Effect of Predation by Colpoda sp. in Nitrogen Fixation Rate of Two Free-Living Bacteria

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Abstract

Biological nitrogen fixation is limited to several groups of prokaryotes, some of them reduce nitrogen as free-living nitrogen-fixing bacteria. Protozoa predation on these latter releases sequestered nitrogen that may enhance the formation of new bacterial biomass and possibly increase nitrogen fixation within soil microbial communities. We aim to evaluate the predation effect of Colpoda sp. on two nitrogen fixers: Azospirillum lipoferum and Stenotrophomonas sp. during their lag, early exponential, and exponential phases. The kinetics of bacterial population growth was determined in the predators’ presence or absence and the effect of predation on the rate of N fixation was evaluated through the reduction of acetylene to ethylene technique. Colpoda sp. showed a non-significant difference in preferences between the two species offered as prey. Consequently, the abundance of A. lipoferum and Stenotrophomonas sp. decreased significantly due to predator’s pressure and both species responded by increasing their specific growth rate. Likewise, predation promoted greater nitrogen fixation rate by CFU during the lag phase in A. lipoferum (0.20 nM/CFU with predation vs 0.09 nM/CFU without predation) and Stenotrophomonas sp. (0.22 nM/CFU vs 0.09 nM/CFU respectively). During early exponential phase (29 h), the rate diminished to 0.13 and 0.05 nM/CFU in A. lipoferum and to 0.09 nM/CFU and 0.05 nM/CFU in Stenotrophomonas sp. Finally, during the exponential phase (52 h), only A. lipoferum without predation produced 0.003 nM/CFU of ethylene. Thus, the nitrogenase activity was higher in the lag and the early exponential phases when predator activity was involved.

Keywords Bacterial specific growth rate · Colpoda predation on bacteria · Free-living nitrogen-fixing bacteria · Nitrogen fixation rate under predation

Introduction

Biological nitrogen fixation (BNF) is the process by which N₂ is reduced to ammonia (NH₃) through the enzyme nitrogenase, a crucial process to assure nitrogen availability in terrestrial ecosystems [1]. The quantification of the nitrogenase’s activity can be accomplished by reduction of acetylene (C₂H₂) to ethylene (C₂H₄) [2], which is a useful and sensitive technique to trace BNF, as the enzyme breaks down the triple bond of acetylene in the same way it breaks the
triple bond of the \( \text{N}_2 \) molecule and adds hydrogen to produce \( \text{NH}_3 \) \cite{3, 4}. BNF can be endosymbiotic (i.e., with nodule formation) or performed by bacteria in a free-living stage, defined as N fixation without the endosymbiotic process between microorganisms and plants \cite{5}.

The group of free-living nitrogen-fixing bacteria (FLNFB) includes the genus \textit{Azospirillum} \cite{6–8}, which also promotes plant growth \cite{9} and the genus \textit{Stenotrophomonas}, which has an important ecological role in the sulfur cycle \cite{10, 11} and participates in an extraordinary range of activities that include beneficial effects on the growth and health of plants such as \textit{S. maltophilia} and \textit{S. rhizophila} \cite{12}.

Early microbial colonization of soils with zero or extremely poor N content needs nitrogen fixing bacteria to support microbial biomass and replenish N losses due to volatilization and denitrification \cite{13}. Thus, soil productivity is based on continuous mobilization of organic nitrogen, which can result from cell lysis, predatory debris, and the decomposition of nitrogenous components trapped in litter. All these activities may lead to N mineralization \cite{14}, while biological fixation means a net input of reduced N allowing the sustainability of the soil system \cite{13}.

On the other hand, protozoa enhance nitrogen mobilization along soil trophic networks \cite{15} and significantly modify the structure of bacterial communities through selective predation \cite{16}. Ciliates are capable of feeding on a wide variety of bacteria, and show a degree of preference towards certain species, depending on the prey size \cite{17, 18}, cell pigmentation, motility, and micro-colony shape \cite{18–20}. \textit{Colpoda} is one of the most abundant genus of soil ciliates showing swimming cells as the active feeding stage. However, this genus makes two different kinds of cysts: the “reproductive” or “dividing cysts” and the “resistance cysts.” The first one is a thin-walled cyst (non-resistant to desiccation) containing a “tomont” able to divide once, twice (Fig. 1), or more times to produce daughter cells \cite{21}. \textit{Colpoda} makes resistant cysts when environmental conditions become adverse, like lack of suitable food, desiccation, or threatening compounds in the surroundings \cite{22}.

Selective predation dramatically decreases the abundances of preferred preys in the short time \cite{22, 23}. Consequently, bacterial species lacking chemical defenses develop extremely high growth rates \cite{24} to survive the negative impact of intense predation pressure \cite{24}. Thus, the reduction in the number of bacteria during predation results in the counter-intuitive situation of increasing bacterial levels of metabolic activity \cite{25}. In this way, predation reduces the bacterial biomass, and increases the levels of reduced nitrogen available and the bacterial growth rate. For this reason, we wonder: How will predation by a ciliate affect the nitrogenase activity of nitrogen-fixing bacteria?

We aim to determine the effect of \textit{Colpoda} sp. in the nitrogenase activity of FLNFB \textit{Azospirillum} \textit{lipoferum} and \textit{Stenotrophomonas} sp. during the exponential and lag phases of population growth.

### Materials and Methods

**Protozoa and Bacteria Isolation and Cultivation**

\textit{Colpoda} sp. and \textit{Stenotrophomonas} sp. were obtained from soil cultivated with corn through the wet but non-flooded soil method from Bamforth \cite{26}. Morphological identification of \textit{Colpoda} sp. was achieved following Foissner and Kohamannkey \cite{27}.

Serial dilutions were prepared for separation of different bacteria growing together in the polyxenic cultures of \textit{Colpoda} sp. One hundred microliters of the liquid medium was diluted in 900 μl of sterile soil extract to make the first dilution \( 10^{-1} \). The procedure was repeated to reach \( 10^{-4} \) dilution from which 40 μl was seeded in Rennie agar (1981) and incubated for 4 days. The most abundant colony forming unit (CFU) recognized by its colony morphology was

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**Fig. 1** Phase contrast photographs of \textit{Colpoda} sp. swimming cells (a) and tomites (b) taken at 40×
purified by sequential streak cultivation. This was a 2- to 4-µm Gram-negative coccoid bacteria able to grow in Rennie and capable of reducing acetylene to ethylene in culture. It was tested for suitability as Colpoda’s prey. The molecular identification of this bacterium was carried out by sequencing the 16S fraction of the rDNA. Bacterial DNA extraction was performed using the GenElute™ Bacterial Genomic DNA Kit. An 800-BP sub-fraction of the 16S ribosomal fraction of DNA was amplified using universal primers FD1 and RD1 [28].

The PCR was carried out in a 50 µl volume containing 0.2 µM of each primer, 0.2 mM of dNTPs, 2.5 mM of MgCl2, 1.25 U of Taq polymerase (Fermentas), 1X PCR buffer, and 50 ng of DNA. The amplification protocol was as follows: initial denaturation for 5 min at 95 °C, followed by 25 cycles under the following conditions: denaturation temperature of 95 °C for 30 s, alignment temperature 57 °C for 40 s, and the temperature of the extension at 72 °C for 2 min. This was followed by a final extension at 72 °C for 5 min. Amplification was performed on a Thermo Scientific™ Piko™ Thermal Cycler. The products were run on 1% agarose gels at 110 V for 30 min, stained with GelRed, and viewed on a KODAK 3.5 1D photo-documentor. Products were cleaned with PureDirex PCR Clean-up & Gel Extraction Kit following the manufacturer’s instructions and sequenced (INTROGEN). The sequences were edited with the BioEdit program and compared with those found in the NCBI-Blast. In this way, it was possible to identify Stenotrophomonas sp. with a similarity rate of 96.97% with the NCBI-Blast sequences (Access No. KX066811).

The second prey offered to Colpoda sp. was Azospirillum lipoferum, obtained from the strain collection of the Soil Hydrology Laboratory of Postgraduate College, campus Montecillo Estado de México. This is a 2- to 5-µm Gram-negative vibroid type and FLNF bacteria.

**Colpoda sp. Food Preference Tests**

The selectivity test of bacterial prey was prepared in Petri dishes as follows: a 4-arm asterisk resin mold was placed on 15 ml of soil extract agar before jellification and removed once the agar gelled. The periphery wells were connected to the central one through channels 5-mm long left by the resin mold [29] (Fig. 2).

About 200 Colpoda sp. cells suspended in 50 µl of sterile yeast extract medium (3 g/l) and NaCl (5 g/l) were inoculated in the central well of the Petri dishes. One hour before inoculation, the polyxenic culture was treated with chloramphenicol (5 mg/ml) to eliminate bacteria growing together with Colpoda. Then, A. lipoferum and Stenotrophomonas sp., and a combination of both were deposited in each of the lateral wells (200 µl of bacteria, 1 × 106 CFU). The control well contained only 200 µl of the sterilized yeast extract and channels were flooded with this medium to allow predators’ movement towards the wells. Subsequently, the Petri dishes were carefully kept without any movement at room temperature (~24 °C) for 3 h. Thereafter, each lateral well was fixed with 4% Lugol (v/v) to count cysts and swimming cells [29]. Thin-walled containing tomites were counted as reproductive cysts and thick-walled were counted as resting cysts.

**Bacterial Growth With and Without Predator**

The kinetics of bacterial population growth, in the presence or absence of Colpoda sp., was achieved as follows: four repetitions for each bacterial species were set up containing 1 ml of bacterial suspension with approximately 5 × 10^5 colony forming units (CFU) inoculated in stoppered flasks containing 60 ml of liquid Rennie medium. Then, 400 µl
of a suspension containing Colpoda’s swimming cells (250 cells/ml) was inoculated and kept at 32 °C incubation along the timespan of the experiment. Bacteria were counted by reading 1 ml in a spectrophotometer set up at 540-nm absorption from 0 to 125 h (22 time intervals). In parallel, serial micro-dilutions were prepared by mixing 10-μl sub-samples (from the 1 ml read at the spectrophotometer) in 90 μl sterile Rennie solution to make dilutions from $10^{-1}$ to $10^{-7}$ in a 96-well microtiter plate, then 20 μl of a $10^{-7}$ dilution was seeded in Rennie agar [30] by triplicate. CFU were counted after 4 days at 32 °C temperature.

### Mathematical Model of Bacterial Growth

The average time for bacteria to perform binary fission during the adapting phase is longer than during exponential growth (reduction of the average time for duplication or growth rate) [31]. A computerized procedure was developed in RStudio (v.1.4) to fit the CFU per milliliter abundances in an adjusted curve to determine the growth parameters. It is considered that during the exponential growth phase, the binary fission develops a geometric progression, and the specific growth rate of bacteria is constant [31]. The model describing the bacteria exponential growth phase is written as follows:

$$\frac{dX}{dt} = \mu X$$

where:

- $X =$ cells/l.
- $t =$ time.
- $\mu =$ specific growth rate in $h^{-1}$ (mass or number).

To summarize:

$$\mu = \frac{\ln 2}{g} = \frac{0.693}{g}$$

The time required to double the number of microorganisms or mass of a population is called generation time and is determined as:

$$g = \frac{t}{n}$$

$g =$ generation time.

$t =$ time interval elapsed between generations No and Nn.

$n =$ number of generations.

Finally, the number of generations was calculated as:

$$n = \frac{\log \left( \frac{N_n}{N_0} \right)}{\log 2}$$

$n =$ number of generations.

$N_n =$ final concentration of organisms.

$N_0 =$ initial concentration of organisms.

### Impact of Predation on Bacterial Abundance

According to Fox [32], the impact of predation (ID) varies between 0 (no predation effect) and 1 (complete extinction of prey). If predation leads to increased prey abundance, the ID value becomes negative. $K_{bac}$ and $R$ are the CFU at the beginning and end of the exponential phase.

$$ID = \frac{K_{bac} - R}{K_{bac}}$$

$K_{bac} =$ final concentration of bacteria without predator.

$R =$ final concentration of bacteria with predator.

### Statistical Analysis

A one-way ANOVA ($P < 0.05$) was performed to determine the existence of significant differences between experimental wells in the food preference test, followed by Tukey’s post hoc test for the comparison between averages (Tukey, $\alpha = 0.05$).

A one-way Student’s $t$ test ($P < 0.05$) was performed to determine the existence of significant differences in the population abundance of both bacteria, with and without predators.

### Nitrogenase Activity

Vial bottles with a capacity of 40 ml were used to test nitrogenase activity. Each bottle contained 20 ml of Rennie medium and 1 ml of bacteria (approximately $5 \times 10^5$ CFU/ml). Thereafter, 200 μl of Colpoda sp. (approximately 55 cells) was added to the treatments in the presence of predation and 200 μl of sterile Rennie solution was added to treatments without predator. Then, 4 ml (10% v/v of the total volume of the vial) of the internal atmosphere of the vials was replaced by 4 ml of acetylene. The gas mixtures from each treatment and the control group were analyzed with mass spectrometry to identify acetylene and ethylene molecules [33]. The experimental design consisted of four treatments with four repetitions each: A. lipoferum with and without a predator, and Stenotrophomonas sp. with and without predator, and two controls without microorganisms: culture medium with acetylene and culture medium with ethylene. All treatments were kept at 32 °C and counting of bacteria and Colpoda sp. was achieved as described in the bacterial growth section.

With the help of acetylene (C2H2) and ethylene (C2H4) standards, the “fingerprints” of both molecules were monitored within a gas mixture to determine their presence and abundance in every phase.

Nitrogen fixation was estimated according to the number of nanomoles (nM) of ethylene produced per unit of bacteria.
Quantification of acetylene and ethylene was done by mass spectrometry (Agilent Technologies brand model 5975 inert XL) of the gas mixture taken from each one of the repetitions at 7 h, 29 h, and 52 h, from control and experimental groups. The nitrogenase activity of *Stenotrophomonas* sp. and *A. lipoferum* first registered after 7 h of incubation at room temperature is the value for the lag phase. Ethylene at 29 h - ethylene at 7 h (lag phase) is the total amount at the early exponential phase, while the exponential phase was obtained by subtracting ethylene at 29 h from ethylene at 52 h.

**Results**

**Food Preference of Colpoda sp.**

*Colpoda* showed significantly higher swimming cell formation ($\alpha = 0.05$) in the wells inoculated with *Stenotrophomonas* sp. ($\alpha = 0.05$) than with *A. lipoferum* (3013 and 2093 swimming cells/ml respectively) as well as when compared with the mixture of these bacteria (1093 swimming cells/ml; Fig. 3), and with the well corresponding to the control group. Also, there was a significantly higher number ($\alpha = 0.05$) of swimming cells than cysts in all treatments inoculated with bacteria, compared to the control wells.

**Population Growth of A. lipoferum and Stenotrophomonas sp. with and Without Predator**

The two bacterial species stayed at least for 26 h in a lag phase, in either the absence or presence of predator. The exponential phase started shortly after this time and lasted until 102 h in the absence of predation. In this regard, *Colpoda* sp. doubled its population after 30 h of contact with the two bacteria separately and in the mixture of both (Fig. 4). It took *A. lipoferum* 102 h to reach its maximum population size with or without a predator, which was the same time that *Colpoda* sp. stopped its division and encysted (Fig. 4a). *Stenotrophomonas* sp. reached its maximum population size at 54 h without predator but reached it earlier (33 h) when cultured with *Colpoda* (Fig. 4b). In this way, the population size of *Colpoda* feeding on *Stenotrophomonas* was twice as big than when feeding on *A. lipoferum* along the 125 h. On the other hand, the population size of *Colpoda* sp. in the mixture of both bacteria species was intermediate between what was observed when cultivated with either bacterium species separately (Fig. 4c).

*Colpoda* sp. reduced the populations of *A. lipoferum* to a lesser extent than those of *Stenotrophomonas* sp. throughout the exponential phase (from 26 to 102 h). Nonetheless, three generations ($n = 3.1$) of *A. lipoferum* elapsed during the exponential phase without *Colpoda* sp., while the presence of this latter allowed it almost 2 generations ($n = 1.7$) during the same time span; that is, predation reduced the specific growth rate from $\mu = 0.02$ without predation to $\mu = 0.01$ and consequently extended the generation time of *A. lipoferum* from $g = 23.4$ h without predation to $g = 40$ h with predation. *Stenotrophomonas* sp. also showed 3 generations during the exponential phase ($n = 3.2$) and shorter generation time ($g = 13.1$ h) than *A. lipoferum* when cultivated alone. However, this species shortened its generation time ($g = 10.4$ h) and the number of generations ($n = 2.3$) under predation (Table 1). Additionally, the specific growth rate of *Stenotrophomonas* sp. increased from $\mu = 0.05$ h$^{-1}$ without predator to $\mu = 0.06$ h$^{-1}$ with predator. On the other hand, the growth rate of *Colpoda* sp. was higher in this strain ($\mu = 3.4$ h$^{-1}$) than that in *A. lipoferum* ($\mu = 3.1$) or in
the mixture of both preys ($\mu = 3.2 \text{ h}^{-1}$). *Colpoda* sp. has a greater positive effect on the biomass formation of *Stenotrophomonas* sp. than in *A. lipoferum*. Therefore, the effect of *Colpoda* sp. (ID) was moderate on *Stenotrophomonas* sp. (0.70) and *A. lipoferum* (0.60).

**Nitrogenase Activity**

The nitrogenase activity of *A. lipoferum* and *Stenotrophomonas* sp. was first registered after 4 h of incubation at room temperature. Then, both bacterial species produced the same rate of ethylene during the lag and early exponential phases (0.09 nM/CFU and 0.02 nM/CFU, respectively) in the absence of predator (Table 2). Both species produced higher rate of ethylene (0.2 nM/CFU) when cultivated with predator during the lag phase. However, *A. lipoferum* showed higher rate of ethylene production (0.9 nM/CFU) during the early exponential phase (29 h) than *Stenotrophomonas* sp. (0.03 nM/CFU) when cultivated with predator. Only *A. lipoferum* cultivated alone kept producing ethylene during the exponential phase, although at a very low rate of 0.003 nM/CFU (Table 2). The production of ethylene per system increased in the lag (75 – 81 μM) and early exponential phase (107 – 137 μM), remaining around the same values during the exponential phase (Table 2).
Table 1 Main parameters of population growth of both bacterial species: \( g \), average time between two successive generations expressed in hours; \( \mu \), intrinsic growth rate; \( n \), number of generations elapsed during exponential growth; \( ID \), impact of predation: values of \( 0 \) are equivalent to having no effect of predation, 1 is equivalent to complete extinction (negative effect on prey population), and negative values mean that predation stimulates prey growth (positive effect on prey population). Number of repetitions = 3. The presence of a predator did not generate significant differences in growth rate \( (p=0.05) \).

| Prey g (h) | \( \mu \) h\(^{-1}\) (UFC) | \( n \) | ID |
|------------|----------------|------|----|
| A. lipoferum | 23.4 | 0.02 | 3.1 |
| A. lipoferum + Colpoda sp. | 40 | 0.01 | 1.7 | 0.75 |
| Stenotrophomonas sp. | 13.1 | 0.05 | 3.2 |
| Steno. + Colpoda sp. | 10.4 | 0.06 | 2.3 | 0.6 |

Predator g (h) \( \mu \) h\(^{-1}\) (cell) \( n \)

| Colpoda sp. + A. lipoferum | 49.4 | 3.1 | 1.48 |
| Colpoda sp. + Sten | 37.1 | 3.4 | 2.5 |
| Colpoda sp. + A. lipo + Steno | 40.4 | 3.2 | 1.81 |

Discussion

Prey Selection

Colpoda sp. has a slightly higher preference for Stenotrophomonas sp. over A. lipoferum, as revealed by the difference in the number of swimming cells and cysts found in the respective wells. However, Colpoda reacted poorly to the mixture of both preys, which could be due to the molecules resulting from the conjugation of the metabolic products of both bacteria [34]. We rule out the masking effect of Stenotrophomonas sp. by A. lipoferum because in such a case, predation would have been like the one shown where this species was offered alone, instead of the marked decrease in preference as seen in Fig. 3.

Predation pressure of Colpoda sp. on A. lipoferum and Stenotrophomonas sp. caused an increase in the intrinsic growth rate of the two bacterial populations during the exponential phase. The contradictory effect of reducing prey abundances while stimulating their growth rate is the bacterial populations’ transitory response to predation [35]. This response results in a dispersal advantage of bacteria reproducing in less time, allowing them to take advantage of the resources in some micro-environments [36–38].

On the other hand, Colpoda sp. produced more cells by consuming Stenotrophomonas sp. (3013 cells ml\(^{-1}\)) than A. lipoferum (2093 cells ml\(^{-1}\)) because the semi-coccus or ovoid shape of Stenotrophomonas sp. and smaller size were more suitable for its intake in comparison to the larger vibroid shape of A. lipoferum [16, 18–20, 39, 40]. As both bacterial species are Gram-negative, we could safely rule out the selection effect of cell wall composition indicated by Griffiths [41], Darbyshire [42], and Drake and Tsuchiya [43].

Table 2 nM of ethylene produced during the lag, early exponential, and exponential phases of population growth by each species of bacteria cultivated alone or with predator. The first row shows the quantity of colony forming units (CFU) recorded at the corresponding time (7 h, 29 h, and 52 h). The second row shows increment (\( \Delta \)) of CFU from the lag to early exponential phase and from the early to the exponential phase. The third row shows the micromole of ethylene (\( \mu \text{M} C_2H_4 \) a surrogate for N fixation) recorded at the corresponding times (7 h, 29 h, and 52 h). The number in parenthesis shows the increment (\( \Delta \)) of \( \mu \text{M} C_2H_4 \) at 29 h and 52 h resulting from subtracting the amount recorded at 7 h (lag phase) from the one recorded at 29 h (early exponential phase) and the amount at 29 h from the one recorded at 52 h. The last row shows the rate of nanomole of ethylene produced per CFU (\( \mu \text{M} C_2H_4/\text{CFU} \)). Numbers outside of the parenthesis resulted from the ethylene recorded at the corresponding time divided between the CFU from the same hour and the number in parenthesis resulted from dividing \( \Delta \) \( \mu \text{M} C_2H_4/\Delta \) CFU/ml meaning the corrected rate of ethylene produced per CFU (\( \mu \text{M} C_2H_4/\text{CFU} \)) during the early exponential and exponential phases.

| Stage growth and culture condition | CFU/ml | \( \Delta \) CFU/ml | \( \mu \text{M} C_2H_4 \) produced | Rate \( \mu \text{M} C_2H_4/\text{CFU} \) |
|-----------------------------------|--------|----------------|------------------|-----------------|
| Lag phase 7 h                     |        |                |                  |                 |
| Stenotrophomonas sp.              | 8.91 \times 10^5 | 81.85 | 0.09 |
| Stenotrophomonas sp. + Colpoda sp.| 3.40 \times 10^5 | 75.55 | 0.22 |
| A. lipoferum                      | 9.70 \times 10^5 | 88.15 | 0.09 |
| A. lipoferum + Colpoda sp.        | 3.80 \times 10^5 | 75.55 | 0.20 |
| Early exponential phase 29 h      |        |                |                  |                 |
| Stenotrophomonas sp.              | 2.3 \times 10^6 | 1.41 \times 10^6 | 119.63 (\( \Delta \) 37.7) | 0.05 (\( \Delta \) 0.02) |
| Stenotrophomonas sp. + Colpoda sp.| 1.2 \times 10^6 | 8.60 \times 10^5 | 107.80 (\( \Delta \) 32.2) | 0.09 (\( \Delta \) 0.03) |
| A. lipoferum                      | 2.6 \times 10^6 | 1.63 \times 10^6 | 128.96 (\( \Delta \) 40.8) | 0.05 (\( \Delta \) 0.02) |
| A. lipoferum + Colpoda sp.        | 1.05 \times 10^6 | 6.70 \times 10^5 | 137.45 (\( \Delta \) 61.9) | 0.13 (\( \Delta \) 0.09) |
| Exponential phase 52 h            |        |                |                  |                 |
| Stenotrophomonas sp.              | 4.34 \times 10^6 | 2.04 \times 10^6 | 119.63 (\( \Delta \) 0) | 0.03 (-) |
| Stenotrophomonas sp. + Colpoda sp.| 1.8 \times 10^6 | 5.99 \times 10^5 | 107.04 (\( \Delta \) -0.7) | 0.06 (-) |
| A. lipoferum                      | 3.90 \times 10^6 | 1.30 \times 10^6 | 133.57 (\( \Delta \) 4.6) | 0.03 (\( \Delta \) 0.003) |
| A. lipoferum + Colpoda sp.        | 2.15 \times 10^6 | 1.10 \times 10^6 | 108.18 (\( \Delta \) -29) | 0.05 (-) |

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Impact of Predation on Bacterial Populations

*Colpoda* sp. impacted stronger on *Stenotrophomonas* sp. population (ID = 0.75) than on *A. lipoferum* (ID = 0.60) due to the higher reproduction rate of the latter during their exponential phase. The intrinsic growth rate of *Stenotrophomonas* sp. got multiplied by 6 times because of *Colpoda* sp. predation, since it increased from 0.05 h⁻¹ without predation to 0.32 h⁻¹ when subjected to this pressure. It follows that a greater nutrient availability is needed to sustain such a growth rate, including nitrogenated compounds. Thus, the doubling time of *Stenotrophomonas* sp. population decreased by 10 h under *Colpoda* sp. predation, and because of this, it would have needed a higher nitrogen availability to satisfy its reproduction needs.

On the other hand, *Colpoda* predation had no effect on the generation time of *A. lipoferum* since the intrinsic growth rate of this bacterium was almost the same in the absence as in the presence of predator (0.02 h⁻¹). It can be assumed that the intrinsic growth rate of *A. lipoferum* was just enough to compensate for the quantity of cells consumed by *Colpoda* sp. Therefore, this bacterium would have needed less nitrogen availability to sustain its population growth.

Effect of Predation on N Fixation

Stimulation of bacterial reproduction due to predation by *Colpoda* sp. shows a pattern similar to the production of new bacterial biomass under conditions of continuous cultivation [44]; the reproduction rate of both bacterial species increased as predation increased too (Fig. 4), thereby stimulating nitrogenase activity from 4 h of incubation. This shows a similarity with the results obtained by Hunt et al. [44] in a chemostat study, where they observed that the predator promotes an increase in the limiting nutrient level by reducing the bacterial biomass, which in turn stimulates a greater bacterial growth rate [44].

*Stenotrophomonas* sp. and *A. lipoferum* registered the highest fixation rate under predator’s pressure than the controls during the lag phase (0.22 and 0.20 nM/CFU vs 0.09 nM/CFU respectively). The N₂ fixation rate of *A. lipoferum* and *Stenotrophomonas* sp. under predation remained above the levels found in the control systems in the early exponential phase (0.03 nM/CFU and 0.09 vs 0.02 nM/CFU respectively). This can be explained by the differential rate of predation, since *Colpoda* sp. feeds more intensely on *Stenotrophomonas* sp. Consequently, the quantity of *Stenotrophomonas* cultivated with the predator was significantly lower than the one found in the control culture, and this produced a drop in the nitrogenase activity falling below the one shown by the control in the exponential phase (107 µM ethylene vs 119 µM respectively) probably because of the availability of nitrogenated molecules released in *Colpoda* wastes. In contrast, *A. lipoferum* took longer to replace its individuals lost by predation of *Colpoda* sp., and nitrogenase activity remained higher per bacteria than in the control, fulfilling the needs of nitrogenated molecules and balancing the smaller proportion of N release due to the lower rate of predation. However, besides the lower predation rate, the kind of nitrogenated molecules should also be important, as the two bacterial species may require different molecules to meet their metabolic needs.

The foregoing highlights the importance of predation by protozoa in nitrogen fixation by free-living bacteria in nature, since comparable results of nitrogenase activity have been found in bacteria associated with the bean rhizosphere (0.0033 to 19 nM) after 24 h of incubation [45], or in the activity of *Azospirillum brasilensis* in non-leguminous plants (0.2 and 0.4 nM) obtained 1 h after colonization [13].

The nitrogen introduced at the microscale may be just enough to balance the losses at that level and become unnoticed in systems reaching a steady state. Distribution of nitrogen between patches poor in nitrogen and plenty of nitrogen is performed by meso- and macrofauna preying along these soils’ mosaics containing fungi, bacteria, and protozoa. Predator wastes allow plants to take the released nutrients keeping the cycle of N going on at the ecosystem scale. However, this may not be the case in early soil development [13], or in soil polluted with hydrocarbons. In these cases, free-living N₂-fixing bacteria such as *A. lipoferum* and *Stenotrophomonas* sp. may become an important source of reduced nitrogen in poor soils or for hydrocarbon metabolization at the patch level. In these cases, mobilization of nitrogen through the soil trophic networks starts when these bacteria are preyed upon by protozoa [13], building up the basic income of this nutrient for increasing soil productivity and facilitating poor-quality organic matter metabolization.

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Data availability  All data are available upon request to the corresponding author.

Declarations

Ethics approval  No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with a reference strain and a bacteria species isolated from soil as well as a microbial eukaryote.

Conflict of Interest  The authors declare no competing interests.

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