

EFFECT OF NITROGEN DEFICIENCY IN THE BIODEGRADATION OF ALIPHATIC AND AROMATIC HYDROCARBONS IN PATAGONIAN CONTAMINATED SOIL

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ABSTRACT

The use of biological method to remediate soil with hydrocarbons as contaminants is possible and nitrogen is important for the process. Incorrect quantities of nitrogen in the soil result in a less efficient remediation process. The main object of this work was the study of the nitrogen effect in aliphatic and aromatic hydrocarbon degradation during 110 days. Five microcosms were designed, the treatments used were natural attenuation, aromatic hydrocarbon mineralization (both microcosms fertilized and unfertilized with nitrogen) and soil systems with diesel oil (both microcosm fertilized and unfertilized with nitrogen). At the beginning and end of the experiment, the hydrocarbons were determined by Soxhlet; nitrate, nitrite and ammonium were analyzed and CO₂ was measured every week. The microbial count was carried out in media with and without nitrogen. The main results show that the presence of nitrogen in the soil favors the aliphatic hydrocarbon degradation and that the nitrogen deficiency favors the aromatic hydrocarbon degradation. In soil with nitrogen deficiency, the aromatic compound produced less nitrogen fixation and nitrification. The nitrogen deficiency produced a decrease in the mineralization, hydrocarbon elimination and biomass. However, remediation is possible because the nitrogen could be fixed due to the presence of nitrogen fixing microorganisms, which can fix the necessary nitrogen for the hydrocarbon remediation.

Keywords: *biodegradation, nitrogen deficit, soil, Patagonia.*

1. INTRODUCTION

Biological methods like natural attenuation, biostimulation, bioaugmentation and bioremediation are efficient and adequate methods to clean up soil with petroleum hydrocarbons as contaminants [1, 2, 3] because those methods do not adversely affect the site. The bioremediation is an economical method compared to the incineration and washing of the soil [3]. The classic methods are biostimulation and bioaugmentation. Both could be used *in situ* and *ex-situ* [3]. To achieve the optimum biodegradation condition, it is important to know the characteristics of the contaminated site before beginning the treatments. Basic information such as residual oil concentration, population density of hydrocarbon degrading microorganisms and the biodegradation potential are key factors to be considered for bioremediation [4, 5].

There are many factors that limit natural attenuation; in Patagonia, one of the most important ones is nitrogen concentration due to its poor concentration in the soil [6]. Nitrogen is essential to microorganism metabolism and it is necessary to biosynthesis of amino acids, protein and nucleic acids [7, 8, 9]. The soil rich in nitrogen has a good metabolic activity and good microbial biomass. Therefore, its presence in the soil for a good bioremediation process is necessary. However, some authors claim that biodegradation in negligible amounts of nitrogen is possible, but the efficiency is lower [10, 11, 12]. The literature quotes different bacterial genera, which have the capacity to degrade hydrocarbons in poor concentration of nitrogen [11]. The genera *Pseudomonas* [10, 13], *Agrobacterium* [14], *Alcaligenes* [14], *Arthrobacter* [15], *Azotobacter* [16] have the capacity to fix nitrogen in soil with deficiency of nitrogen.

The effects of biostimulation on petroleum hydrocarbon degradation have been investigated in different conditions [5, 17]. However, the effects of nitrogen affect the biodegradation of hydrocarbon classes, i.e. aliphatic, aromatic and polar hydrocarbons have not been completely studied in the Patagonian soil, which has a poor concentration of nitrogen and it is an important oil production area.

In this work, the effects of presence and absence of nitrogen on the biodegradation of aliphatic and aromatic hydrocarbons were evaluated in a study conducted in microcosms during a 110 day period.

2. MATERIALS AND METHODS

2.1. Soil samples.

The soil samples were collected from a landfarming area belonging to a petroleum refinery in the Patagonia Region. The samples were taken at depths between 10 and 30 cm. The soil was characterized according to standard method of soil analysis, having the following characteristics: moisture 7.0%; pH 7.0; organic matter 9.9%; total nitrogen 11%; porosity 45%; water retention capacity 38%; Ca^{+2} 260.3 ± 15.6 ppm; Mg^{+2} 136.5 ± 9.8 ppm; CO_3^{-2} 0 ppm; HCO_3^{-} 575.1 ± 30.5 ppm; SO_4^{-2} 996.7 ± 59.6 ppm; Cl^- 898.5 ± 49.3 ppm; S^{-2} 1.1 ± 0.2 ppm; ammonium 0.41 ± 0.01 ppm; NO_2^- 0.92 ± 0.06 ppm; NO_3^- 2.23 ± 0.08 ppm; PO_4^{-3} 5.42 ± 0.41 ppm; urea 0.61 ± 0.02 ppm; total petroleum hydrocarbons 5.8%: 31.8% $\pm 1.91\%$ aliphatic hydrocarbon, 28.7 $\pm 2.06\%$ aromatic hydrocarbons and 39.5 $\pm 2.48\%$ polar hydrocarbon; total aerobic bacteria $3.80 \pm 0.57 \times 10^6$ CFU g^{-1} ; nitrogen fixing bacteria $0.45 \pm 0.01 \times 10^6$ CFU g^{-1} ; hydrocarbon degrading bacteria $0.27 \pm 0.01 \times 10^6$ CFU g^{-1} and hydrocarbon degrading bacteria in absence of nitrogen $0.37 \pm 0.12 \times 10^6$ CFU g^{-1} .

2.2. Chemistry analysis.

Hydrocarbon concentration was determined by Soxhlet extractor using trichloroethane as the extraction solvent. The extracted hydrocarbons were quantified on a mass difference basis [18]. The extracted hydrocarbons were separated into class fractions by silica gel column chromatography. Aliphatic, aromatic and asphaltic oil fractions were eluted with 250 mL of hexane, 150 mL of benzene and 150 mL of chloroform-methanol 1:1 respectively [6].

Twenty grams of soil were placed into Erlenmeyer Flasks containing 50 mL of distilled water. Suspensions were homogenized and centrifuged for 20 min at 3500 rpm. Nutrients (nitrate, phosphate, nitrite and ammonia) and pH were determined [19] in the supernatant solution.

2.3. Bacterial count.

The numbers of the bacteria were examined using the spreading plate method on four different media. Heterotrophic bacteria were estimated on R2A-agar [20], diazotrophic bacteria on DM-agar [6], Hydrocarbon degrading bacteria (HDB) on MM PGO-agar [18] and diazotrophic hydrocarbon degrading bacteria (DHDB) were estimated on MM PGO medium without nitrogen compounds (MM2 PGO).

The results are expressed in CFU. g^{-1} and we used two ratio: one is DHDB/HDB, this ratio indicated the quantitative number of hydrocarbon degrading microorganisms in a nitrogen deficiency medium versus the same quantitative number of hydrocarbon degrading microorganisms in a medium with nitrogen; the other ratio is DM/TAB, and this ratio indicated the quantitative number of nitrogen fixing microorganisms versus the same quantitative number of total aerobic bacteria.

2.4. Soil biodegradation experiments.

Laboratory microcosms were used for the incubation experiment. The sample was separated into 100 g (dry weight) portions, and each portion was transferred to a sterile 1,000 mL brown bottle. Triplicate microcosms were prepared for each treatment (Table 1).

Table 1 Composition of microcosms. The benzoate solution used was 0.5M.

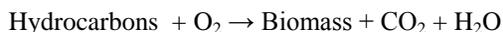
Microcosm	Soil ^a	(NH ₄) ₂ SO ₄ ^a	K ₂ HPO ₃ ^a	H ₂ O ^b	Benzoate ^b	Diesel oil ^b	C:N:P
NA	100	-	-	10	-	-	100:0.003:0.003
AL1	100	0.5	0.06	10	-	1	100:2:0.22
AL2	100	-	0.06	10	-	1	100:0.003:0.22
AR1	100	0.5	0.06	10	1	-	100:2:0.22
AR2	100	-	0.06	10	1	-	100:0.003:0.22

^a Expressed in grams. ^b Expressed in mL. S/N₂: without nitrogen, C/N₂: with nitrogen.

Ammonium was added in the form of (NH₄)₂SO₄, and phosphate was added as KH₂PO₄ to prevent any limitation of activity caused by nutrient imbalance. The microcosms were incubated for 110 days at 28°C in the dark and they were weekly aerated to maintain aerobic conditions. Total mineralized carbon contents were measured twice per week by titrating the CO₂ trapped during incubation in NaOH [21]. At the beginning and end of each experiment, the content of hydrocarbons was determined by Soxhlet extractor and fractionated by silica gel column chromatography, the bacterial counts were in R2A, DM, MM PGO and MM2 PGO medium. Nitrate, nitrite and ammonium were analyzed every week. On day 33 ammonium salt was added.

The treatments were NA (intrinsic or natural attenuation), 2 systems with soil having 0.1 % of diesel oil acting as inductor for aliphatic metabolic ways, fertilized with nitrogen (AL1) and unfertilized (AL2), and 2 systems with soil having 0.1% of benzoate acting as inductor for aromatic metabolic way [22], fertilized (AR1) and unfertilized (AR2) with nitrogen.

The CO₂ data and total petroleum hydrocarbons were utilized to calculate the theoretical biomass. For the calculation, we used the approximate composition of oil extracted in San Jorge Gulf region with 85% of carbon [23] and the approximate proportion of microorganism biomass with 46.5% of carbon and 10.85% of nitrogen [24].



2.5. Statistical analysis.

Results were analyzed using ANOVA with BIOM program (Applied Biostatistics Inc., 11711 USA). The results are shown in a graphical form and the tables include the average value of triplicates with their standard deviations.

3. RESULTS

3.1. Biodegradation of hydrocarbons.

The values of mineralization in AL1 and AL2 microcosms showed higher values than the values of mineralization in aromatic hydrocarbons microcosm's (Table 2). Consequently, there was a significant difference ($p < 0.05$) between both aliphatic hydrocarbon microcosms. On the other hand, there was no difference between aromatic microcosms. At the end of the experiment, the low nitrogen concentration in soil produced a decrease in the mineralization rate in about 25.8% and 7.4% in microcosms AL and AR respectively (Table 2). The measure of TPH and hydrocarbon fraction biodegradation percentage was less in unfertilized microcosm than in fertilized microcosms. And the theoretical ratio ARO/ALI increased in nitrogen absence.

3.2. Nutrients.

Nitrate ions increased significantly ($p < 0.05$) in relation to the contact time in fertilized microcosms, whereas ammonium decreased (Fig. 1). This increase could have been produced by the nitrogen added.

Nitrate ions increased significantly in microcosms AL2 and AR2 (Fig. 1.B). The concentration of nitrite ions was either constant or had a slight variation during the 110 days of experiments, in all microcosms. The ammonium ions concentration did not change in the soil when nitrogen was not added (Fig. 1.A). However, when nitrogen was added to microcosms, independent of the type of contamination, the ammonium concentration decreased during the first 10 days and, consequently, the concentration further decreased on day 33 after nitrogen was added again.

Microcosm AL1 had an increase of approximately $18.1 \pm 2.3 \text{ mg.kg}^{-1}$ in nitrate ions, whereas microcosm AR1 increased to $16.7 \pm 0.9 \text{ mg.kg}^{-1}$. The contaminants present in both microcosms affected the nitrifying bacteria in a similar way. Nitrate ions concentration in microcosms without nitrogen increased in a different way: in those microcosms presenting benzoate, the nitrate ions concentration increased to $10.7 \pm 1.4 \text{ mg.kg}^{-1}$, whereas in microcosms with diesel oil, the nitrate ions increased to $19.2 \pm 2.3 \text{ mg.kg}^{-1}$. These showed that aromatic hydrocarbons produced an inhibition of soil-nitrifying microorganisms.

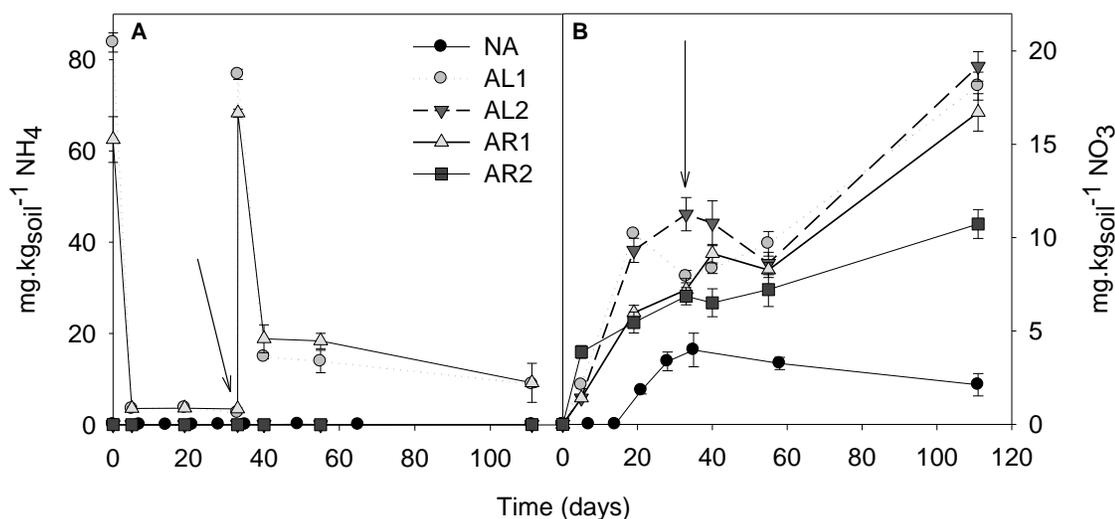
Although there was an increase of total nitrogen in all microcosms, fertilized microcosms showed a better capacity to incorporate this nutrient. Whereas the values of nitrogen fixation were $1242 \pm 137 \text{ mg.kg}^{-1}$ and $1197 \pm 195 \text{ mg.kg}^{-1}$ in AL1 and AR1 respectively, in unfertilized microcosms, the nitrogen fixation was $600 \pm 24 \text{ mg.kg}^{-1}$ in AL2 and $432 \pm 15 \text{ mg.kg}^{-1}$ in AR2.

Fertilized microcosms that are rich in aromatic hydrocarbons, i.e. AR microcosms, produced a $3.62 \pm 0.1 \%$ nitrogen fixation reduction and a $7.73 \pm 0.9\%$ increase in nitrate ions; this is a high value compared to microcosms rich in aliphatic hydrocarbons.

Unfertilized microcosms showed a $28 \pm 4.6 \%$ reduction in nitrogen fixation and a $44.3 \pm 6.5 \%$ increment in nitrate ions.

Table 2 Biodegradation percentage, bacteria count, ratio count and biomass.

	NA	AL1	AL2	AR1	AR2
Mineralization rate (mgCO₂.kg⁻¹.day⁻¹)	30.1	117.5	87.2	90.5	83.8
% Biodegradation of hydrocarbons					
Total	8.9	54.1	32.7	39.5	16.4
Aliphatic	13.3	89.5	47.2	57.1	19.4
Aromatic	12.3	58.2	43.9	67.1	26.4
Polar	2.5	14.5	9.3	5.3	4.1
Microorganisms (CFU.g⁻¹.10⁶)					
TAB	6	650	210	160	99
DM	2	450	160	89	71
HDB	0.6	120	38	100	17
DHDB	0.8	77	90	57	62
Ratios					
Aro/Ali	0.84	0.48	0.70	1.09	1.22
DM/TAB	0.33	0.69	0.76	0.56	0.72
DHDB/HDH	1.33	0.65	2.35	0.56	3.60
Biomass (mg%)	241	4748	2516	2722	408
Nt (mg%)	102	454	170	449	153
N-Biomass (mg%)	24	475	251	272	41

**Figure 1.** Monitoring of nutrients in the microcosms. The arrow indicates the addition of nitrogen on day 33 in microcosms AL.

3.3. Microorganisms.

The total bacteria counts in all microcosms showed an increment in their number of cultivable bacterial population after 110 days (Table 2).

The absence of nitrogen in polluted soil produced greater values on ratio DM/TAB and DHDB/HDB (Table 2). The highest values were found in the aromatic microcosms.

Unfertilized soil had a decrease in the theoretical biomass of about 42% and 67% on microcosms AL and AR respectively.

On the other hand, in NA, AR2 and AR1 the quantity of nitrogen found in the biomass was 67 ± 8 ; $85 \pm 12.2\%$ and $88 \pm 5.8\%$ of the total nitrogen respectively. In microcosms AL2 and AL1, the calculated nitrogen value was higher in the biomass than the calculated nitrogen value originally found in the soil.

In AR1 and AR2 the nitrogen fixed were 120 and 43 mg respectively. In AR1, the nitrogen included in the biomass was higher than the total nitrogen in 105 mg. It is believed that the fixed nitrogen could have been used by the organisms to produce biomass. The nitrogen fixation in these types of systems is possible when the soil has a deficiency in nitrogen. This situation occurs before adding fertilizer. In AR2, the difference between total nitrogen and calculated biomass was the same as the value of fixed nitrogen. This can be due to the same reasons that produce natural attenuation, for example, the fact that the nutrient is not bioavailable because it is forming an organic complex, like oil, and the microorganisms take the nitrogen and fix it to the system.

AL1 and AL2 fixed 124 and 60 mg of nitrogen respectively. The nitrogen included in the biomass was higher than the total nitrogen. The difference between total nitrogen and calculated biomass was 100 mg and 150 mg in AL1 and AL2 respectively. The atmospheric nitrogen fixation is a process that needs a lot of energy. The nitrogen fixation is used to form biomass and the energy that the process needs is taken from the carbon compound oxidation [25]. This may mean that microorganisms could utilize energy by mineralization to support the metabolic activity and not to form biomass [12], this was greatly marked in soil with nitrogen deficiency.

4. DISCUSSION

4.1. Hydrocarbon elimination.

The recommended optimal ratio C:N is in the order of 100:10 to 100:1 [26]. Our results agree with these findings. Chaîneau et al. in 2005 [27], who experimented with soil contaminated with hydrocarbon, most of which were aliphatic hydrocarbons, found an inhibitory effect of high nutrient concentration on hydrocarbon degradation and also found that the deficiency of nitrogen concentration could eliminate 47% of aliphatic hydrocarbons; this ratio was less than C:N 100:13, which showed 62% of degradation. Our results showed by the aliphatic hydrocarbon microcosms were similar to the results obtained by Chaîneau et al. in 2005 [27]. The biodegradation was about $54.1 \pm 7.1\%$ and $32.7 \pm 4.6\%$ in AL1 and AL2 respectively. In both studies, the bioremediation of the total amount of hydrocarbons produced interesting values when there was a deficiency of nitrogen in soil. On the other hand, the results in AR1 and AR2 were similar. In accordance with Pucci and Pucci [18], those aromatic hydrocarbons are more difficult to be degraded by microorganisms than aliphatic hydrocarbons.

The hydrocarbon elimination percentage in AL1 and AL2 is compared in Table 2. The nitrogen deficiency produced a decrease of $21.4 \pm 5.1\%$ in this parameter. Toccalino et al. in 1993 [28] demonstrated how the nitrogen is the only limiting factor in the biodegradation process of butane and propane; they also observed that when the nitrogen concentration is low, the biodegradation of these hydrocarbons is low as well.

The values of hydrocarbon elimination were better when aromatic compounds were present in the medium; this was evidenced by AR2, where the elimination of hydrocarbon was $23.1 \pm 1.7\%$ higher than the elimination in AR1. The aromatic compound greatly inhibited hydrocarbon degradation in soil with nitrogen deficiency. The stress produced by nitrogen deficiency was greater in presence of aromatic compound in the soil than in the presence of aliphatic compound. Many authors studied the toxic effect of aromatic hydrocarbons on cellular membrane [29] and the problems of the nitrogen deficiency in the biodegradation process [30]. Both effects could produce an inhibition in hydrocarbon elimination.

When the contamination was rich in aromatic compounds, the biodegradation of the aromatic group in the oil mixture was stimulated. The ratio C:N of 100:2 favors the removal of aliphatic compounds and the ratio C:N 100:0.003 favors the removal of aromatic compounds. The aliphatic fraction present in soil was more sensitive to nitrogen deficiency than the other soil conditions; in accordance with Chaîneau et al. [27]. This increase of biodegradation of aromatic compounds could be explained by the enzymatic competition given between the aromatic compound, the ammonia monooxygenase and nitrite oxidoreductase; as a result, the bacteria cannot be used for nitrification.

The CO₂ production showed how nitrogen was important in the biodegradation process. Bento et al. in 2005 [5] worked with different microcosms, such as natural attenuation, biostimulation and bioaugmentation. They concluded that the biostimulation produced a higher number of microorganisms and that their metabolic activity was improved, thus producing a better result in the biodegradation of hydrocarbons. The nitrogen could work as a stimulant in the

use of this compound [30, 31, 32]. In spite of the fact that nitrogen is in low concentration, some microorganisms can use it.

4.2. Microorganisms.

Deni and Penninckx in 1999 [33] observed the presence of bacteria in hydrocarbon contaminated soil, which had a capacity to oxidize ammonium and nitrite. They concluded that soil with a large history of contamination was poor in nitrogen and that nitrogen deficiency selected bacterial nitrifying communities with a high affinity to ammonium. We can attribute the increase of nitrate, in AL2 and AR2, to the presence of this microorganism, or to the presence of nitrogen fixing organisms, which can develop their metabolism in presence of a high concentration of aliphatic more than in the presence of aromatic compounds. These microorganisms used hydrocarbons to the expenses of atmospheric nitrogen fixation [12], and they are also beneficial to microorganisms, which are nitrifying, adapted and produce an increase of nitrates in the soil. Due to this result, we suppose that the ammonium is necessary to nitrify in AL2 and AR2 and this ammonium can be obtained by fixation. The result showed a good count in free nitrogen media. Eckford et al. in 2002 [10] isolated 5 strains of hydrocarbon contaminated soil, which could fix nitrogen. Two of them were *Pseudomonas*. In our work, hydrocarbons in nitrogen deficient soil produced an enrichment of diazotrophic bacteria, which can use oil derivatives.

The chemical determination of total nitrogen and nitrate indicated that the chemical composition of hydrocarbons has a different pressure over bacterial communities. Aromatic compounds in presence of nitrogen do not show a significant difference ($p > 0.05$) between nitrogen fixation and the nitrification. But both chemical determinations in the soil with nitrogen deficiency and aromatic compounds as contaminant had a significant decrease ($p < 0.05$) on the biodegradation. Fuller and Scow in 1996 [34] demonstrated how toluene produced an important inhibition in the ammonium oxidation due to a competition in active occupation sites in ammonium monooxidase enzyme, which is responsible for the process. In microcosms fertilized with nitrogen, this process did not occur; however in unfertilized microcosms we could observe a decrease in nitrification, which can be the consequence of a competitive process or hydrocarbon degrading microorganisms, which use fixed nitrogen as ammonium to introduce it in the cell and this produces an inhibition of the nitrifying genes [33]. The mineralization rate and the decrease of hydrocarbons in unfertilized microcosms were less than the same parameters in fertilized microcosms. The increase of total nitrogen occurred due to the presence of microorganisms, which were able to biodegrade this organic compound.

The aliphatic microcosms, AL1 and AL2, have a higher biodegradation percentage than aromatic microcosms, however the microbial count was able to grow in both, in presence and absence of nitrogen. The use of hydrocarbons has a significant difference ($p < 0.05$). The aromatic compound, unlike aliphatic compounds, produces a greater decrease of biomass in nitrogen deficiency.

In bacterial ecology studies, the environmental conditions are responsible for the selection of microorganisms [35, 36]. The bacterial count in unfertilized microcosms presented a higher count in nitrogen free media and the microorganism count in the fertilized microcosms was higher in normal media. Soil microcosms have hydrocarbon degrading microorganisms, which were more affected by the presence of aromatic compounds than by the presence of aliphatic compound. When the nitrogen was not bioavailable, the microorganisms could use hydrocarbons in slow velocity.

The natural attenuation of soil fixed 10 mg of nitrogen. The total ultimate nitrogen was 120 mg, therefore, 70 mg of them were biomass. As a result, the nitrogen was not bioavailable to microorganisms or it was less fixed because the requirement of microorganism was supported by soil.

The main results show that the presence of nitrogen in the soil favors the aliphatic hydrocarbon degradation and that the nitrogen deficiency favors the aromatic hydrocarbon degradation. In soil with nitrogen deficiency, the aromatic compound produced a higher decrease in nitrogen fixation and nitrification compared to soil with presence of nitrogen. The nitrogen deficiency produced a decrease in the mineralization, hydrocarbon elimination and biomass. However, remediation is possible because the nitrogen could be fixed due to the presence of nitrogen fixing microorganisms, which can fix the necessary nitrogen for the hydrocarbon remediation.

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