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

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Effect of predation by *Colpoda* sp. in nitrogen fixation rate of two free-living bacteria

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Abstract

Biological nitrogen fixation (BNF) is limited to several groups of prokaryotes, which can reduce nitrogen through complex endosymbiotic relationships or as free-living nitrogen-fixing bacteria (FLNFB). Predation of FLNFB by protozoa releases reduced nitrogen, enhancing the formation of plant and bacterial biomass as well as nitrogen (N) mineralization within soil microbial communities. We aim to evaluate the predation effect of *Colpoda* sp. on two FLNFB *Azospirillum lipoferum* and *Stenotrophomonas sp.* during their exponential and lag phase. The likelihood of *Colpoda* sp. to feed on the former species was needed to ensure there is a predation effect. The kinetics of bacterial population growth was determined in the predators' presence or absence and the effect of predation on the biological fixation of N 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. However, it had a higher positive effect on the formation of new bacterial biomass on *Stenotrophomonas* sp.as revealed by the increase of its specific growth rate. Likewise, predation promoted greater nitrogen fixation in *A. lipoferum* and *Stenotrophomonas sp*. during the lag phase (0.34 nM and 0.38 nM) than in the exponential phase (0.27 nM and 0.17 nM). We concluded that predation by *Colpoda*sp stimulates the rate of nitrogen fixation of *A. lipoferum* and *Stenotrophomonas* sp.

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

1. Introduction

Biological nitrogen fixation (BNF) is the process by which N_2 is reduced to ammonia (NH3) through the enzyme nitrogenase, a crucial process to assure nitrogen availability in terrestrial ecosystems [1]. BNF can be endosymbiotic (i.e., with nodule formation) or reduced by bacteria in a free-living stage, defined as N fixation, which occurs without the formal endosymbiotic process between microorganisms and plants [2]. The group of free-living nitrogen-fixing bacteria (FLNFB) includes the genus *Azospirillum* [3–5], which also promotes plant growth [6] and the genus *Stenotrophomonas*, which has an important ecological role in the

sulfur cycle [7,8] and participates in an extraordinary range of activities that include beneficial effects on the growth and health of plants, such as *S. maltophilia* and *S. rhizophila.* [9].

Soil productivity is based on a continuous mobilization of organic nitrogen, which can be a result of cell lysis, predatory debris, microbial biomass and the decomposition of nitrogenous components trapped in litter. All these activities may lead to N mineralization [10], while biological fixation means a net input of reduced N allowing the sustainability of the soil system [11]. BFNVL are also among the early colonizers of locations with zero nitrogen content or extremely poor reduced nitrogen content. Once the trophic networks of the soil ecosystem have been established, BFNVL provide the necessary nitrogen to cover losses due to volatilization and denitrification [11].

The quantification of the nitrogenase's reducing activity can be accomplished by reduction of acetylene (C_2H_2) to ethylene (C_2H_4) [14], which provides a useful and sensitive assay, as this enzyme breaks dawn the triple bond of acetylenein the same way it breaks the triple bond of the N_2molecule and adds hydrogen to produce (NH_3) [12,13].

On the other hand, protozoa enhance nitrogen mobilization along soil trophic networks [15] and significantly modify the structure of bacterial communities through selective predation [16]]. Although protozoa are capable of feeding on a wide variety of bacteria, they also show a degree of preference towards certain species, depending on the prey size [17,18], cell pigmentation, motility and micro-colony shape [18-20]. In this sense, selective predation dramatically decreases the abundances of preferred preys in short time [21,22]. Consequently, bacterial species lacking chemical defenses develop extremely high growth rates [23] to survive the negative impact of intense predation pressure [23]. Thus, the reduction in the number of bacteria during predation results in the counter-intuitive situation of increasing bacterial levels of metabolic activity [24]. In this way, predation reduces the bacterial biomass, increases the levels of reduced nitrogen available and the bacterial growth rate. For this reason, we wonder: How will predation by protozoa affect the nitrogenase activity of nitrogen-fixing bacteria?

We aim to determine the effect of *Colpoda* sp. in the nitrogenase activity of FLNFB *Azospirillum lipoferum* and *Stenotrophomona s*sp during the exponential and lag phases of population growth.

2. Materials and Methods

2.1 Protozoa and bacteria isolation and cultivation

Colpoda sp. and *Stenotrophomonas* sp. were obtained from soil cultivated with corn through the wet but non-flooded soil method from Bamforth [25]. Morphological identification of *Colpoda*sp was achieved following Foissner and Kohamannkey [26].

The most abundant bacteria in the polyxenic cultures of *Colpoda* sp. in liquid medium was a 2 to 4 µm Gram-negative coco bacterium able to grow in Rennie medium (1981) and capable of reducing acetylene to ethylene in culture. It was selected as suitable free-living nitrogen fixing bacterial (FLNFB) 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[27].

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), 1 X 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 110V 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 Estadode Mexico. This is a 2 to 5 µm Gram-negative vibroid type and a (FLNF) bacteria.

2.2 *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 on15 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 [28] (figure 1).

About 200 *Colpoda* sp. cells were inoculated in 50 µl in the central well of the Petri dishes (one hour before inoculation, the polygenic culture was treated with Chloramphenicol (5 mg ml^{-1}) to eliminate the foreign bacteria from the polygenic culture). *A. lipoferum, Stenotrophomonas sp*, and a combination of both were deposited in each of the lateral wells (200 µl of bacteria, 1 x 10^6 CFU). The control well contained only 200 µl of the sterilized yeast extract. Channels were flooded with yeast extract medium (3 g / l) and NaCl (5 g/ l) to allow predators' movement towards the bacteria in suspension wells. Subsequently, the Petri dishes were carefully kept without any movement at room temperature (~ 24 ºC) for 3 hours. Thereafter, each lateral well was fixed with 4% Lugol (v/v) to count cysts and trophozoites [28].

2.3 Bacterial growth with and without predator

The kinetics of bacterial population growth, in the presence orabsence of *Colpoda* sp., was achieved by counting bacteria at 22-time intervals from 0 h to125 h. Bacterial counts were done by spectrophotometer readings set up at 540 nm absorption.

Parallelly, colony-forming units (CFU) were determined from serial micro-dilutions in a 96-well plate to corroborate the population size determined by spectrophotometer. For the CFU count, dilutions of 10^{-7} were considered and 20 µl were inoculated in triplicate in Petri dishes with Rennie agar [29], an extremely low-content of reduced-nitrogen medium.

2.4 Mathematical model of bacterial growth

The following model was used to calculate the exponential growth phase, where the binary fission of bacteria occurs at regular intervals and increasing velocity [31]; under exponential conditions, bacterial growth is described as:

132 $$\frac{dX}{dt} = \mu X \qquad (1)$$

133 Where:

134 $X = organic\ matter\ \frac{g}{l}\ or\ cells/l$

135 $t = time$

136 $\mu = \boldsymbol{specific\ growth\ rate\ in\ h^{-1}(mass\ or\ number)}$

137 To summarize:

138 $$\mu = \frac{Ln2}{g} = \frac{0.693}{g}$$

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

141 $$g = \frac{t}{n}$$

142 $\boldsymbol{g = genrational\ time}$

143 $t = time\ interval\ elapsed\ between\ generations\ No\ and\ Nn$

144 $n = number\ of\ generations$

145 Finally, the number of generations was calculated as:

146 $$n = \frac{\log\left(\frac{Nn}{No}\right)}{\log 2}$$

147 $\boldsymbol{n = number\ of\ generatios}$

148 $Nn = final\ concentration\ of\ organisms$

149 $No = initial\ concentration\ of\ organism$

150 **2.5 Impact of predation on bacterial abundance**

151 According to Fox [32], the impact of predation (ID) varies between 0 (no predation effect) and 1
152 (complete extinction of prey). If predation leads to increased prey abundance, the ID value becomes negative

153 $$ID = \frac{Kbac - R}{Kbac}$$

154 $$Kbac = final\ concentration\ of\ bact.without\ predator$$

155 $$R = final\ concentration\ of\ bact.with\ predator$$

156 **2.6 Statistical analysis**

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

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

162 **2.7 Nitrogenase activity**

163 Vial bottles with a capacity of 40 ml were used, which contained 20 ml of Rennie medium and 1 ml of
164 bacteria (approximately 2 x 10^7 CFU / ml). 200 µl of *Colpoda* sp. (55 cells / ml approximately) were added to
165 the treatments in the presence of predation. 4 ml (10% v / v of the total volume of the vial) of the internal
166 atmosphere of the vials were replaced by 4 ml of acetylene. The gas mixture from each treatment and the
167 control group were analyzed with mass spectrometry to identify acetylene and ethylene molecules. [30]. The
168 experimental design consisted of four treatments with four repetitions each: *A. lipoferum* with and without a
169 predator, and *Stenotrophomonas* sp. with and without predator, and two controls without microorganisms:
170 culture medium with acetylene and culture medium with ethylene.

171 With the help of acetylene (C_2H_2) and ethylene (C_2H_4) standards, the identities of both molecules were
172 determined by monitoring the presence and abundance of the ions that give it the "fingerprint" within a gas
173 mixture, to identify the presence or absence of the two molecules in question.

174 Nitrogen fixation rate was estimated according to the number of nanomoles of ethylene produced per
175 unit of bacteria. Quantification of acetylene to ethylene transformation was done by mass spectrometry

(Agilent Technologies brand model 5975 inert XL) of the gas mixture from each experiment and control group at the 22-time intervals. The nitrogenase activity of *A. lipoferum* and *Stenotrophomonas* sp. was first registered after 4 h of incubation at room temperature.

3. Results

3.1 Food preference of *Colpoda* sp.

Colpoda showed significantly higher trophozoite formation ($\alpha = 0.05$) in the wells inoculated with *Stenotrophomonas* sp. ($\alpha = 0.05$) and with *A. lipoferum*, ($\alpha=0.05$), as well as in the mixture of these bacteria, than in the well corresponding to the control group. *Colpoda* sp. had a greater presence of trophozoites in the well of *Stenotrophomonas* sp. than in that of *A. lipoferum* (3013 and 2093 trophozoite cells / mL respectively). However, *Colpoda* was significantly less numerous in the well containing the mixture of both bacteria (1093 trophozoite cells / mL; Figure 2). There was a significantly higher number ($\alpha = 0.05$) of trophozoites than cysts in all treatments inoculated with bacteria, compared to the control wells.

3.2 Population growth of *A. lipoferum* and *Stenotrophomonas* sp. with and without predator

The two bacterial species reached the Lag phase at26 h, either in the absence or presence of predator. In this regard, *Colpoda* sp doubled its population after 30 h of contact with the two bacteria separately and in the mixture of both (Figure 3). It took *A. lipoferum* 102 h to reach its maximum growth with or without a predator, which was the same time that *Colpoda* sp stopped its division and encysted (Figure 3-a). *Stenotrophomonas* sp. reached its maximum population growth at 54 h without predator but shortened it to 33 h after culture with *Colpoda* (Figure 3-b). In this way, the predator maintained its growth trend up to 125 h. On the other hand, the growth of *Colpoda* sp. in the mixture of both bacteria species was intermediate between what was observed when cultivated with either bacteria species separately (Figure 3-c).

Colpoda sp. reduced the populations of *A. lipoferum* to a lesser extent than those of *Stenotrophomonas* sp throughout the entire experiment. Nonetheless, *A. lipoferum* reached the exponential phase in three generations ($n = 3.1$), while the presence of the predator almost did not modify this value ($n = 2.9$); that is, predation extended the generational time of *A. lipoferum* from $G = 24.5$ h without predation to $G = 26$ h with predation. By contrast, *Stenotrophomonas* sp. Extended its time to reach the exponential phase from two and a

half generations in the absence of the predator (n = 2.4) to three and a half generations (n = 3.4) under the grazing of *Colpoda* sp. Predation decreased the bacteria generational time from 12 h (G = 12.3) to just 2 h (G = 2.1 h). Consequently, the predation of *Colpoda* sp. did not modify the specific growth rate of *A. lipoferum* ($\mu = 0.02\ h^{-1}$), while that of *Stenotrophomonas* sp. increased the specific growth rate (from $\mu = 0.02\ h^{-1}$ to $\mu = 0.32\ h^{-1}$). On the other hand, the growth rate of *Colpoda* was higher in this strain ($\mu = 3.4\ h^{-1}$) than in *A. lipoferum* ($\mu = 3{,}1\ h^{-1}$) and even in the mixture of both preys ($\mu = 3.2\ h^{-1}$). *Colpoda*has a greater positive effect on the biomass formation of *Stenotrophomonas* sp. than in *A. lipoferum*. Therefore, the effect of *Colpoda* sp. (ID) on numbers of *A. lipoferum*was moderate (0.43), while the predation of *Colpoda* sp. on *Stenotrophomonas* sp. was nearly null (0.23) (Table 1).

3.3 Nitrogenase activity

The nitrogenase activity of *A. lipoferum* and *Stenotrophomonas* sp. was first registered after 4 h of incubation at room temperature. Then it was carried out hourly during the first 12 h. Both bacteria species produced a higher quantity of ethylene after 24 h, although nitrogenase activity continued to be registered until 102 h of incubation. The highest fixation rate per bacterial unit (nM / CFU) was observed during the lag phase of bacterial growth of both bacterial populations subjected to predation (Figure 5), while the lowest yields of fixation (nM / CFU) were presented during the exponential phase. However, the synthesis of ethylene per unit of bacteria, both in the lag and in the exponential phase of *A. lipoferum*, was greater in the presence of predator than in its absence, while the fixation rate per unit of *Stenotrophomonas* sp. was the same in the lag and exponential phases growth, when it was subjected to predation (Table 2).

4 Discussion

4.1 Prey Selection

Colpoda sp had higher preference for *Stenotrophomonas* sp. Over *A. lipoferum, as* revealed by the difference in the number of trophozoites 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 [44–46]. We rule out the masking effect of *Stenotrophomonas* sp by *A. lipoferum*, because in such a case, predation would have been similar to the one shown where this species was offered alone, instead of the marked decrease in preference as seen in Fig. 2.

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 phaseof their population development. The contradictory effect of reducing prey abundances while stimulating their growth rate is the bacterial populations' transitory response to predation [33]. This response has been found to result in a dispersal advantage of bacteria reproducing in less time in some micro-environments [34,35, 36].

On the other hand, the semi-coccus orovoid shape of *Stenotrophomonas* sp. And smaller size in comparison to the larger vibroid shape of *A. lipoferum*, explains why *Colpoda sp*. had consumed greater quantity of *Stenotrophomonas* (3013 cells mL^{-1}) than *A. lipoferum* (2093 cells mL^{-1}) [16, 40, 41, 18–20]. As both bacteria species are Gram-negative, we could safely rule out the selection effect of cell wall composition indicated by Griffiths [37], Darbyshire [38], Drake and Tsuchiya [39].

4.2 Impact of predation on bacterial populations

Colpoda sp impacted stronger on *A. lipoferum* population (ID=0.43) than on *Stenotrophomonas* sp. (ID = 0.23) 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* predation, since it moved 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 hours, producing at least one generation more under *Colpoda* predation, and because of this, it would have needed a higher nitrogen fixation rate to satisfy its reproduction needs.

On the other hand, Colpoda predation had no effect on the generational time of *A lipoferum*, since the intrinsic growth rate of this bacterium was 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 fixed nitrogen to sustain its population growth.

4.3 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 [42], the reproduction rate of

256 both bacterial species increased as predation increased too (Figure 4), thereby stimulating nitrogenase activity
257 from 4h of incubation. This shows a similarity with the results obtained by Hunt et al [4] in a chemostat study,
258 where they observed that the predator promotes an increase in the limiting nutrient level by reducing the
259 bacterial biomass, which in turn stimulates a greater bacterial growth rate [4].

260 *Stenotrophomonas* sp. and *A lipoferum* registered the highest fixation rate under predator's pressure
261 than the controls during the lag phase (0.38 and 0.34 nM/CFU vs. 0.17 nM/CFU respectively) (Fig. 5).
262 Conversely, the N_2 fixation rate of *Stenotrophomonas* sp, with predation, was significantly higher than the
263 one shown by the control in the lag phase (0.38 vs. 0.17 nM/CFU) and returned to match the control one
264 during the exponential phase (0.17 nM/CFU). While the N_2 fixation rate of *A lipoferum* under predation
265 remained above the one obtained by the control during the lag (0.34 vs. 0.17 nM/CFU) and the exponential
266 phase (0.27 vs. 0.18 nM/CFU). This can be explained by the differential rate of predation, since *Colpoda* sp.
267 Feeds more intensely on *Stenotrophomonas* sp. Consequently, the quantity of *Stenotrophomonas* cultivated
268 with the predator was significantly lower than the one found in the control culture, and this produced a drop in
269 the N_2 fixation to match the one shown by the control in the exponential phase, notwithstanding the
270 availability of nitrogenated molecules released in *Colpoda* sp wastes. In contrast, *A. lipoferum* took longer to
271 replace its individuals lost by predation of *Colpoda* sp., because these are larger cells. Consequently, N_2
272 fixation must remain higher per bacteria than in the control, to fulfill the needs of nitrogenated molecules, as
273 the proportion of reduced N release in *Colpoda* wastes are smaller due to the lower rate of predation.
274 However, besides the lower predation rate, the kind of nitrogenated molecules should also be important, as the
275 two bacterial species may require different molecules to meet their metabolic needs [47,48].

276 The foregoing highlights the importance of predation by protozoa in nitrogen fixation by free-living bacteria,
277 since comparable results of nitrogenase activity have been found in bacteria associated with the bean
278 rhizosphere (0.0033 to 19 nM) after 24 h of incubation [43], or in the activity of *Azospirillum brasilensis* in
279 non-leguminous plants (0.2 and 0.4 nM) obtained one hour after colonization [11].

280 Further, *A. lipoferum* and *Stenotrophomonas* sp. demonstrated their ability to fix nitrogen without
281 endosymbiotic association to any plant species, which highlights their importance as pioneers for colonization
282 of nitrogen-poor soils [11]. On the other hand, these free-living N_2-fixing bacteria become the source of

reduced nitrogen by being preyed upon by protozoa during their most active phase. Thus, predation mobilizes nitrogen through the soils-trophic networks [11] and increase soils productivity of agro ecosystems.

These results highlight the importance of protozoa predation of nitrogen-fixing bacteria as a valuable service helping improve the bioremediation strategies (when pollutants produce nitrogen-poor soil layers) and for reclamation of agricultural, grassland and forest soils that have deteriorated due to poor management practices.

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Declarations

Author Contributions: Conceptualization: Nathalie Cabirol, Alejandro Alarcón Carlos Manuel Martínez Reyes and Salvador Rodríguez Zaragoza**; Methodology feeding preferences:** Salvador Rodríguez Zaragoza and Carlos Manuel Martínez Reyes**; Methodology Nitrogen fixing and Predator prey counts:** Nathalie Cabirol, Ma Remedios Mendoza López, Alejandro Alarcón and Carlos Manuel Martínez Reyes**; Formal analysis and investigation:** Nathalie Cabitol, Ma. Remedios Mendoza López, Alejandro Alarcón, Carlos Manuel Martínez Reyes, Salvador Rodríguez Zaragoza**; Writing - original draft preparation**: Alejandro Alarcón, Carlos Manuel Martínez Reyes**; Writing - review and editing:** Nathalie Cabirol, Ma. Remedios Mendoza López, Salvador Rodríguez Zaragoza**; Funding acquisition:** Salvador Rodríguez Zaragoza**; Resources: PAPIIT IN222618, Dirección General de Asuntos del Personal Académico (DGAPA), UNAM; Funding acquisition:** Carlos Manuel Martínez Reyes; **Resources**: CONACyT Scholarship granted through posgrado en Ciencias Biológicas de la UNAM

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Ethical 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

Availability of data and material: All data are available upon request to the corresponding author

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415 atmosférico en rizósfera de frijol contaminado con queroseno.TERRA Latinoamericana 21: 81–89.
416

417 FIGURES.

418 Figure Captions

419 Fig.1 Resin molds (left) were used for printing wells and communication channels in soil-extract agar (right)
420 to perform the food preference tests. Each bacteria species was deposited in one peripherical well (200 µl of
421 bacteria, 1 x 10^6 CFU), a mixture of both bacteria strains was deposited in a 3rd well (200 µl of bacteria, 1 x
422 10^3 CFU from each strain) and the 4th well was filled with 200 µl sterilized yeast extract (3 g / l) as control.
423 Communication channels were filled up with yeast extract.

424

425 Figure 2. Counting of trophozoites and *Colpoda*sp cysts in wells inoculated with A. *lipoferum* and
426 *Stenotrophomonas* sp., and in a mixture of both species of bacteria, a control well (bacteria-free) was also
427 used. The bars represent the standard deviation and only the cysts show significant differences between them
428 ($\alpha = 0.05$).
429

430 Figure 3. Population growth *Stenotrophomonas* sp (a) and *A. lipoferum* (b) in the absence (bridged lines)
431 and presence (dashed line) of the predator (grey line) and population growth of *Colpoda* sp (c) grown in the
432 presence of both bacteria. n = 3. Scale of X axis is the time in hours after setting up experiments.
433

434 Figure 4. Role of predation in nitrogen fixation and the development of prey populations
435

436 Figure 5. Nitrogenase activity. The abundance of bacteria in CFU / mL during lag (dark bars) and
437 exponential phase (grey bars). The dark bars indicate the fixation rate during the Lag phase and the light bars
438 during the phase of the exponential phase. The presence of the predator did not generate a significant
439 difference in ethylene production ($p = 0.05$).
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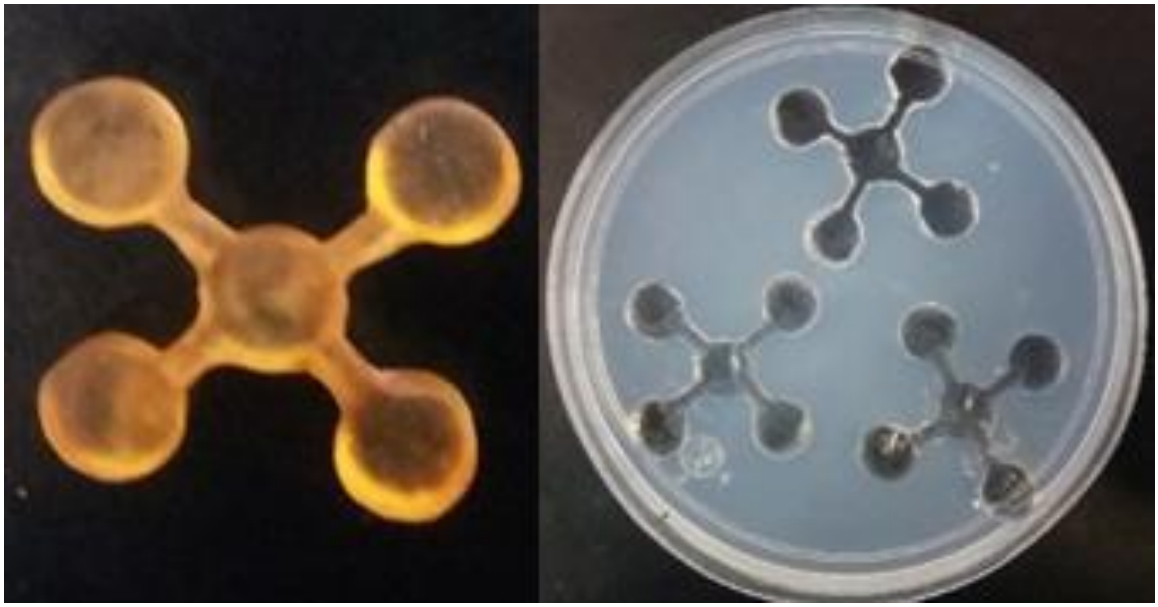


Figure 1. Resin mold (left) and agarose gel printing (right).

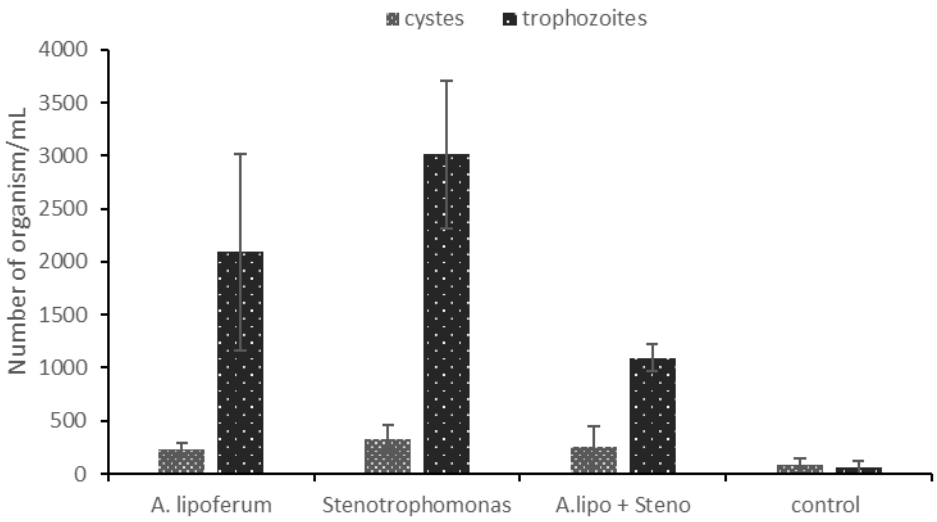

cystes
trophozoites
Number of organism/mL
4000
3500
3000
2500
2000
1500
1000
500
0
A. lipoferum
Stenotrophomonas
A.lipo + Steno
control

Figure 2.

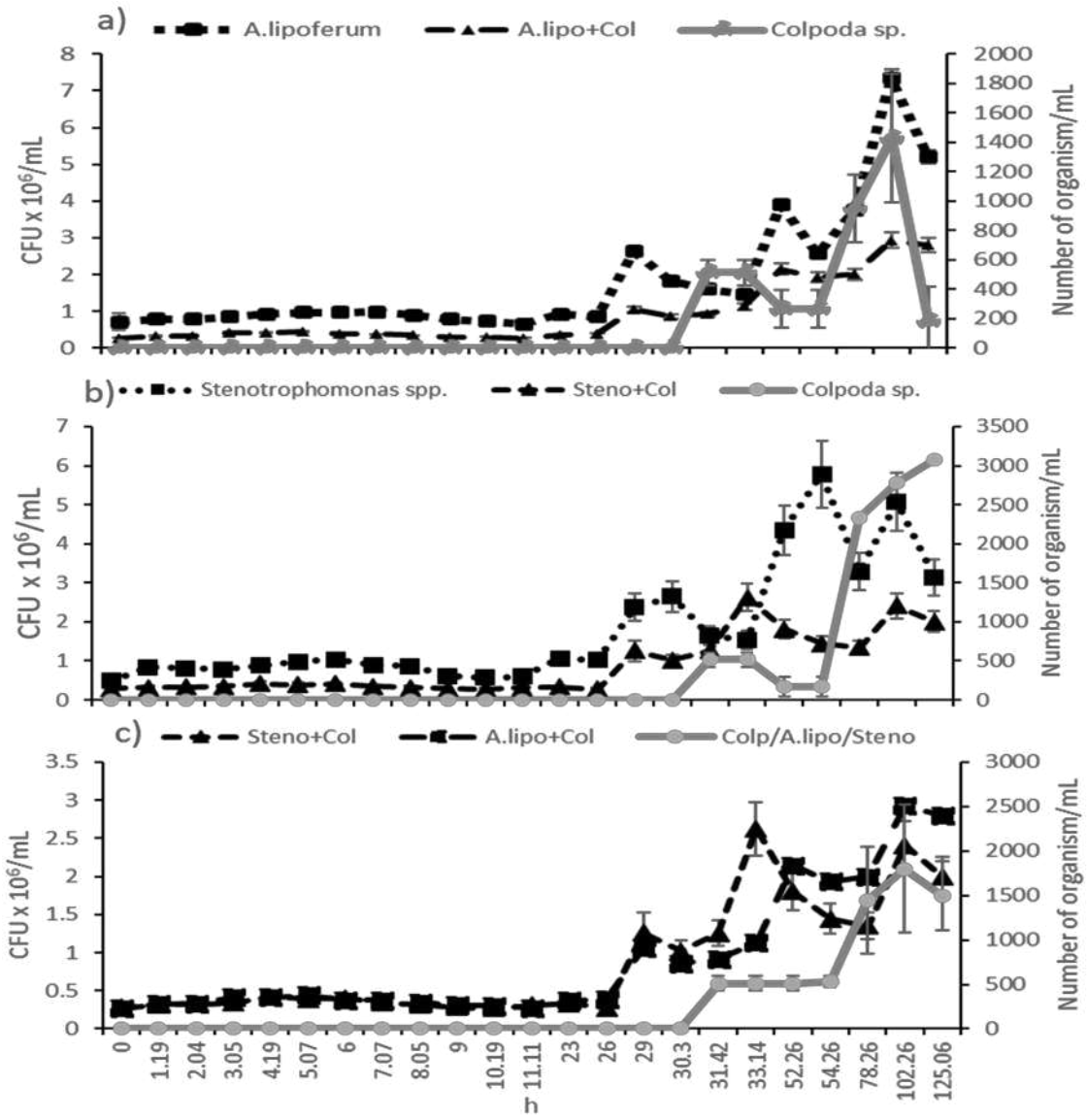
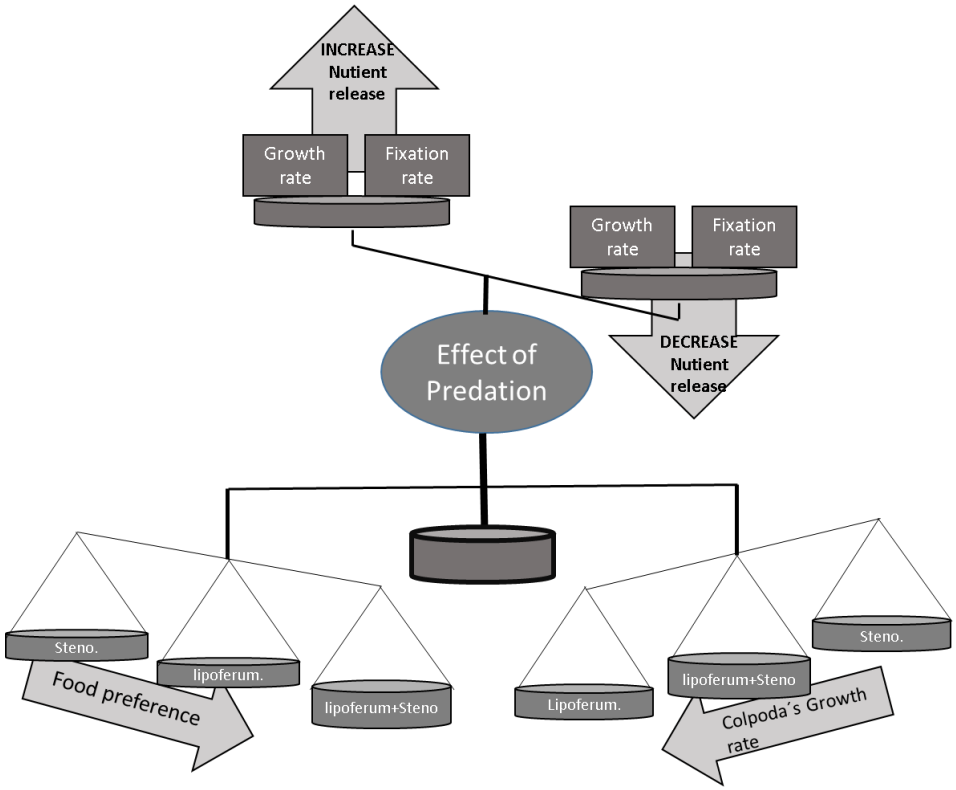

a)
A.lipoferum
A.lipo+Col
Colpoda sp.
CFU x 10⁶/mL
Number of organism/mL
b)
Stenotrophomonas spp.
Steno+Col
Colpoda sp.
CFU x 10⁶/mL
Number of organism/mL
c)
Steno+Col
A.lipo+Col
Colp/A.lipo/Steno
CFU x 10⁶/mL
Number of organism/mL
0
1.19
2.04
3.05
4.19
5.07
6
7.07
8.05
9
10.19
11.11
23
26
29
30.3
31.42
33.14
52.26
54.26
78.26
102.26
125.06
h

Figure 3.



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486 Figure 4.

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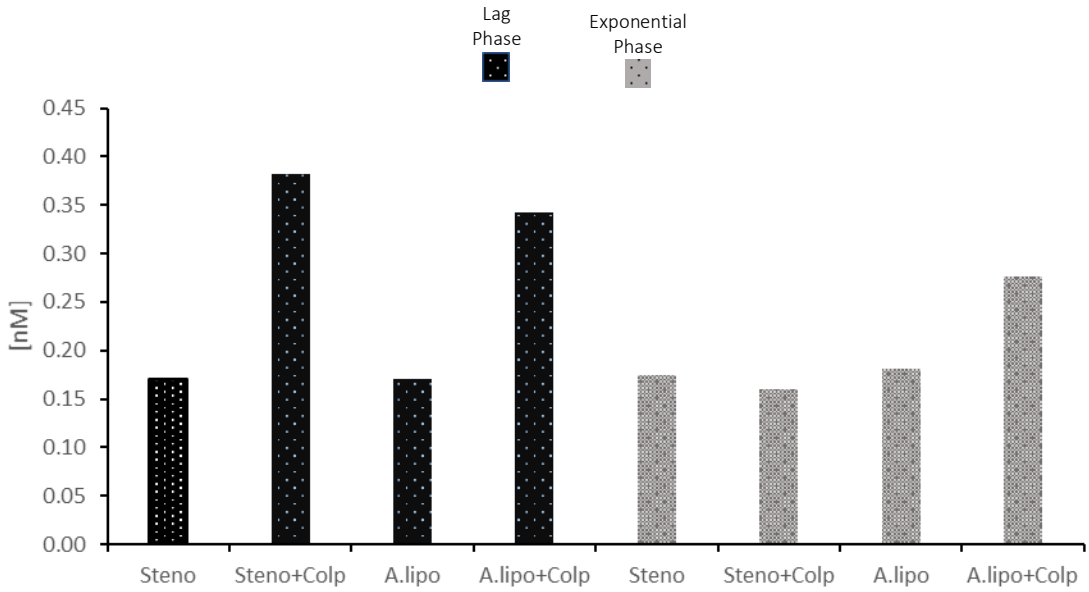
Lag Phase
Exponential Phase
[nM]
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Steno
Steno+Colp
A.lipo
A.lipo+Colp
Steno
Steno+Colp
A.lipo
A.lipo+Colp

Figure 5.

Table Captions

Table 1. Main parameters of population growth of both bacterial species: g = average time between two successive generations expressed in hours, μ = 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 means 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).

Table 2. nM of ethylene produced during the Lag phase and the exponential phase of bacterial growth by each species of bacteria cultivated alone or with predator. The last row shows the nM of fixed N per UFC of bacteria. *Stenotrophomonas sp* reach a higher rate of N fixation at exponential growth when cultivated together with *Colpoda sp*.

Table 1.

PREY	**G (h)**	**μ**	**n**	**ID**
		h^{-1}(UFC)		
A. lipoferum	24.4	0.02	3.1	0.43
***A. lipoferum* + *Colpoda*sp**	26	0.02	2.91	
Stenotrophomonassp	12.3	0.05	2.43	0.23
Steno+*Colpodasp*	2.15	0.32	3.24	
PREDATOR	**G(h)**	**μ**	**n**	
		h^{-1}(CELL)		
Colpoda*sp + *A. lipoferum	49.4	3.1	1.48	
***Colpoda*sp + Steno.**	37.1	3.4	2.5	
***Colpoda*sp+A. lipo+Steno**	40.4	3.2	1.81	

Table 2.

Samples	**Growth phase**	**CFU/mL**	**Fixation rate [nM]/UFC**
***Stenotrophomonas*sp**	Lag.	8.90E+05	0.17
Steno+Colpoda	Lag.	3.40E+05	0.38
***Stenotrophomonas*sp**	Exponential	2.30E+06	0.17
Steno+Colpoda	Exponential	1.20E+06	0.17
A. lipoferum	Lag.	9.70E+05	0.17
***A. lipo*+Colpoda**	Lag.	3.80E+05	0.34
A. lipoferum	Exponential.	2.60E+06	0.18
***A. lipo*+Colpoda**	Exponential.	1.05E+06	0.27

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Figures

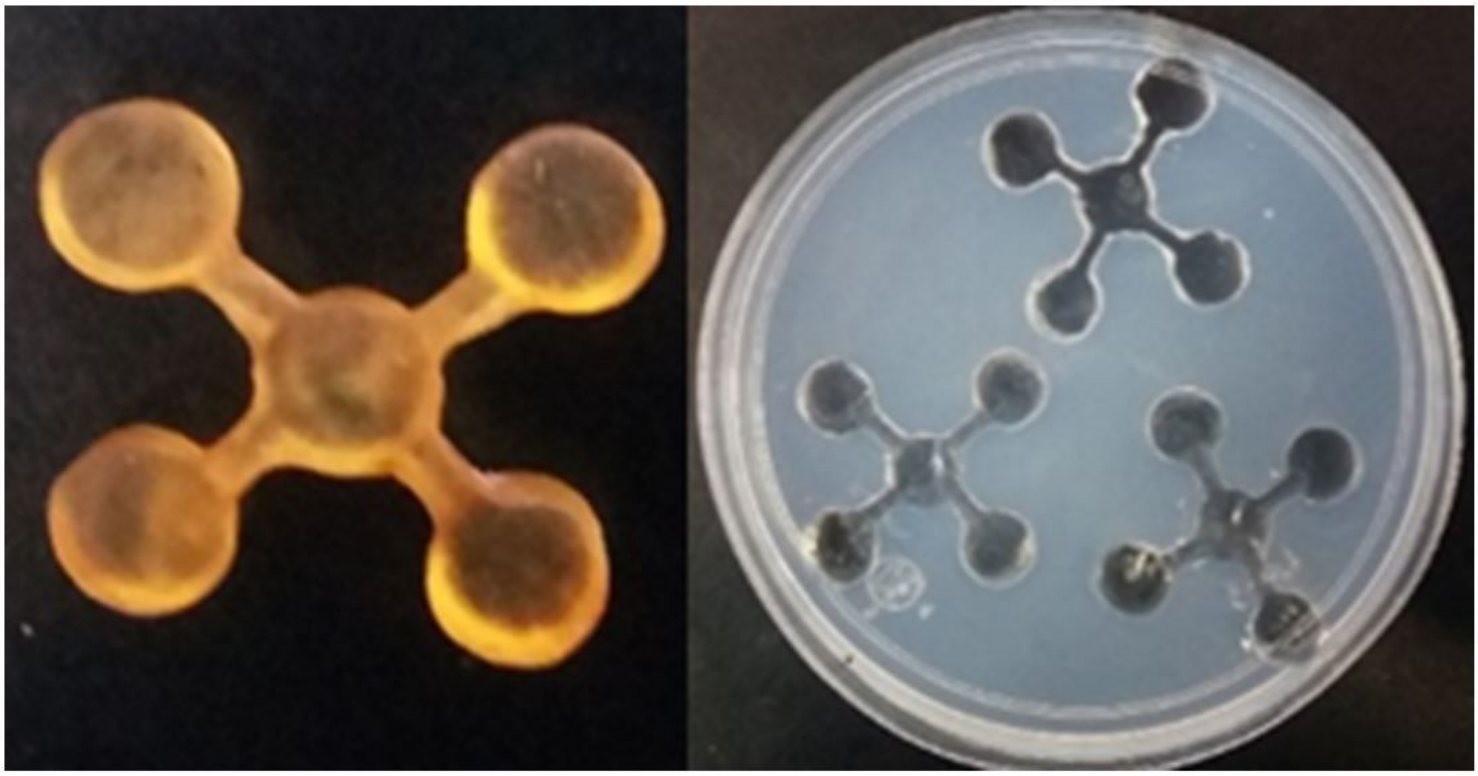

Figure 1

Resin molds (left) were used for printing wells and communication channels in soil-extract agar (right) to perform the food preference tests. Each bacteria species was deposited in one peripherical well (200 µl of bacteria, 1 x 106 CFU), a mixture of both bacteria strains was deposited in a 3rd well (200 µl of bacteria, 1 x 103 CFU from each strain) and the 4th well was filled with 200 µl sterilized yeast extract (3 g / l) as control. Communication channels were filled up with yeast extract.

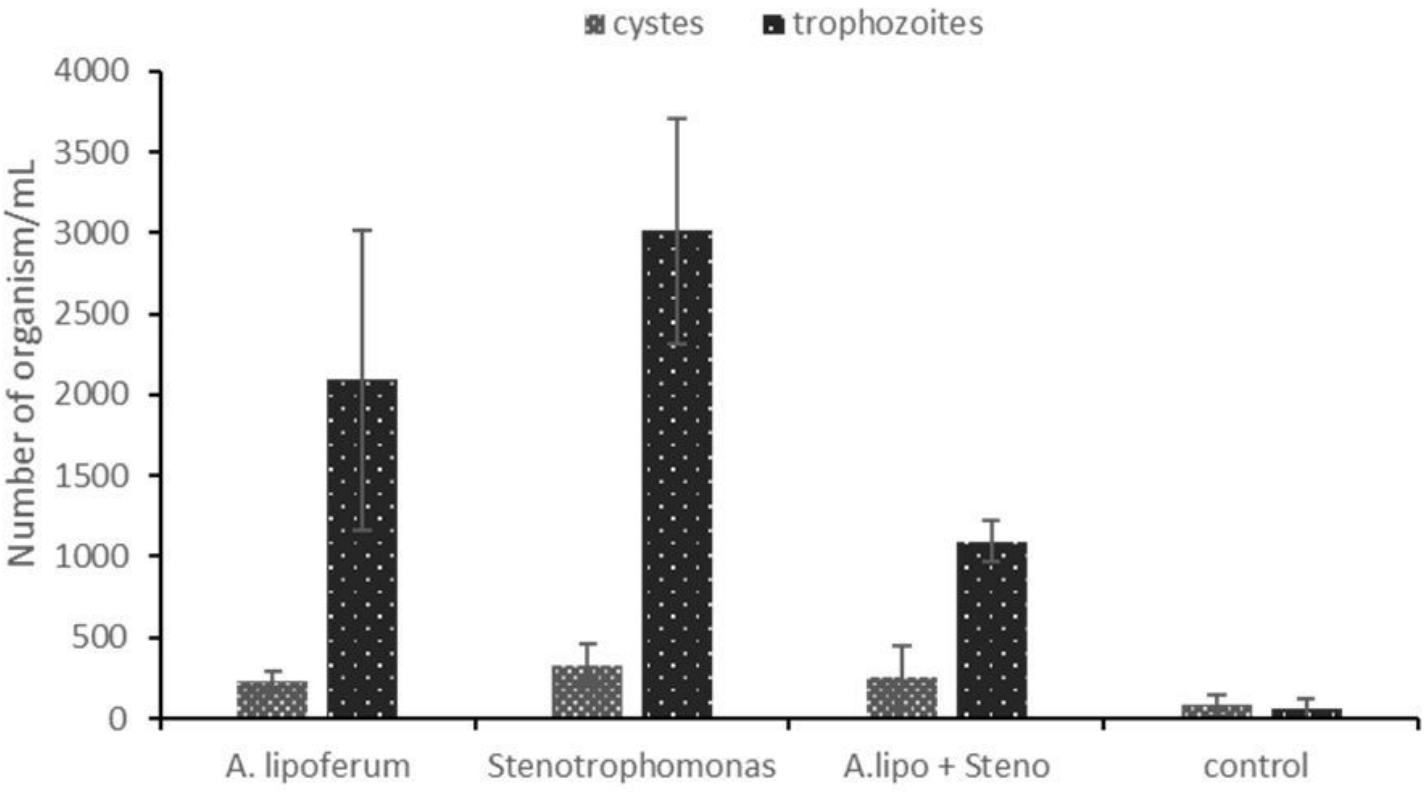

Figure 2

Counting of trophozoites and Colpodasp cysts in wells inoculated with A. lipoferum and Stenotrophomonas sp., and in a mixture of both species of bacteria, a control well (bacteria-free) was also used. The bars represent the standard deviation and only the cysts show significant differences between them ($\alpha = 0.05$).

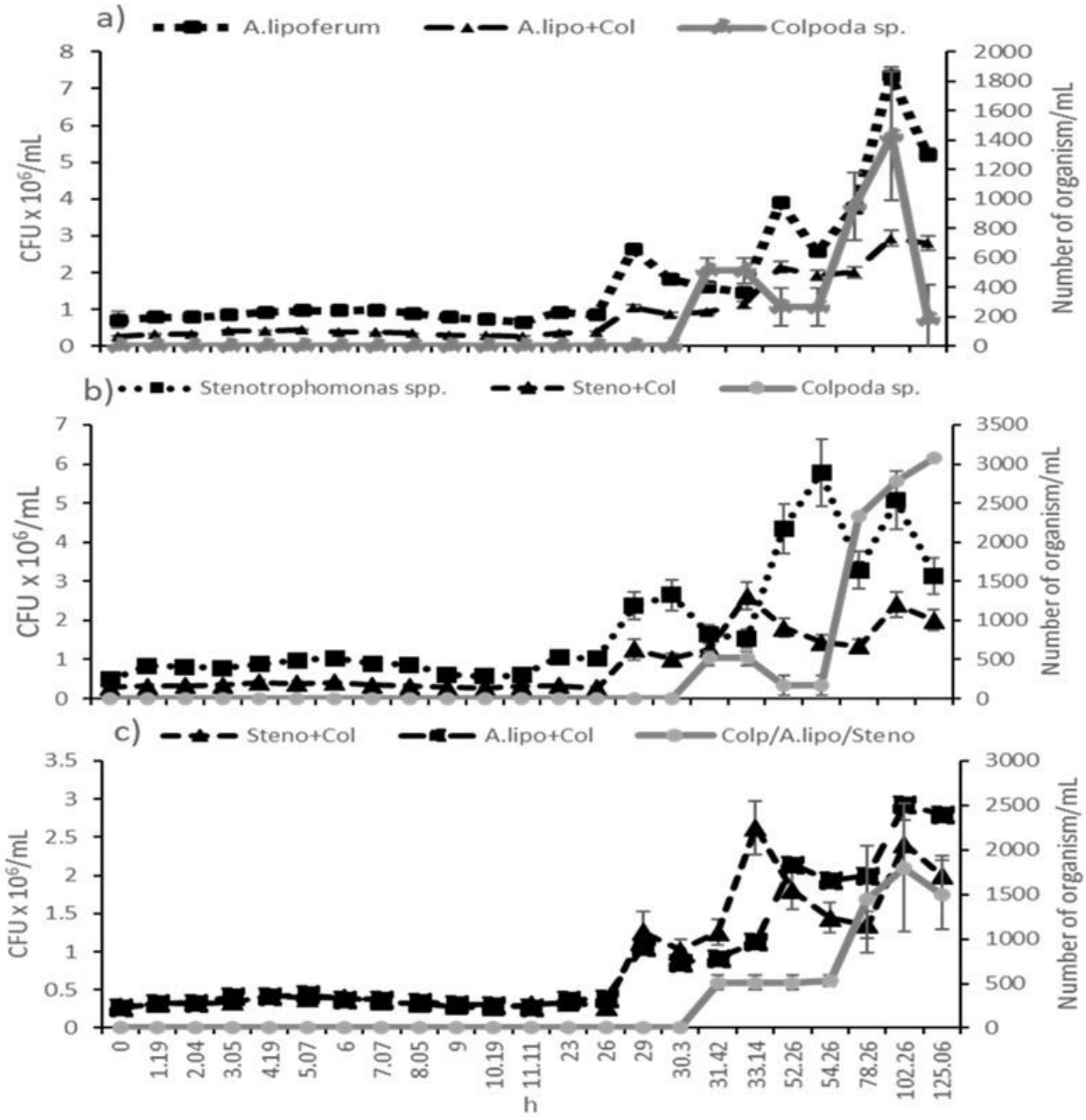

Figure 3

Population growth Stenotrophomonas sp (a) and A. lipoferum (b) in the absence (bridged lines) and presence (dashed line) of the predator (grey line) and population growth of Colpoda sp (c) grown in the presence of both bacteria. n = 3. Scale of X axis is the time in hours after setting up experiments.

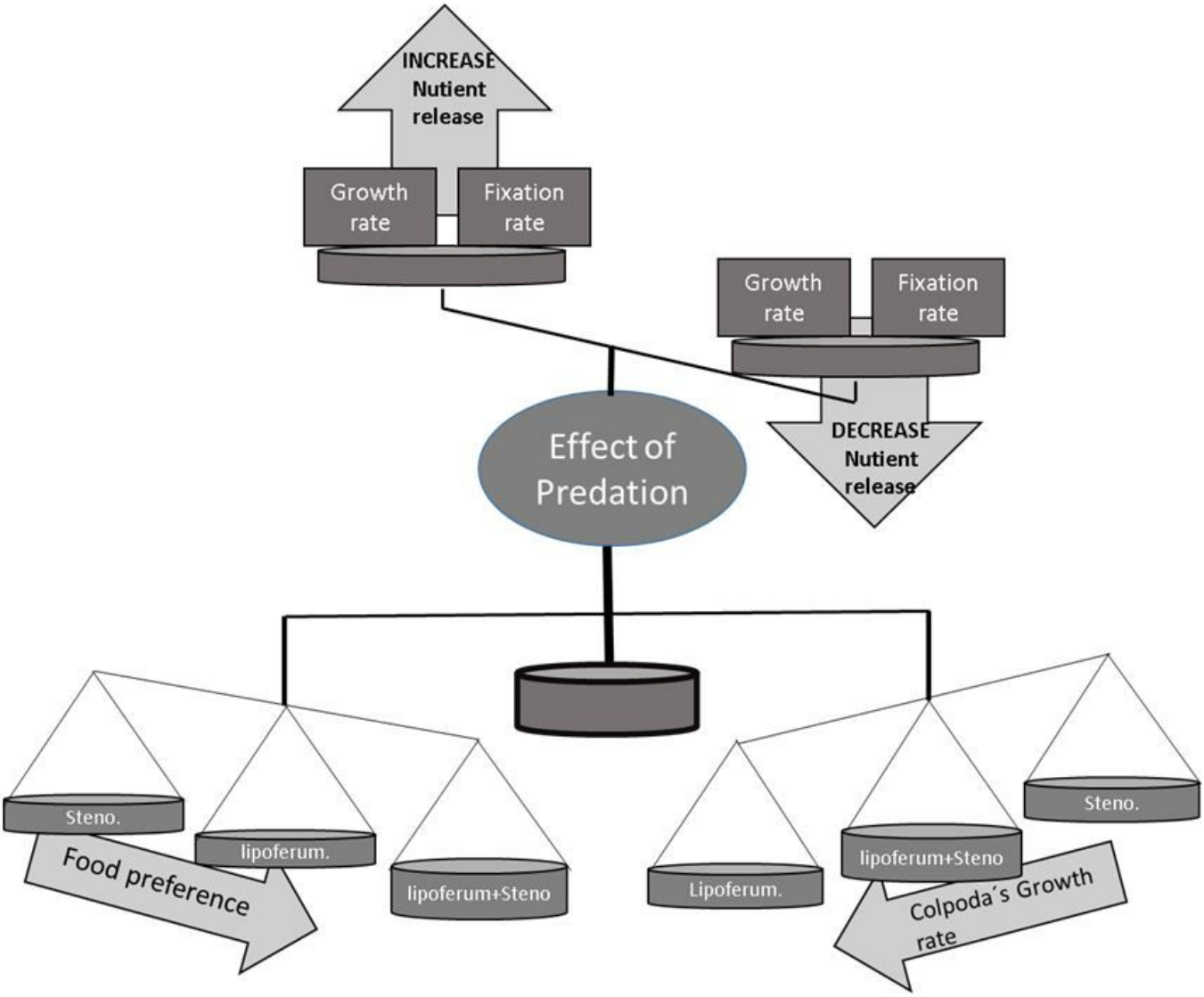

Figure 4

Role of predation in nitrogen fixation and the development of prey populations

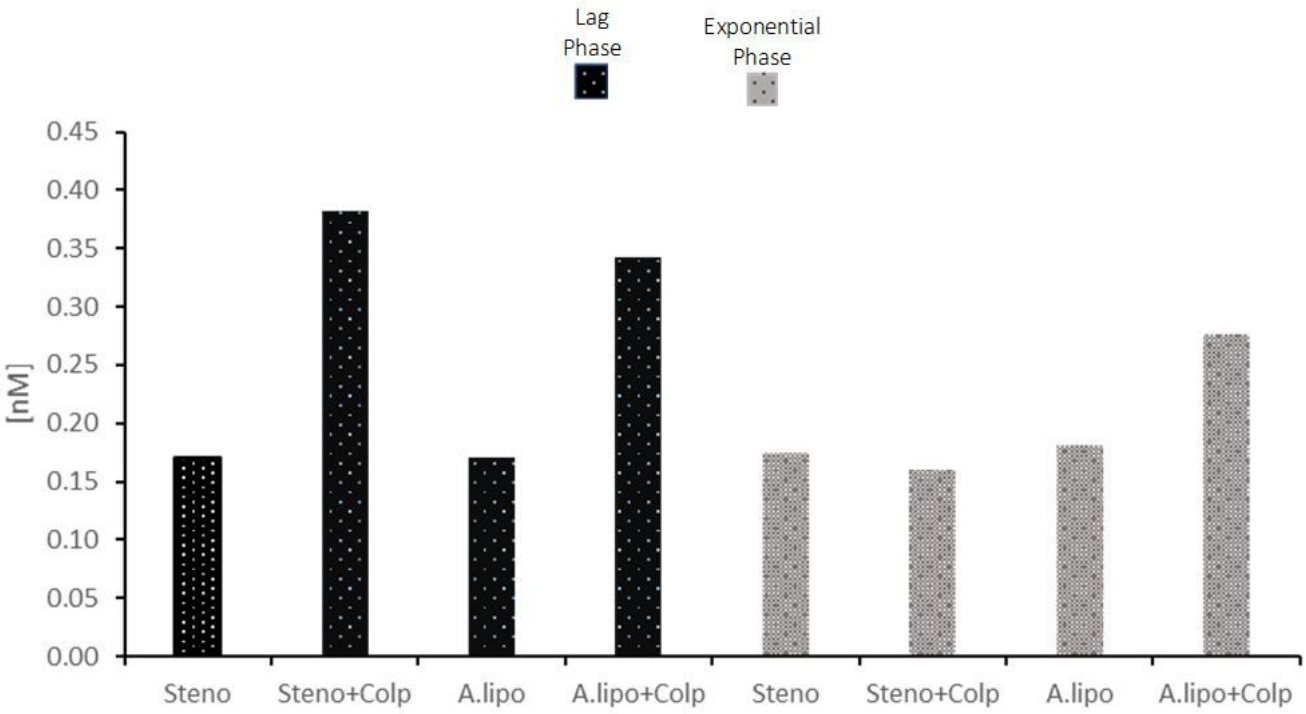

Figure 5

Nitrogenase activity. The abundance of bacteria in CFU / mL during lag (dark bars) and exponential phase (grey bars). The dark bars indicate the fixation rate during the Lag phase and the light bars during the phase of the exponential phase. The presence of the predator did not generate a significant difference in ethylene production ($p = 0.05$).