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Viruses and bacteria microfauna of the soil and the digestive tract of earthworms (Oligochaetes annelids) of Cameroon: highlighting, quantification and importance

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Abstract

The flow cytometry technique, based on the enumeration and multi-parametric analysis of suspended particles in a liquid medium, has enabled us to identify an population of viruses and bacteria in the soil and the digestive tract of earthworms. It appears that the passage of the ground through the digestive tract of oligochaetes annelids would regulate bacterial and viral biomass of the soil as well as soil physico-chemical parameters. The soil microfauna is involved in the decomposition of the organic material and the nutrient bioavailability to plants and soil microorganisms. It also plays an important role in the creation and conservation of the soil structure. Apart from their role as "engineers", earthworms through their internal microfauna, participate in the regulation of physical and chemical parameters such as nitrogen mineralization. Earthworms thus participate in the processes of decomposition of organic material improve the bioavailability of minerals, maintain the porosity of the soil and increase the stability of the aggregates. Oligochaetes are expected to play a central role in the implementation of biological regulation in the agro-ecosystems essential to the development of ecological and intensive agriculture. Another reason to be interested in these soil organisms is that they are good candidates for bio-indicators. Indeed, the level of their population is sensitive to cultural practices, chemical products that are spread, quality and quantity of the resource in carbon, which are important characteristics of the sustainability of agricultural systems.

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Introduction

The flow cytometry technique, based on the enumeration and multi-parametric analysis of suspended particles in a liquid medium, has enabled us to identify an population of viruses and bacteria in the soil and the digestive tract of earthworms. It appears that the passage of the ground through the digestive tract of oligochaetes annelids would regulate bacterial and viral biomass of the soil as well as soil physico-chemical parameters. The soil microfauna is involved in the decomposition of the organic material and the nutrient bioavailability to plants and soil microorganisms. It also plays an important role in the creation and conservation of the soil structure. Apart from their role as "engineers", earthworms through their internal microfauna, participate in the regulation of physical and chemical parameters such as nitrogen mineralization. Earthworms thus participate in the processes of decomposition of organic material improve the bioavailability of minerals, maintain the porosity of the soil and increase the stability of the aggregates. Oligochaetes are expected to play a central role in the implementation of biological regulation in the agro-ecosystems essential to the development of ecological and intensive agriculture. Another reason to be interested in these soil organisms is that they are good candidates for bio-indicators. Indeed, the level of their population is sensitive to cultural practices, chemical products that are spread, quality and quantity of the resource in carbon, which are important characteristics of the sustainability of agricultural systems.

The soil is a place where various microorganisms with their enzymatic arsenal are involved in mineralization. Soil fauna, which is usually divided according to the size of it organisms comprises three separate groups, micro, meso and macro fauna, that covers many taxa. These taxa contain hundreds or even thousands of species.

Oligochaetes Annelids are grouped into three ecological categories: epigeic, anecic and endogeic (Bouché 1972, 1977). They represent a major component of soil macrofauna because they dominate in biomass in the most terrestrial ecosystems. They dig many galleries, conduct vertical transport of the soil, providing a mixture of organic debris with the mineral part of the soil (soil recycling and material flow), thereby promoting burrowing activity (West *et al.,* 1991). In addition, earthworms ingest the soil containing various organic remains in decomposition which pass through their gastrointestinal tract.

The digestive fluid contains enzymes that release amino acids, sugars and other small organic particles. This fluid also contains protozoan ciliates (Fokam *et al.,* 2012; Nana *et al.,* 2012A), bacteria, and other microorganisms as well as organic material partially decomposed. The parameters reminiscent of the quality of a soil result not only from the action of "engineers" organisms (earthworms and ants), from the action of the microflora (bacteria, fungi) through the production of enzymes (mineralization), but also from the action of viruses. These actions are therefore considered as the potential indicators of the soil quality (Bloem *et al*., 2006).

We set out to determine whether, next to mineralizing bacteria, viruses were present in soil of the banks of the Sanaga River (Cameroon). Besides, we estimated the amount of each microorganism in the soil in an attempt to appreciate its contribution to the process of soil mineralization. Eventually, we quantified viruses and mineralizing soil bacteria in the digestive tract of the oligochaete *Alma emini*, aiming to highlight the importance of the passage of the ground through the digestive tract of this Oligochaete.

Materials and methods

Earthworms harvest and soil extract

The collection of worms and soil were performed in June 2012 on the banks of the Sanaga River, at Ebebda (11°30' and 11°50' of East longitude and 4°00' and 4°30' of North latitude) located at 60 km North of the Yaoundé city in Cameroon (Fig. 1). These harvests were realized by profound and rapid bleeding in the sandy clay acid soil of the banks of the Sanaga River, near the worm cast.

Annelids Oligochaetes waterlogged of the Glossoscolecidae family are members of the genus *Alma*: *Alma emini*, specimens which were 51 cm long and weighed 3.8 g on average, collected and preserved in humid ground. They were identified according to the Omodeo (1958) and Sims & Gerard (1999) keys.

Treatment of samples

The extraction involves milling of each piece of the worm (anterior, middle and posterior) and the soil in a porcelain jar. Then, 6 grams of each homogenate were mixed with 6 mL of distilled water. Thereafter, Tween 80 (Prolabo, France) was added at 10 % in combination with sodium tetrapyrophosphate (BDH Laboratory, England) at 10 mM in 14 mL water. The mixture was stirred for 15 minutes and then centrifuged during 1 minute at 1000 g. The supernatant was filtered through syringe filter of 5 microns (Whatman, UK) after 1 hour and 30 minutes settling at 4° C. Staining with SYBR-Green (Molecular Probes Europe, Netherlands) of the diluted extract at 1/1000 with TE buffer (5 minutes at room temperature, 10 minutes at 80 ° C and 5 min at room temperature) was made prior to flow cytometry assay.

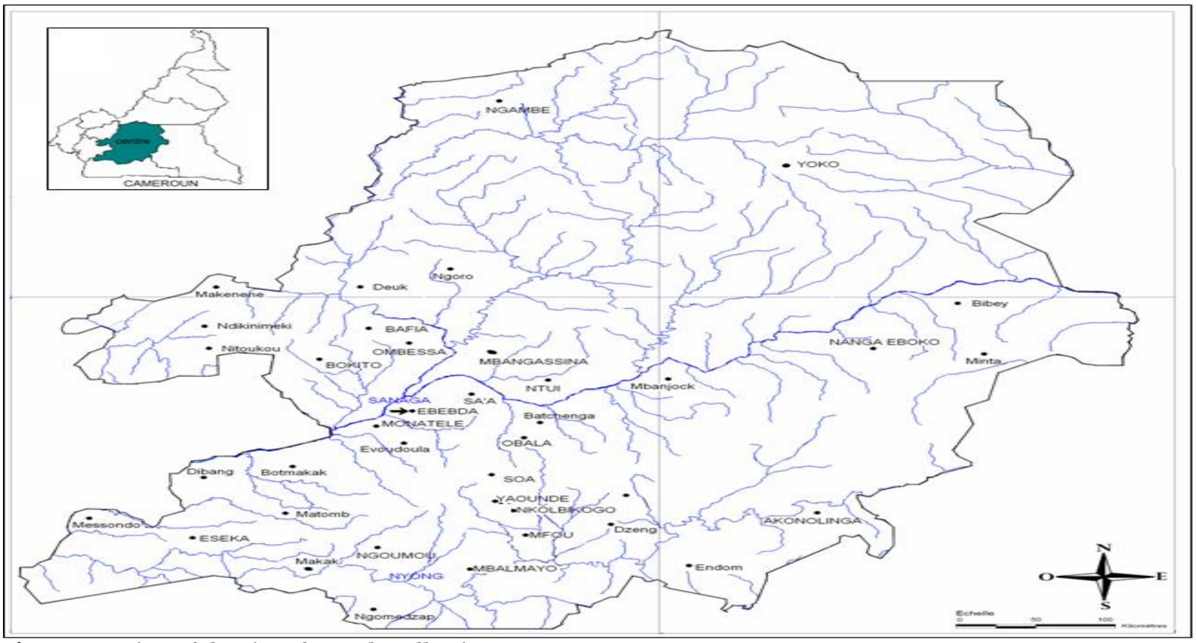

Fig. 1. Location of the site of sample collection.

Sample Analysis

The samples were analyzed using a FACS Calibur cytometer (Beckton Dickinson, USA) with an acquisition time of 60 seconds at a speed of 22.8 $\mu L.min^{-1}$. The routine parameters for the samples analysis are the structure and the FL1 green fluorescence for the cells labeled with Sybr-Green I. The structure parameters and the fluorescence are collected in logarithmic mode FL1 using the photomultiplier tube as a trigger. Windows and following settings (AUC = 650, FL1 = 467, threshold = 10) are used to discriminate bacteria from viruses in the worms and estimate their quantitative importance on the cytograms.

Transmission electron microscopy

Observation of the samples at the transmission electron microscopy has permitted to achieve photographs showing particles with phage appearance in soil extracts and in the worm too.

Results

Highlighting soil and oligochaete digestive tract viruses

The observation of the soil and earthworm in transmission electron microscopy (TEM) allowed highlighting particles that look like phage and morphologically varied (Fig. 2). Like all viruses, phages are classified according to the nature of the nucleic acid and their structure (presence or absence of an envelope). Phages are divided into families, those classified in the Caudovirales order are however the most numerous (more than 90% of known bacteriophages) and they present an original symmetry described as binary. The virions have a head with cubic symmetry containing the DNA and an asymmetric tail. The photographs in Figure 2 therefore allow us to classify viruses of the *Alma emini* digestive tract in three families:

The Cystoviridae, characterized by a circular head and a tapered tail, very short (approximately 15 nm) (Fig. 2A and 2D).

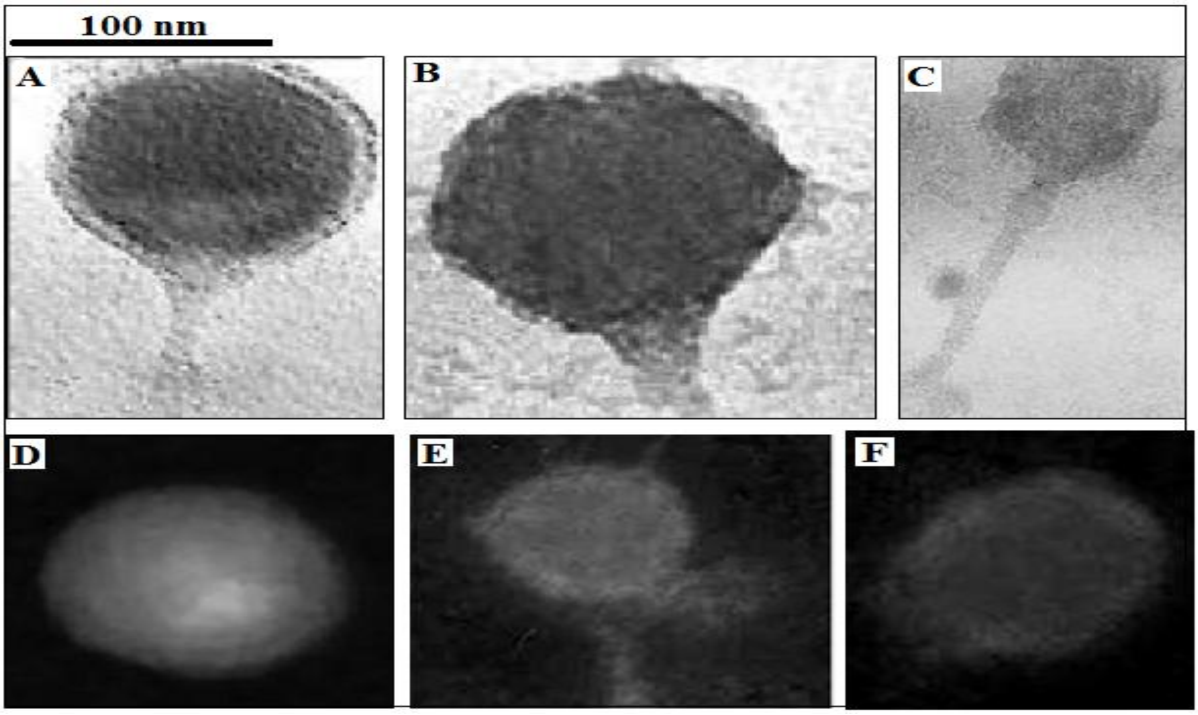

Fig. 2. Micrographs of viral particles of soil and crushed oligochaetes after observation in transmission electron microscopy (TEM). A and D: Cystoviridae; B and E: Podoviridae; C and F: Myoviridae.

The Podoviridae (Fig. 2B and 2E.), Characterized by a short tail (approximately 20 nm) and non-contractible. Bacteriophages T7 and P22 can be classified in this family.

The Myoviridae characterized by a blocky head and a very long tail (about 100 nm) (Fig. 2C and 2F.). Viral particles belonging to this family have a contractile sheath surrounding the central cylinder of the tail, which allows them to inject their DNA into the bacterial cell. Some of these viruses are very well known, in the case of phages T2, T4, T6 and μ.

Viruses and soil bacteria abundance variation in the digestive tract of oligochaete

Flow cytometer analysis of various extracts allowed us to highlight two windows (R1 and R2) in which bacteria and viruses are counted respectively for samples of soil and crushed whole worm (Fig. 3A and 3B).

Micrographs (Fig. 2A and 2B) show the appearance of the phage particles in the soil and the crushed worm. We noticed that the viral population is subservient to a large bacterial population. Bacterial concentrations range from about $1.2.10^{11}$ bacteria / g in the soil to $7.2.10^{8}$ bacteria / g in the crushed worm. The viral concentration varies from $2.1.10^{10}$ viruses / g in the soil to $2.7.10^{9}$ viruses / g in the crushed worm. The ratios viruses/bacteria are respectively 0.2 in the soil and 3.7 in the ground from the worm digestive tract.

When the worm gut was divided into three equal portions (anterior, middle and posterior), and each portion of the crushed worm analyzed by flow cytometer, it was found that contrary to the bacterial cells, the virus concentration increases dramatically from the foregut to the hindgut (Fig. 4).

In the foregut, there were about 81 bacterial cells / g against 119 viral particles / g with a VBR (viruses/bacteria Ratio) = 1.47. In the middle portion of the intestine, the VBR = 4.66. Indeed, in this part of the digestive tract of the worm, there were 32 bacteria / g and 149 viral particles / g. In the hindgut, bacterial concentration becomes very low in contrast to the viral concentration with a VBR of 22.25.

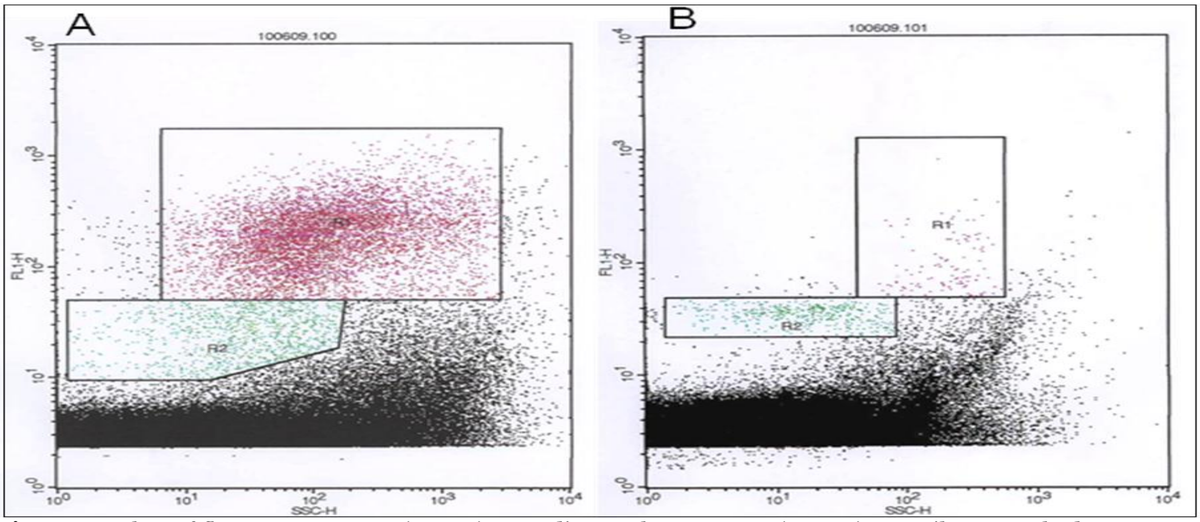

Fig. 3. Number of fluorescent events (FL1-H) according to the structure (SSC-H). A: soil; B: crushed worm; R1: Bacteria; R2: Viruses.

Discussion

Analysis of cytograms obtained, combined with data from transmission electron microscopy (TEM) permit to confirm the existence of a significant concentration of bacteriophages and mineralizing bacteria in the soil and in the digestive tract of earthworms. The same result had already been revealed by Brito-Vega & Espinosa-Victoria (2009) who, working on *Eisenia fetida* worms and *Lumbricus rubellus*, revealed the presence of an important bacterial concentration in their digestive tract. The work of Singleton *et al.* (2003); Valle-Molinas *et al.* (2007); Byzov *et al.* (2007) on the same species of oligochaetes have distinguished several kinds of bacteria that contribute to the decomposition of organic material: *Bacillus, Pseudomonas, Klebsiella, Azotobacter, Serratia, Aeromonas, Morganella* and *Enterobacte*r. In addition, some authors (Dash *et al.*, 1986; Parthasarathi & Ranganathan, 1998, 2002; Wolter & Scheu, 1999; Houjian Cai *et al.*, 2002; Parthasarathi *et al.*, 2007.), working on oligochaetes *Lampito mauritii, Perionyx excavatus, Eudrilus eugeniae Lumbricus terrestris* and *Eisenia fetida* showed the coexistence of this meso bacterial flora and other microorganisms including: fungi (*Aspergillus fumigatus, Aspergillus flavus, Aspergillus ochraceous, Mucor plumbeus, Rhizopus* sp), the actinomycetes (*Streptomyces albus, Nocardia asteroides, Streptomyces albus, Streptomyces somaliensis, Nocardia asteroides, Nocardia caviae, Thermophilic actinomycetes, like Saccharomonosporia*) and protozoa (*Amoeba proteus, Amoeba terricola, trichium Paramecium, Euglena viridis, Euglena orientalis, Vorticella picta, Trichomonas hominis*). Viruses of the digestive tract of the *Alma* genus would be a peculiarity and could allow the regulation of the bacterial charge. In fact, viruses, obligates parasites and without cellular machinery are closely dependent on their host cells (bacteria). It seems clear that bacteriophages dominate the viral community. Knowledge on the virus's diversity of the worm's digestive tract is still limited. Current ecological data on bacterial populations in aquatic environments indicate a wide variety of survival strategies and environmental niches (Colombet & Sime-Ngando, 2010).

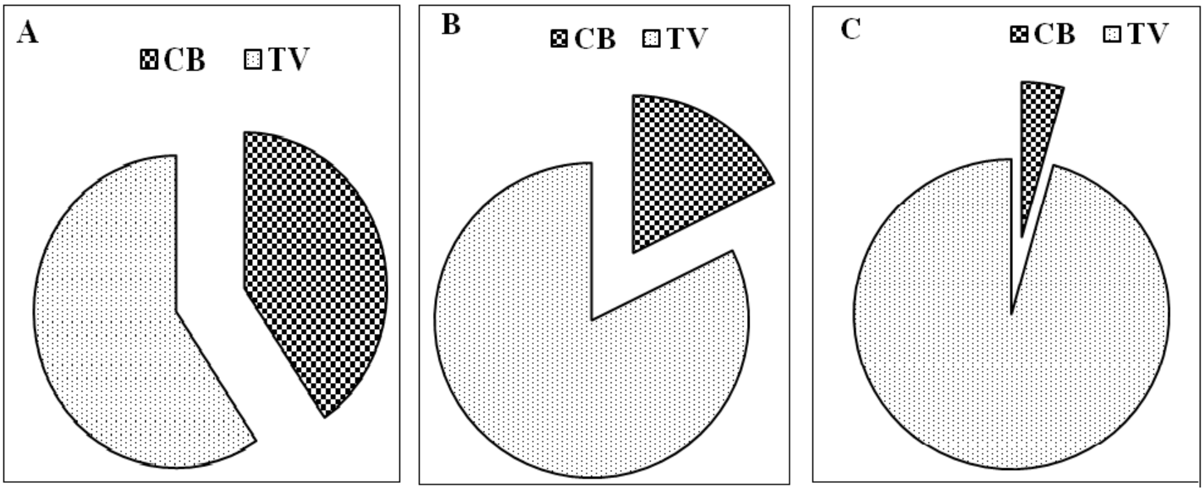

Fig. 4. Abundance of viruses and bacteria along the digestive tract of *Alma emini*. A: foregut; B: midgut; C: hindgut; CB: concentration of bacteria; TV: total viruses.

Quantitatively, concentration's variations of these two groups of organisms in the soil and from one end to the other of the digestive tract of earthworms, can be explained by the fact that the virus shelter under a lysogenic form in the bacterial population, form in which their genetic material is integrated in the host one and cannot be expressed, and may remain like that for generations. On the other hand, the physico-chemical and biological conditions (Nana *et al.*, 2012 B; 2014) offered in each portion of the digestive tract of the worm would be favorable to the emergence of the virus through the bacterial lysis, following the expression of the viral genes (viral protein synthesis) and then encapsidation phenomena and release; or a production of indigenous viruses in the digestive system. This allows hypothesizing for the main function of this virus in the biological system. The impact on bacterial viruses seems to vary in the temporal and spatial scale.

Through advanced identification techniques, our data permit the enumeration and measurement of metabolic activity; on glimpse the incredible diversity of telluric microorganisms, the extent of their living conditions and their abundances that was largely underestimated. In addition, the significant improvement in separation methods allowed us to describe the biochemical composition of communities and address the opportunities of material transfer in trophic network at a quality angle. It is because of these technological advances that smaller organisms that we know today represent the largest reservoir of diversity, of carbon and functions in the soil, were taken into account in different ecosystems (Pomeroy, 1974; Colombet, 2008). From this point of view, the development of the concept of microbial loop (Azam *et al.,* 1983) was structured for the development of the aquatic microbial ecology or mesofauna gut worms. This notion of microbial loop has recently expanded the concept of microbial trophic network by taking into account the pico- and nanoplanctoniques autotrophic microorganisms that form the core of aquatic primary producers, and are also given as prey for protozoa (Rassoulzadegan, 1993; Amblard *et al,*. 1998). If these well-established concepts in the aquatic environment could be transposed into microbial mesofauna worm's gut in terms of results, we think this microhabitat is a food chain that remains to be seen but in which the phage, as in aquatic ecosystems play a regulatory role.

In regard of the abundance variations of viruses and bacteria along the digestive tract of earthworms, we can consider, like Pedersen & Hendriksen (1993) and Peter *et al.* (2010) that the passage of the soil in the digestive tract influence not only the physical and chemical properties, but also the microbial biomass which acts in the degradation of organic material in the soil. Monroy *et al.* (2008) have also revealed changes in density of nematodes, protozoa and total coliforms soil after transit through the gut of epigeic earthworms. In the same vein, Brito-Vega & Espinosa-Victoria (2009) showed that the passage of the soil in the digestive tracts of earthworms could stimulate or inhibit the growth of microorganisms and mineralizing bacteria (Byzov *et al.,* 2007).

Conclusion

In regard of the variations of viruses and bacteria abundance in the soil and along the digestive tract of earthworms, we can consider like other authors, that the passage of the soil in the digestive tract influences not only the physical and chemical properties, but also the soil microbial biomass. We remind that the soil microbes interact and participate in the decomposition of organic material and nutrient availability for the growth of plants and other organisms in the soil through the flow of materials and energy. The transit of the ground through the digestive tract of oligochaetes contributes to the regulation of microbial concentration in the soil. It is therefore urgent to consider oligochaetes in general and *Alma emini* particularly in agricultural policies for healthy production free from environmental aggressions, thereby advocating sustainable agricultural development.

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