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Original Article

Measurement of Microbial activity and Applicability of Dissolved DNA as indicator of microbial activity in pasturage soil

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ABSTRACT

A method was developed for the determination of dissolved DNA in the pasturage soil. The method is 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. Fluorescence not attributable to DNA was corrected for by DNase I digestion of the extracts and averaged 25% of the total fluorescence for all samples. Concentration of dissolved extracellular DNA from a variety in 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 DNA dissolved. The method Hoefer TKO 100 DNA Mini-Fluorometer is simple, for extracellular DNA and 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, each strand containing several million nucleotides linked by 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. Although many of the low molecular-weight compounds of the dissolved organic matter in soil have been identified and quantified [12].

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. [10].Since humic substances become major concern upon the amplification of target DNA extracted from soil or sediment samples, it is important that humic substances be 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 [11]. 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 [20].

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 [17]. Most methods for the identification of biological activity in the soil involve use of Microbial biomass and mineralisation property, Ammonification, Nitrification, and enzymes activity. The 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 [9-16-19]. In this study we have optimized the DNA extraction protocol, at small scale level for the pasturage soil samples.

MATERIAL & 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, 100 Mg ha-1] as secondary treatments. Soil samples were taken from the 0-15 and 15-30cm depth. The moist soil samples were sieved at 6mm in the field; roots and stubble were removed. Samples were kept at 4°C until microbial activity analysis was performed.

Determination of the contribution of bacteria, fungi and actinomycetes to soil respiration.

A technique using selective inhibitors was used to estimate the relative contributions of bacteria, fungi and actinomycetes to soil respiration [CO2 production]. The CO2 produced by the different microbial groups was determined by titration method. Glucose was the substrate used. Streptomycin and actidione were used to inhibit bacteria or fungi, respectively [2].

Microbial biomass C, N and P were determined using the chloroform-fumigation-incubation method [4]; and were estimated from the difference between organic C, N and P extracted with 0.5M potassium sulfate from chloroform fumigated and unfumigated soil samples, using Kc factors of 0.38, 1.85 and 0.4 respectively. Microbial biomass levels were expressed as kg ha-1 soil.

Soil pH was measured in a soil:water ratio of 1:2. The organic C content was determined by wet oxidation procedure [Walkley and Black 1934]. Total N was estimated by Kjeldhahl digestion [13]. Soil water-soluble C was assessed as previously described.

A nitrogen and Carbon mineralization measurement was determined by method incubation [18]. The soil carbon and nitrogen mineralization potentials were determined by kinetic models [3]. Arginine ammonification was carried out as described by [1].

Concentration and measurement of DNA Dissolved

50 ml Na2SO4 [120mM] sodium phosphate buffer [pH=8] was mixed with Soil samples [10g] and the samples were shaking at 150 rpm for 15 min is pass through a filter combination consisting of a GF/D filter [watman, 40 and 42] and filter under a vacaum of < 150 mm Hg [< 20 KPa], with the filtration flask immerse in an ice bath. The DNA in the filtrate is precipitating by the addition of 2 volumes of 200-proof [100%] ethanol. After 48 h at -20oC the precipitate is collecting by centrifugation with two volumes in a GS 10 rotor at 6.800 x g for 20 min. The precipitate was then dialyzed at 4 0C for 48h against deionized water and then for 24 h against 1 x SSC [0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0]. Triplicate samples of the dialysate 2ml with SSC and 1 ml of 6 x10-7 M solution stock Hoechst 33258 in distilled, filtered H2O[concentration Dye Stock [1mg/ml H2O]] is adding. DNase I is dissolving in 0.02M sodium acetate pH 5, with 5 mM MgSO4 and 100 ml of the DNase solution. The samples are warming to room temperature, and the fluorescence is determining as previously described spectroflurometer and TKO 100 Mini-fluorometer. Little measurements is correct for fluorescence from material other than DNA by DNase I treatment. Calf thymus DNA ng/ml [0,40,60,80,100,250,500,750] DNA using 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 microorganisms as means to monitor DNA in soil environments. john et al. [5] concluded that dissolved DNA showed seasonal variation with minimal values in December and January and maximum values in summer months. The DNA in concentration of dissolved DNA and particulate DNA is quantifying by the fluorescence of Hoechst 33258-DNA complexes.

RESULTS & DISCUSSION

The results of DNA of soil are presented in [Table 1]. The range of DNA is 29.8 ng/gr to 131.26 ng/gr soil. The rang soil DNA dissolve in the surface soil [0to-15cm] was 29.80 to 131.26 ng DNA in 10 gr of soil . The results were similar tothe results of Ogram et al. [17], that they observed in the 1 mgr soil there was 1 gr DNA Dissolved. We obtained that there was not significant effect of soil tillage management practices on soil DNA dissolve in surface soil. Also we did not see significant effect of manure application on soil dissolve DNA in surface soil . Furthermore we did not see any interaction of manure application rates and soil tillage management practices on soil DNA dissolved in this layer. But we obtained that differences level of manure application and soil tillage management practices affected soil DNA dissolved in surface soil. The results suggested that soil DNA dissolve decline whit tillage management practices, the results indicated remarkably that soil DNA dissolved was higher in no-till soil than plow soil [32.50%] in this study may be in plow soil there is other elements such as methal ions and fluvic acid could also contribute to the inhibiting effect. The present results is common with those of other authors indicate that on entry to soil DNA are rapidly and extensivly degraded samply in plow treatment. Measurments of DNA with pasturage soil suspension resulted in a rapid increase in bacterial numbers cause tillage management practices resulted a small release inorganic P in soil [Inorganic P in this study was higher in no-till soil than tille soil] and increase in bacterial number that they want inorganic P for their activities Greaves et al [8] reports microbial biomass P increased in no-till soil on comparison tillage system, in our work 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 whit ethidium bromide [electrophoresis technique], [Fig 1]. Another reason may be soil tillage management practices influences closely soil coloids, soil bacterial cells associated with soil coloid in the separating process are differences resistances for liberation soil microorganism cells and soil DNA that there is forming in the Matter organic or microbial biomass that are in the soil. Soil tillage management practices influences on soil texture, and clay%, sand%, and silte% so it's one strategy for increasing of DNA in the soil. Another reason may be soil tillage management practices influences DNase in soil, therefore we know that

enzyme responsible for degradation DNA in the soil. Quality of DNAase can influences closely soil DNA Dissolved. In our study we observed variation in soil pH in no-till soil and plow soil, Humic substances, mainly Humic acid and felvic acid can affected by soil pH, The trace amounts of Humic acid and felvic acid influences DNA recover in the soil samples. Tsai et al. [20] used polymerase chain reaction [PCR] for recovery and separation of microorganism DNA in soil. Other elements for example metal ions can affect soil DNA recovery in the soil.

Measurement of bacterial, fungal and actinomycetes contribution to microbial respiration [CO2]

The results of the soil respiration studies in presence of inhibitors are presented in [Tables 2]. After, 1032 hours after incubation, total soil bacterial and actinomycetes respiration were influenced by soil depth: F=14.25** and F=28.28**, respectively . A significant interaction [F=10.01**] between manure applications and soil depth on actinomycetes total respiration activity after 1032 hours was observed.

Tillage management practices influences weight moleculars DNA in the soil results affected the quality and the quantity the bases in the DNA as sush as Adenin and thymidine [AT] this bases are very important bases for fluorometric method, because the greatest flurescence occurs in portion of DNA rich in adenine plus thymine A+T; the region that contain guanine plus cytosine possess only 50% of the fluorescence of the A+T region. In general Dissolved DNA was produced by actively growing heterotrophic bacterioplankton, but in our study not only DNA dissolved was produced by actively 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 influences closely soil Dessolved 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 contain guanine plus cytosine possess only 50% of the fluorescence of the A+T regions. Hoechst 33258 dye is thought to bind 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 to DNA, DNase I [80 dornanse unites mg of protein-1] is using for degrade DNA in extracts. The result of this study present that DNase I is dissolving in 0,02m sodium acetate buffer, pH 5,0 containing 5 mM MgSO4 for final concentration of 2 mg ml-1. To a 2 ml DNA extract in SSC [pH 7.0] 30 ml of 1 M acetic acid, 5 ml of 2 m Mg SO4 and 100 ml of the DNase solution. 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 posses 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. We obtained that to determine the effect of RNA on Hoechst 33258-DNA fluorescence, 10 mg of RNase is adding in the presence and absence of DNase.

Novitsky [14] reports that soil RNA degradation was more rapidly than soil DNA, there for after tillage management practices may be soil microorganism use Inorganic P cause by degradation RNA by using RNAase results the number bacteria increased after this time soil microorganisms use DNA in soil this is another reason that we observed DNA dissolved was higher in no-till soil than plow treatments. Novitsky [14] report that 30% of soil microbial biomass C and significant portion of specific cellular components are degraded quickly after cell death. Our results declin that soil microbial biomass C degraded rapidly but microbial biomass P was higher in no-till soil than plow soil, so DNA dissolved must higher in no-till soil than plow soil. We observed that soil Dissolved DNA increased whit 50 [0.99%] and 100 [22.29%] tone manure application in plow treatments in this study. The reason for this differences between control and the tratments that we using 50 and 100 manure application, whit manure application the DNA synthsis rates and the heterotrophic uptake activity of sample increased. Our results showed that soil microbial biomass P also changed such as DNA Dissolved in plow treatments. This is in agreement whit results from other studied.

The results was similar to the results of [15], who found rate of nucleic acid synthesis variation is as such as heterotrophic activity or microbial heterotrophy activities in surrounding sediment that affected by sewage Outfalls. The greater number heterotrophy microorganisms and phytoplankton population in the 50 and 100 tones

manure application influences DNA dissolved in soil. The application of manure in soil influences molecular DNA , because in the optomum condition soil biodiversity microorganisms increased results we can to see DNA with isotope 15 in the DNA composition bacteria for example Meselson that in the composition this bacteria there is N15 so this DNA have weight molecular more than the DNA whit N14. However application of different level of manure rate show different results that we observed in plow soil the results suggested that soil DNA dissolved increased whit 50 [30.74%] ton manure application and decreased by 100 [5.68%] tone manure application in no-till soil.

The range of soil microbial biomass P was from 5.8 to 160 mg/kg soil. The range 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, chemicals and some biologicals properties show in [Table 3-4]. We did not see 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]. We did not see relationship between 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]. We have relationship between soil DNA dissolved and microbial biomass C r=0.78**. The reason for this effect may be We have relationship between soil DNA dissolved and soil microbial biomass N, r=0.61*. We did not see relationship between soil DNA dissolved and soil microbial biomass P, r=-0.22. In this study we did not see relationship between soil DNA dissolved and different enzymes activities. We did not see relationship between soil acid phosphatase activity and DNA dissolved in this study . We did not observe relationship between soil alkalin phosphatase activity and soil dissolved DNA in our work r=0.11.

We studied relationship between soil some chemicals properties. But we did not see relationship between soil DNA dissolved and soil pH r=-0.19. We did not observe relationship between soil DNA dissolved and total N r=-0.23. We did not see relationship between soil DNA dissolved and total organic C r=-0.04. We did not observe relationship between soil DNA dissolved and soil carbon soluble [Cs] r=0.22. We did not observe relationship betwwen soil DNA dissolved and C/N r=-0.10. In our work we studied relationship between soil physical property and DNA dissolved, for this study we had been selection Mean weight diameter as such as soil physical property, we did not see significant relationship between soil phisical activity [Mean weight Diameter] and DNA dissolved in surface soil r=0.36. There was not relationship between soil humidity and DNA dissolved r=-0.52.

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

We showed that there is relationship between soil DNA dissolved and arginin ammonification r=0.67*. The results suggested since arginin ammonification frequently have applicability of microbial activity in soil, we found it necessary to demonstrate that DNA dissolved it is association whit living cells. [Fig 2], demonstrated linear regression of the amount of dissolved DNA measurement with gr volume soil was using. Amendment of soil with C sources usually manure application results in a marked increase in microbial metabolic activities. We think measurements of DNA dissolved may be used as a expensive and relatively difficult method for estimation of soil microbial activity potentials, Two factors might limit its application. Measurement of DNA dissolved may be is relatively expensive and relatively it is not fast method for routine estimation of soil microbial activities.

CONCLUSION

Verification of method for measurment DNA Dissolved in the soil

[Flurescencent Probe Specific for AT Concentration in Chromosomal DNA by using TKO 100 Mini-Fluorometer]

The results suggested that the amount of DNA dissolved as a function of volume filtered and volume of spoil that we was using in this studies [Fig1]. This results indicated that dissolved DNA concentration are a function of the volume of soil but not a function of the volume of water filtered. We used in this studies for measurements of fluorescence spectra, a model TKO 100 mini-fluoremeter was using to measure the fluorescence spectra of Hoechst 332558-DNA complex. For excitation spectra emission was 472nm, for emission spectra, excitation was at 342 nm. The variability of the method sample was filtered by the standard procedure and precipitated in 100 ml volumes of water, from soil sample. We think measurements DNA dissolved may be used

as a expensive and relatively difficult method for estimation of soil microbial activity potential and estimation soil fertility. But whit this method we can measurement some concentration DNA dissolved in soil that is very difficult and very expensive with another method. Finally in this method we study the development of a method for the determination of DNA in soil.

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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

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 respirartion after 1032 hours incubation of a meadow soil.

Treatment	Bacteria activities	Fungi activities	Actinomycets 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 $p \leq 0.01$.

Table 3. Linear correlation coefficient some chemicals and physical parameters and DNA Dissolved in soil.

Coefficient	PH (H2O)	%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 wieght Diameter(mm)
*. Significant at ≤ 0.05

Table 4. Linear correlation coefficient some biologicals 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 µg/gr dwt^{-1} h^{-1}
DNA Dissolved Ng/10gr 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 $p \leq 0,05$ and $p \leq 0,01$ respectively.

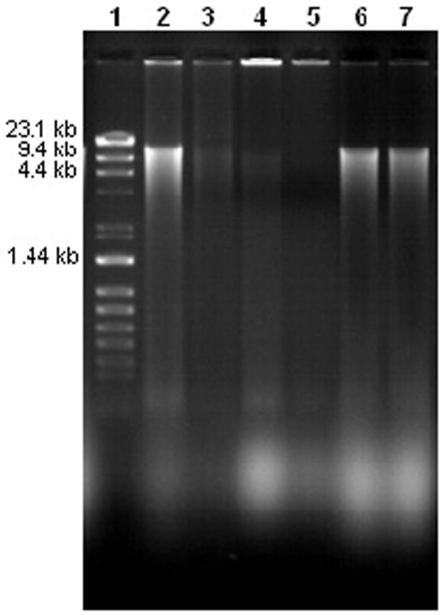

Fig 1. Agarose gel electrophoretogram of the total DNA isolated from three sampling soil.

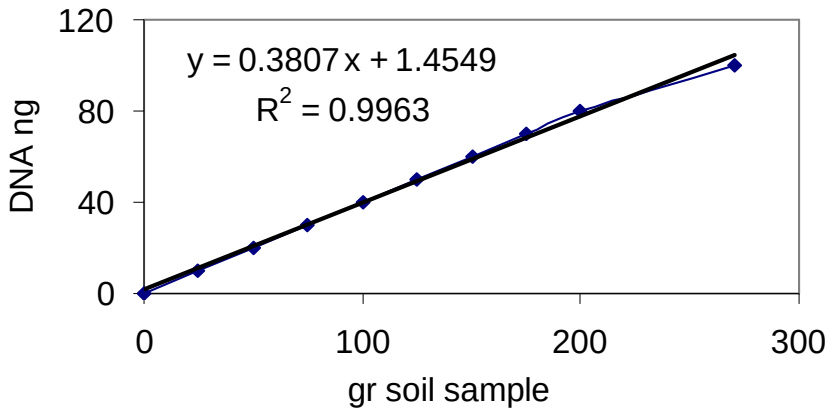

Fig2. Linear regression of the amount of Dissolved DNA measurment with gr soil was using.