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*Full Length Research Paper*

# Measurement of microbial activity and applicability of dissolved DNA as indicator of microbial activity in pasturage soil

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**A method was developed for the determination of dissolved DNA in pasturage soil and was based on the concentration of dissolved DNA by ethanol precipitation of 0.2 µm - pore size filtered water. The DNA concentrated extracts were quantified by the fluorescence of Hoechst 33258-DNA complexes. The fluorescence that was not attributable to DNA was corrected by DNase I digestion of the extracts and it averaged 25% of the total fluorescence for all samples. Concentration of dissolved extracellular DNA from a variety in the soil ranged from 131.26 to 29.08 DNA ng/ml in 10 gr soil. The agarose gel stained with ethidium bromide (electrophoresis technique) was used for soil dissolved DNA. The method of Hoefer TKO 100 DNA Mini-Fluorometer is simple for extracellular DNA and is more sensitive than previously described methods for the determination of extracellular DNA. The TKO 100 Mini-Fluorometer is designed specifically for the accurate quantitation of DNA in dilute samples. The unit can also be used for assaying protease, glucuronidase (GUS) and 1-galactosidase activity.**

**Key words:** Extracellular DNA, fluorescence, electrophoresis technique.

## INTRODUCTION

In cells, DNA is present in double-stranded form. Each cellular chromosome contains two strands of DNA and each strand contains several million of nucleotides linked to phosphodiester bonds. Nucleic acids are long polymers in which nucleotides are covalently bonded to one another in a defined sequence forming structures called polynucleotides.

Approximately, 90% of the organic substance in seawater exists as dissolved compounds, even though many of the low molecular-weight compounds of the dissolved organic matter in soil have been identified and quantified (Markov and Ivanov, 1974).

As a constituent common to all living cells, DNA is a potential component of the dissolved macromolecular fraction in aquatic environments. The polymerase chain reaction (PCR) is a very powerful and sensitive analytical technique with applications in many diverse fields, including molecular biology (Lemanceau et al., 1994). Since humic substances have now become a major concern upon the amplification of target DNA extracted from soil or sediment samples, it is important that humic substances are removed or attenuated from the nucleic acid extracts to avoid inhibition of the PCR.

Little information exists on the temporal and spatial distribution of particulate DNA and dissolved DNA in soil. DNA is a compound rich in nitrogen and phosphorus, which could be an important source of microbial nutrition (Mary et al., 1986). Dissolved DNA could also be a source of nucleic acid precursors, which are energetically expensive for microorganisms.

Recently, numerous studies have investigated new methods to improve extraction, purification, amplification and quantification of DNA from soils (Tsai and Olson, 1991).As a constituent common to all living cell, DNA is a potential component of the dissolved macromolecule fraction in aquatic environments. The presence of extracellular DNA in the soil has been known for some time (Ogram et al., 1987). Most methods for the identification of biological activity in the soil involve the use of microbial biomass and mineralisation property, ammonification, nitrification and enzymes activity. The

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increasing importance of molecular biological techniques in microbial ecology and activity is in the use of these techniques for extraction of microbial DNA from soil.

The persistence of measurable concentrations of extracellular DNA in soil is important for several reasons: As a compound rich in nitrogen and phosphorus, DNA could be an important source of microbial nutrition. Dissolved DNA could also be a source of nucleic acid precursors, which are energetically expensive for microorganisms to synthesize *de novo*. The measurement of extracellular DNA may also be important in light of the environmental use of genetically engineered microorganisms, as a means to monitor DNA in aquatic environments. In the past decade, applications of new molecular biology methods, based primarily on amplification of soil-extracted nucleic acids, have provided a pertinent alternative to classical culture-based microbiological methods, providing unique insight into the composition, richness and structure of microbial communities (Stackebrandt, 1993; Nusslein and Tiedje, 1998; Kuske et al., 1997).

In this study, we have optimized the DNA extraction protocol at small scale level for the pasturage soil samples.

## MATERIALS AND METHODS

### Soil sampling

This long term experiment on a meadow soil began in 1978. The study was carried on a silt-loam at the MAPAQ experimental farm in St. Lambert, Quebec Canada.

Treatments in a split-plot design consisted of no-till and tillage as principal treatments and three rates of manure (0, 50 and 100 Mg $ha^{-1}$) as secondary treatments. Soil samples were taken from 0 to 15 and 15 to 30 cm depth. The moist soil samples were sieved at 6 mm in the field, while roots and stubble were removed. Samples were kept at 4°C until microbial activity analysis was performed.

### Determination of bacteria, fungi and actinomycetes contribution to soil respiration

A technique (using selective inhibitors) was used to estimate the relative contributions of bacteria, fungi and actinomycetes to soil respiration ($CO_2$ production). The $CO_2$ produced by the different microbial groups was determined by titration method, while glucose was the substrate used. Streptomycin and actidione were used to inhibit bacteria or fungi, respectively (Anderson and Domsch, 1974).

Microbial biomass C, N and P were determined using the chloroform-fumigation-incubation method (Jenkinson and Powlson, 1976) and were estimated from the difference between organic C, N and P, extracted with 0.5 M potassium sulfate from chloroform fumigated and unfumigated soil samples using $K_C$ factors of 0.38, 1.85 and 0.4, respectively. However, microbial biomass levels were expressed as kg $ha^{-1}$ soil.

Soil pH was measured in a soil: water ratio of 1:2, while the organic C content was determined by wet oxidation procedure (Walkley and Black, 1934). Subsequently, the total N was estimated by Kjeldhahl digestion (Nelson and Sommers, 1982) and the soil water-soluble C was assessed as previously described by Dormaar et al. (1984).

A nitrogen and carbon mineralization measurement was determined by incubation method (Stanford and Smith, 1972), while the soil carbon and nitrogen mineralization potentials were determined by kinetic models (Ellert and Bettan, 1988) and arginine ammonification was carried out as described by Alef and Kleiner (1987).

**Table 1.** The effect of tillage practices and manure application rate on soil DNA dissolved in surface soil.

| DNA ng/gr soil | Treatments |
|---|---|
| 29.8 | Plow soil with 0 ton |
| 30.1 | Plow soil with 50 ton |
| 131.26 | Plow soil with 100 ton |
| 44.15 | No-till soil with 0 ton |
| 63.75 | No-till soil with 50 ton |
| 41.64 | No-till soil with 100 ton |

### Concentration and measurement of DNA dissolved

50 ml $Na_2SO_4$ (120 mM) of sodium phosphate buffer (pH = 8) was mixed with soil samples (10 g) and the samples were shook at 150 rpm for 15 min and they passed through a filter combination consisting of a GF/D filter (watman, 40 and 42) and were filtered under a vacuum of < 150 mm Hg (< 20 KPa), with the filtration flask immerse in an ice bath. The DNA in the filtrate was precipitated by the addition of 2 volumes of 200-proof (100%) ethanol. After 48 h at -20°C, the precipitate was collected by centrifugation with two volumes in a GS 10 rotor at 6.800 × g for 20 min. The precipitate was then dialyzed at 4°C for 48 h against deionized water and then for 24 h against 1 × SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). Triplicate samples of the dialysate 2 ml with SSC and 1 ml of $6 \times 10^{-7}$ M solution stock Hoechst 33258 in distilled, filtered $H_2O$ [concentration dye stock (1 mg/ml H2O)] were added. DNase I was dissolved in 0.02 M sodium acetate pH 5, with 5 mM $MgSO_4$ and 100 ml of the DNase solution. The samples were heated to room temperature and the fluorescence was determined as previously described by spectroflurometer and TKO 100 Mini-fluorometer. A small part of the measurements from the material was correct for fluorescence other than DNA by DNase I treatment. Calf thymus DNA ng/ml (0, 40, 60, 80, 100, 250, 500 and 750) is the DNA used by the standard assay in the TKO 100 Mini-fluorometer.

The measurement of extracellular DNA may also be important in light of the environmental use of genetically engineered micro-organisms as means to monitor DNA in soil environments. John et al. (1988) concluded that dissolved DNA showed seasonal variation with minimal values in December and January and maximum values in summer months. Moreover, the DNA in concentration of dissolved DNA and particulate DNA was quantified by the fluorescence of Hoechst 33258-DNA complexes.

## RESULTS AND DISCUSSION

The results of the soil's DNA are presented in Table 1, while the range of DNA is 29.8 to 131.26 ng/gr soil. However, the range of the soil's DNA dissolved in the surface soil (0 to -15 cm) was 29.80 to 131.26 ng DNA in 10 gr of soil. The results were similar to the results of Ogram et al. (1987), who observed that in 1 mgr soil, 1 gr DNA was dissolved. We observed that there was no significant effect of soil tillage management practices on the soil DNA dissolved on the surface of the soil. Also, we did not observe any significant effect of manure application on soil dissolved DNA on the surface of the soil. Furthermore, we did not observe any interaction of

manure application rates and soil tillage management practices on soil DNA dissolved in this layer, but it was obtained that the differences in the level of manure application and soil tillage management practices affected soil DNA dissolved on the surface of the soil. The results suggested that dissolved soil DNA decline with tillage management practices and indicated remarkably that soil dissolved DNA was higher in no-till soil than plow soil (32.50%). In this study, it may be that it is in plow soil that there are other elements such as methal ions and fluvic acid, which could also contribute to the inhibiting effect. The present results, which are in line with those of other authors, indicate that on entry to soil DNA, they are rapidly and extensively degraded simply in plow treatment. Measurments of DNA with pasturage soil suspension resulted in a rapid increase in bacterial numbers, which cause tillage management practices that resulted in a small release of inorganic P in soil (inorganic P in this study was higher in no-till soil than till soil) and an increase in bacterial number that they want inorganic P for their activities. Greaves et al. (1970) report that microbial biomass P increased in no-till soil on comparison with tillage system, while in this study, microbial biomass P was higher in no-till soil (44.15%) than plow soil. We observed that soil DNA dissolved was higher in no-till soil than plow soil by using agarose gel stained with ethidium bromide (electrophoresis technique) (Figure 1). Another reason may be that soil tillage management practices influence closely, soil colloids and soil bacterial cells associated with soil colloids. In the separating process, there are different resistances for liberating soil microorganism cells and soil DNA that are formed in the matter organic or microbial biomass that are in the soil. The influence of soil tillage management practices on soil texture, that is, clay (%), sand (%) and silt (%) is one strategy for increasing DNA in the soil, while another reason may be that soil tillage management practices influence DNase in the soil. Therefore, we know that enzyme is responsible for the degradation of DNA in the soil and as such, the quality of DNAase can influence closely, soil DNA dissolved. In this study, we observed a variation in soil pH in no-till soil and plow soil, while humic substances, mainly humic and felvic acid can be affected by soil pH. As a result, the trace amounts of humic and felvic acid influence DNA recovery in the soil samples. Tsai et al. (1991) used polymerase chain reaction (PCR) for recovery and separation of microorganism DNA in soil. However, it should be noted that other elements (for example metal ions) can affect soil DNA recovery in the soil.

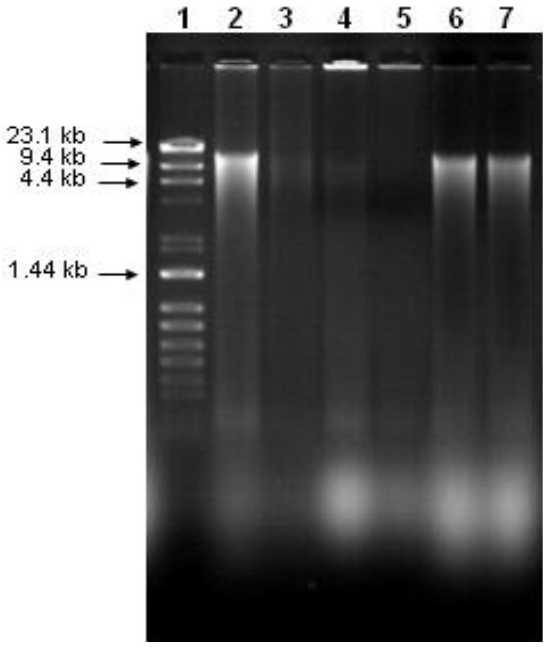


**Figure 1.** Agarose gel electrophoretogram of the total DNA isolated from three sampling soil.

## Measurement of bacterial, fungal and actinomycetes contribution to microbial respiration ($CO_2$)

The results of soil respiration studies in the presence of inhibitors are presented in Table 2.

Afterwards, 1032 h after incubation, the total soil bacterial and actinomycetes respiration were influenced by soil depth: F = 14.25** and F = 28.28**, respectively, while a significant interaction (F =10.01**) between manure applications and soil depth on actinomycetes total respiration activity was observed.

Tillage management practices influence weight molecular DNA in the soil, which results to an effect of the quality and quantity of the bases in the DNA such as adenin and thymidine (AT). These bases are very important bases for fluorometric method, because the greatest fluorescence occurs in portion of DNA rich in adenine plus thymine A+T, while the region that contains guanine plus cytosine possesses only 50% of the fluorescence of the A+T region. In general, dissolved DNA was produced by actively growing heterotrophic bacterioplankton, but in this study, dissolved DNA was not only produced actively by the microorganisms, but also could be produced by soil organic matter or soil microbial biomass after their degradation in soil. Therefore, soil tillage management practices can influence closely soil dissolved DNA.

The result of this study suggested that Hoechst 33258 dye which is weakly fluorescent increases in fluorescence in the presence of DNA. The greatest fluorescence occurs in portions of DNA rich in adenine plus thymine (A+T). The region that contains guanine plus cytosine possesses only 50% of the fluorescence of the A+T regions. It is thought that the Hoechst 33258 dye would be bound without intercalation in the major groove of the double helix of A+T rich regions, perhaps, by hydrophobic interaction with the methyl of thymidine. To verify that the fluorescence observed is for DNA, DNase I (80 dornanse unites mg of protein$^{-1}$) is used to degrade

**Table 2.** Analyses of variance (F values) of the effect of tillage practices and manure application rates on bacteria, fungi and actinomycetes on the cumulative total respiration after 1032 h incubation of a meadow soil.

| Treatment | Bacteria activities | Fungi activities | Actinomycetes activities |
|---|---|---|---|
| Tillage (T) | 1.25 | 0.69 | 2.86 |
| Manure (M) | 1.34 | 1.47 | 2.06 |
| Depth (D) | 14.25** | 2.82 | 28.28** |
| T X M | 0.19 | 1.29 | 0.33 |
| T X D | 2.41 | 0.02 | 0.38 |
| M X D | 1.80 | 3.01 | 10.01** |
| T X M X D | 1.43 | 0.55 | 0.49 |

**,*, Significant at $p \leq 0.01$.

DNA in extracts. The result of this study presents that DNase I is dissolved in 0.02 m sodium acetate buffer and pH 5.0 containing 5 mM $MgSO_4$ for final concentration of 2 $mgml^{-1}$ while 30 ml of 1 M acetic acid, 5 ml of 2 m Mg $SO_4$ and 100 ml of the DNase solution are dissolved in a 2 ml DNA extract in SSC (pH 7.0). Hoechst 33258, which is weakly fluorescent, increases in fluorescence in the presence of DNA binding. Specifically and quantitatively, the greatest fluorescence occurs in portions of DNA rich in adenine plus thymine (A+T). The regions that contain guanine plus cytosine possess only 50% of the fluorescence of the A+T regions. CoDNase I treatment of extracts of natural microbial populations remove 95 to 100% of the observation of fluorescence. However, we obtained that to determine the effect of RNA on Hoechst 33258-DNA fluorescence, 10 mg of RNase is added in the presence and absence of DNase.

Novitsky (1986) reports that soil RNA degradation was more rapid than soil DNA, in that, after tillage management practices, the use of inorganic P by soil microorganism cause the degradation of RNA with RNAase, which results to an increase in the number of bacteria. After this time, soil microorganisms use DNA in soil. This is another reason buttressing the observation that DNA dissolved was higher in no-till soil than plow treatments. Also, he reports that 30% of soil microbial biomass C and significant portions of specific cellular components are degraded quickly after cell death. The study's results decline that soil microbial biomass C degraded rapidly, but microbial biomass P was higher in no-till soil than plow soil. So, DNA dissolved must be higher in no-till soil than plow soil. However, it was observed that soil dissolved DNA increased with 50 (0.99%) and 100 (22.29%) tone manure application in plow treatments in this study. The reason for these differences between the control and treatments was that we used 50 and 100 manure application. With manure application, the DNA synthesis rates and the heterotrophic uptake activity of the sample increased. Our results showed that soil microbial biomass P also changed, such as DNA dissolved in plow treatments. This is in agreement with results from other studies.

The results were similar to the results of Novitsky et al. (1985), who found that the rate of nucleic acid synthesis varies as such in heterotrophic activity or microbial heterotrophy activities in the surrounding sediment that is affected by sewage outfalls. However, the greater number of heterotrophy microorganisms and phytoplankton population in the 50 and 100 tones manure application influences DNA dissolved in soil, while the application of manure in soil influences molecular DNA, because in the optimum condition, there is an increase in soil biodiversity of microorganisms. The results show DNA with isotope 15 in the DNA composition bacteria, for example Meselson stipulates that in the composition of these bacteria, there is N15 and as such, this DNA have molecular weight more than the DNA with N14. However, application of different level of manure rate showed different results that were observed in plow soil. The results suggested that soil DNA dissolved increased with 50 (30.74%) ton manure application and decreased by 100 (5.68%) ton manure application in no-till soil.

The range of soil microbial biomass P was from 5.8 to 160 mg/kg soil, while the range of microbial biomass N was from 42 to 181.5 mg/kg soil.

## Relationship between soil DNA dissolved and some soil properties (DNA dissolved as indicator of soil microbial activity in the soil samples)

Relationship between soil DNA dissolved and physical, chemical and some biological properties are shown in Tables 3 and 4.

In this study, we did not see a relationship between soil DNA dissolved and soil mineralization N parameters (cumulative N mineralization Nm over 270 days incubation r = 0.18, potential N mineralization N0, r = -0.004 and initial N mineralization Ne, 0.36) and soil DNA dissolved and mineralization C parameters (cumulative C mineralization Cm over 270 days incubation r = 0.40, potential C mineralization C0, r = 0.36), but a relationship was observed between soil DNA dissolved and microbial biomass C r = 0.78** The reason for this effect may be

**Table 3.** Linear correlation coefficient of some chemical and physical parameters and DNA dissolved in soil.

| Coefficient | PH ($H_2O$) | %N | %C | C/N | Cs mg/kg | MWD (mm) | P µg/gr |
|---|---|---|---|---|---|---|---|
| DNA dissolved ng/10gr soil | -0.19 | 0.11 | -0.04* | -0.10 | -0.22 | 0.36 | 0.23 |

Cs: Carbon soluble; MWD: Mean weight diameter (mm); *: significant at ≤ 0.05.

**Table 4.** Linear correlation coefficient of some biological parameters and DNA dissolved in soil.

| Coefficient | Nm µg/gr | N0 | Ne µg/gr | Cm µg/gr | C0 | MBN µg/gr | MBC µg/gr | MBP µg/gr | NH4 $\mu g/grdwt^{-1}\ h^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|
| DNA dissolved ng/10 gr soil | 0.18 | -0.004 | 0.36 | 0.40 | 0.36 | 0.61** | 0.78** | -0.22 | 0.67* |

N0: N mineralization potential, Nm: total amount of N mineralization; Ne: N mineralized over 10 days; C0: C mineralization potential, Cm: total amount of mineralized carbon*, **, significant at p ≤ 0.05 and p≤ 0.01, respectively.

attributable to the fact that a relationship was observed between soil DNA dissolved and soil microbial biomass N, r = 0.61*. However, we did not see a relationship between soil DNA dissolved and soil microbial biomass P, r = -0.22. Also, a relationship was not seen between soil DNA dissolved and different enzymes activities, soil acid phosphatase activity and DNA dissolved and soil alkalin phosphatase activity and soil dissolved DNA r = 0.11 in this study.

We studied the relationship between soil and some chemical properties, but we did not see a relationship between soil DNA dissolved and soil pH r = -0.19. Likewise, a relationship was not observed between soil DNA dissolved and total N r = -0.23, soil DNA dissolved and total organic C r = -0.04, soil DNA dissolved and soil carbon soluble (Cs) r = 0.22 and soil DNA dissolved and C/N r = -0.10. In our work, we studied the relationship between soil physical property and DNA dissolved, while for this study, we selected the mean weight diameter such as soil physical property, but did not see a significant relationship between soil physical activity (mean weight diameter) and DNA dissolved in surface soil r = 0.36. As a result, there was no relationship between soil humidity and DNA dissolved r = -0.52.

## DNA dissolved as indicator of soil microbial activity in the soil samples

In this study, we showed that there is a relationship between soil DNA dissolved and arginin ammonification r = 0.67* and as such, the results suggested that since arginin ammonification frequently have applicability of microbial activity in soil, it is necessary to demonstrate that DNA dissolved is in association with living cells. Figure 2 demonstrated that linear regression for the amount of dissolved DNA measurement with gr volume soil w as used, whereas amendment of soil with C sources, usually manure application, results in a marked increase in microbial metabolic activities. We think that measurements of DNA dissolved may be used as an expensive and relatively difficult method for estimation of soil microbial activity potentials. Moreover, two factors might limit its application, and as such, measurement of DNA dissolved may be relatively expensive and may not be a fast method for routine estimation of soil microbial activities.

## Verification of the measurement method of DNA dissolved in the soil (Concentration of fluorescent probe specific in chromosomal DNA, using TKO 100 mini-fluorometer)

The results suggested that the amount of DNA dissolved is a function of the volume filtered and the spoilt volume that we used in this study (Figure 2). These results indicated that dissolved DNA concentration is a function of the volume of soil, but not a function of the volume of water that was filtered. In this study, we used a model TKO 100 mini-fluorometer to measure the fluorescence spectra of Hoechst 332558-DNA complex.

For excitation spectra, emission was 472 nm, while for emission spectra, excitation was at 342 nm. The variability of the method sample was filtered by the standard procedure and was precipitated in 100 ml volumes of water from the soil sample. We think that the measurements of DNA dissolved may be used as an expensive and relatively difficult method for estimation of soil microbial activity potential and the estimation of soil fertility, but with this method, we can measure some concentration of DNA dissolved in a soil that is very difficult and expensive with another method. Finally, with this method, we studied the development of a method for the determination of DNA in soil.

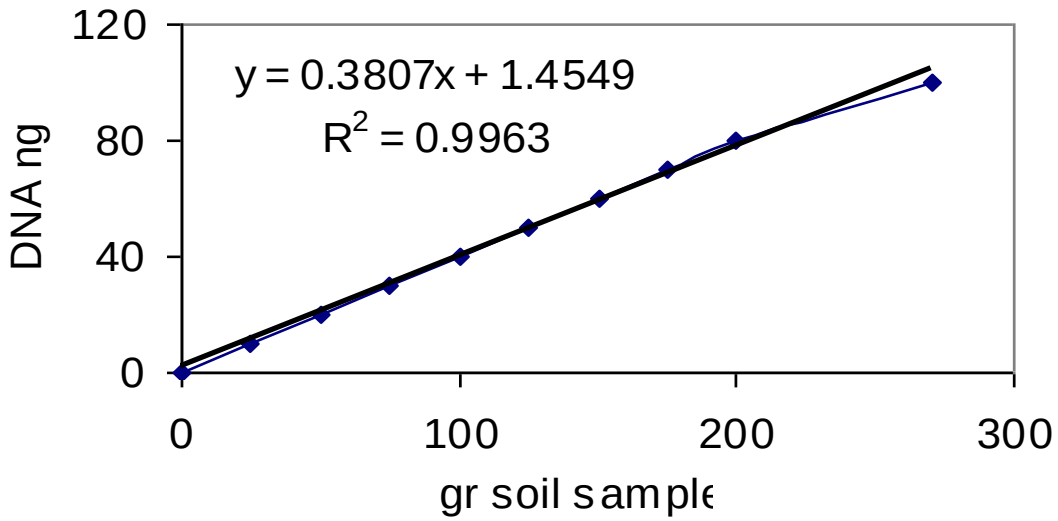


**Figure 2.** Linear regression of the amount of dissolved DNA measurement with gr soil that was used.

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