can be expected. The original fumigation-incubation method to measure biomass (Jenkinson ,et.al., 1976) is still standard procedure to measure biomass. Sample of soil is sieved and placed in a container and CO_2 output is measured over 20 days. There is typically a period of very rapid respiration followed by a much lower but stable respiration rate. If the organisms are killed by fumigation with chloroform, the initial flush of activity does not occur and the cells are killed. If chloroform is removed there will be bigger flush or rapid respiration because dead microbial cells will contribute to total substrates available (they were killed by the chloroform treatment). The difference between normal rapid respiration and the greater amount after chloroform treatment is due to amount of microbial biomass originally in the system.

Now days this method is superseded by fumigation-extraction technique. The fumigation of soil with $CHCl_3$ followed by extraction with various salt solutions has been used to measure microbial S (Saggar, et. al., 1981). P (Brooks, et. al., 1982; Hedley, et.al., 1982), N (Brooks, et. al., 1985; Amato, et.al., 1988; Joergensen, et.al., 1990). The fumigation-extraction method was first used to measure microbial biomass P by Brooks, et. al., 1982, then biomass N (Brooks, et. al., 1985) then probably most importantly, microbial C by Vance, et. al., 1987. An improved analytical procedure was introduced by Chaussod, et.al., 1988 and developed for use in laboratory by Wu, et. al., 1990. Chander, et. al., 2002 found out that soil fumigated with chloroform, with a much smaller microbial biomass than the corresponding non-fumigated soil, respired about the same amount of ^{14}C-CO_2 from labeled straw as the non-fumigated soil. They also found that pollution with zinc-affected respiration of non-fumigated and fumigated soils in the same way, indicating that the ratio between substrate C-to-microbial biomass C (larger in fumigated than non-fumigated soil) was not important. The ratio of CO_2 to ^{14}C-to-microbial biomass ^{14}C was linearly related to Zn pollution. Griffith, et. al., 2000, found out that soil fumigated with chloroform, with greater microbial diversity, was more resistant and resilient than soil with a less diverse community to perturbations such as heating at 40^0C for 18 hours or treatment with 500μg Cu g^{-1} soil. However, they suggested that the observed effects were due to the physiological influence of $CHCl_3$ fumigation on the microbial community rather than differences in microbial diversity.

d) Determination of ATP content of soils

Adenosine tri phosphate is an important energy compound in the metabolism of all living organisms. ATP is considered to be a useful indicator of life in soil (Dinesh, et. al., 2006). ATP can be measured with great sensitivity. Though dependent on physiological state, ATP concentrations are fairly uniform relative to cell carbon for many bacteria, algae, and protozoa. ATP is an accepted indicator of microbial biomass in aquatic and terrestrial environments (Martins, et. al., 1985; Dobrowolski, et. al., 1991; Gonzalez, et. al., 1992). The primary reasons for measuring this cellular constituent include (i) ATP is lost rapidly following the death of cells, a measurement of ATP concentrations can be used to estimate living biomass (Holm-Hansen., 1969; Paerl ,et.al., 1976; Deming, et. al., 1993; Stevenson, et. al., 1972), (ii) ATP is considered to be present in a uniform concentration in all microbes (Martins, et. al., 1985; Dobrowolski, et. al., 1991) and (iii) the analytical techniques for the extraction and assay of ATP are specific, relative quick, easy to perform, and inexpensive. The ATP monitoring concept was developed in the mid seventies (Lundin, et. al., 1976). According to this concept, the firefly luciferin-luciferase system could be added to an ATP converting reaction mixture, enabling continuous monitoring of the ATP concentration by measuring the intensity of the light emission. The usefulness of this concept was based on the fact that rate of the individual steps of the firefly catalytic cycle could be regulated by stimulators and stimulators to obtain a light emission rather than a peak light emission (Lundin., 1982).

The ATP monitoring concept has been used to develop assays of e.g. photophosphorylation (Lundin, et.al., 1978), adenylate kinase (Lundin., 1985), ATP/ADP/AMP (Lundin et al., 1986), glycerol (Lundin, et. al., 1989; Hellmer, et. al., 1989), inorganic phosphate (Nyren., 1985), oxidative phosphorylation (Wibom, et. al., 1990) and ATP/phosphocreatine (Wibom, et. al., 1990). Fireflies or lightening bugs make light within their bodies. This process is bioluminescence and is shared by many other organisms, mostly sea living organisms. The cells contain a chemical called luciferin (Fig.1) and make an enzyme called luciferase (Fig.2). To make light, the luciferin combines with oxygen to form an inactive molecule called oxyluciferin. The luciferase speeds up the reaction, which occurs in two steps (Fig 3 a & b):

1. The luciferin combines with adenosine tri phosphate (ATP), which is present in cells, to form luciferyl adenylate and pyrophosphate (PPi) on the surface of luciferase enzyme. The luciferyl adenylate remains bound to the enzyme (Lundin., 1993).

Luciferin + ATP $\longrightarrow$ **Luciferyl adenylate + PPi**

2. The luciferyl adenylate combines with oxygen to form oxyluciferin and adenosine mono phosphate (AMP). Light is given off and oxyluciferin and AMP is released from the enzyme's surface:

Luciferyl adenylate + O_2 $\longrightarrow$ **oxyluciferin + AMP + PPi**

The wavelength of light given off is between 510 and 670 nm (pale reddish green color). Amount of light emitted in this reaction is directly proportional to the ATP concentration. Measurements of ATP can be used to estimate microbial biomass (Lundin, et. al., 1979; Lundin.,

1982). A factor of 250-286 is often used for conversion of ATP to cellular carbon for aquatic samples (Hamilton, et.al., 1965; Holm-Hansen, et.al., 1966; Holm-Hansen., 1973 a, 1973 b; Paerl, et.al., 1976); for soil, a factor of 120 is used to convert ATP to biomass carbon (Ausmus., 1973; Jenkinson, et.al., 1979; Oades, et.al., 1979). A high correlation has been found between soil ATP and biomass determined by fumigation method (Table.1), (Oades, et.al., 1979).

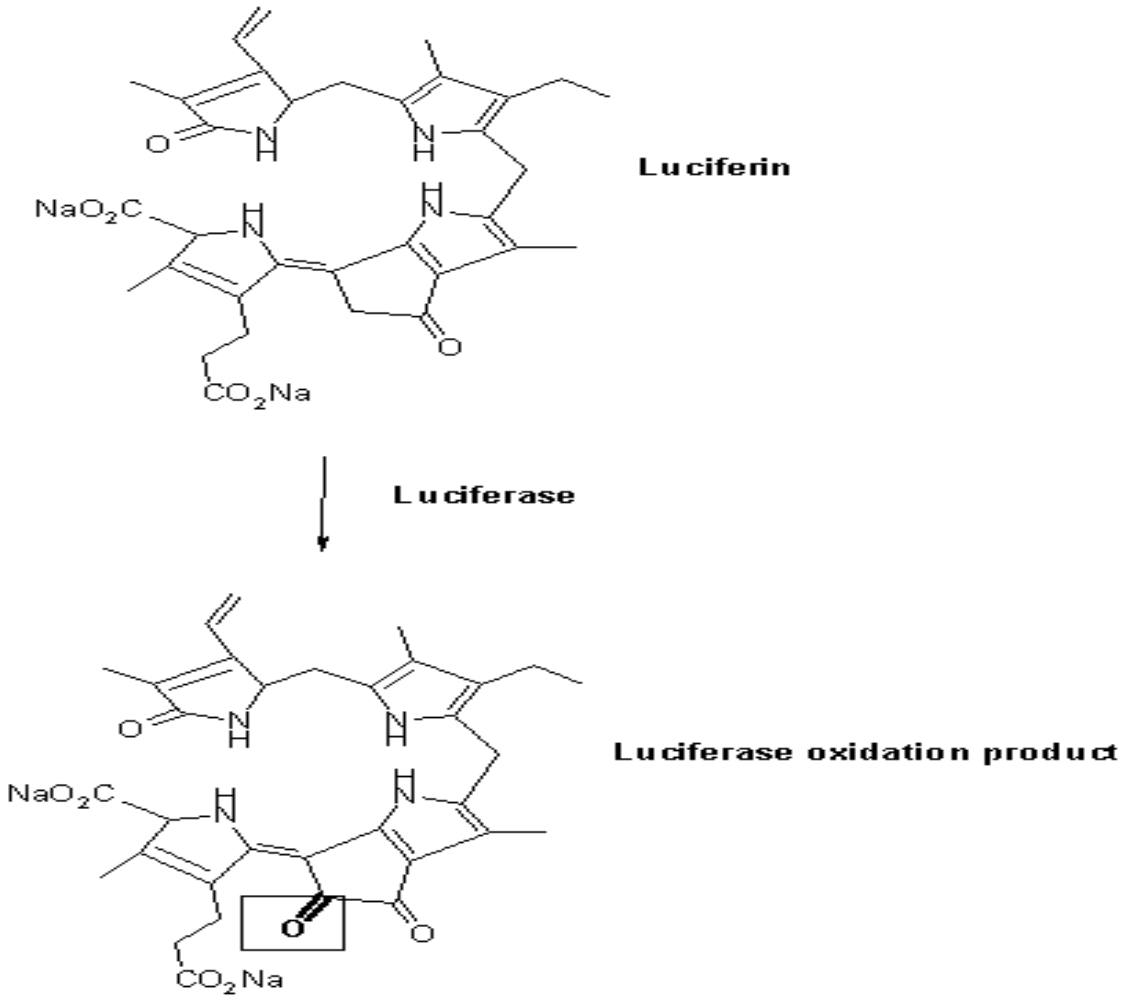


Fig .1 Chemical Structure of Luciferin.

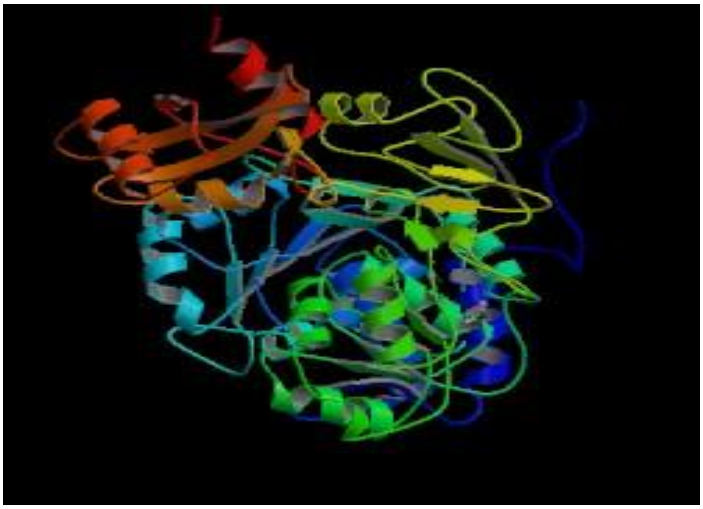
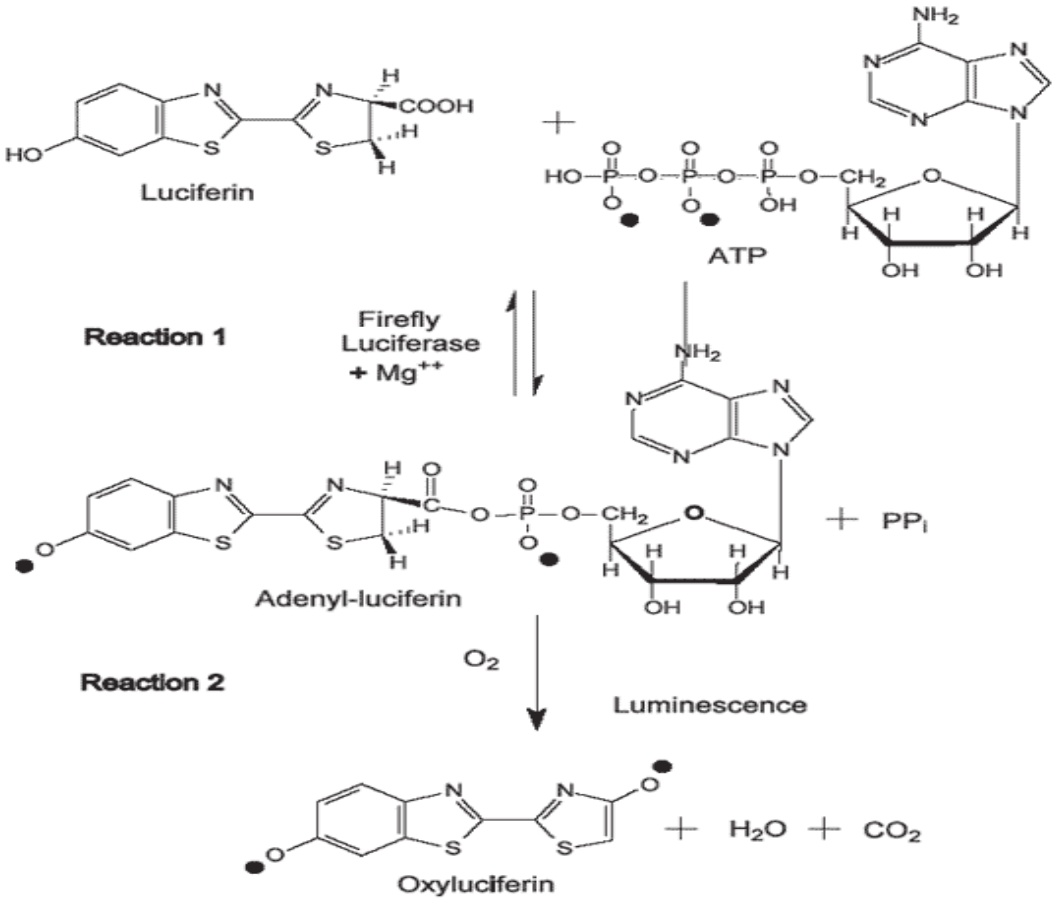


Fig.2 Ribbon structure of luciferase enzyme.



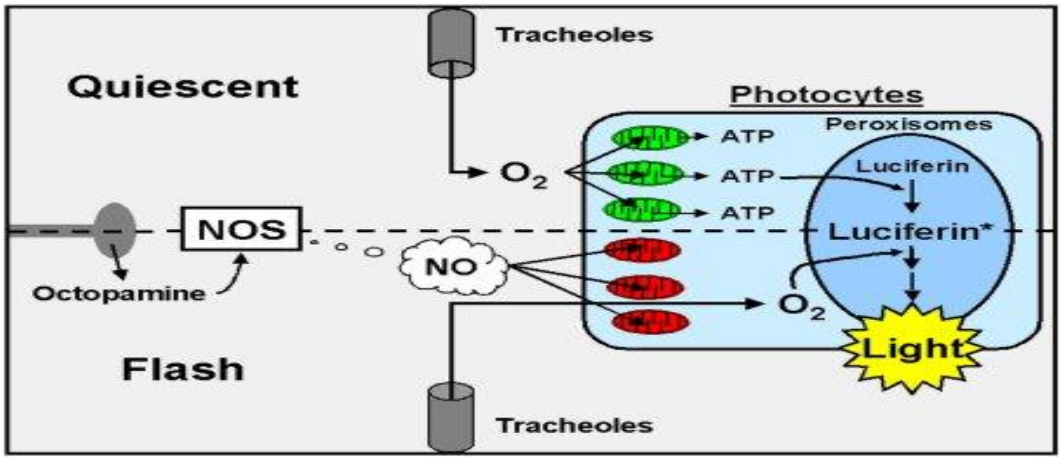


Fig.3 a & b. Steps of Luciferin-Luciferase light reaction

Table.1 Comparison of biomass estimated by soil fumigation and ATP in various soils.

Soil Sample	CO_2 carbon evolved by unfumigated soil in 10 days (µg g^{-1})	CO_2 carbon evolved by fumigated soil in 10 days (µg g^{-1})	Biomass carbon (µg g^{-1})	ATP in soil (µg g^{-1})
1	70	114	88	0.75
2	205	315	220	2.07
3	131	240	218	3.14
4	20	43	48	0.64
5	236	613	754	9.03
6	108	158	99	0.90
7	117	341	448	2.97
8	264	565	603	4.56

9	**76**	**143**	**135**	**1.37**
10	**259**	**642**	**766**	**7.00**
11	**257**	**646**	**778**	**6.55**
12	**85**	**189**	**208**	**1.32**
13	**229**	**525**	**591**	**4.20**

Source: Oades and Jenkinson, 1979.

The bacterial activity measured in terms of biochemical approaches such as, ATP content, significantly proves that the community structure in the soil is directly correlated with availability of oxygen and organic matter. Thus, by measuring the above mentioned parameters there is a better understanding of the relations between microbial diversity and soil functions.

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