Effect of Oxygen on Bacterial Denitrification (Aerobic Denitrification)

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The polyfactorial effects of environmental conditions on the quantity and quality of denitrification are still incompletely understood. Generally, the rate of denitrification enhances with increasing temperature, amount of easily decomposable organic matter, nitrate concentration and pH, whereas aeration is believed to decrease nitrate respiration /SMITH and TIEDJE, 1979; KNOWLES, 1982/. Since oxygen and nitrate are regarded as alternative electron acceptors for aerobic bacteria, the absence of oxygen is considered as a prerequisite to initiate denitrification processes. In soils and sediments denitrification is thought to occur mainly at anoxic sites /"hot spots"/ with restricted oxygen diffusion and high respiration rates due to a relatively high supply of easily decomposable organic matter. At present, the determining effect of the amount of easily mineralizable organic matter for the onset of denitrification has been well recognized /EL DEMERDASH and OTTOW, 1983; ABOU SEADA and OTTOW, 1985; OTTOW et al., 1985/. With respect to the nitrate concentration, denitrification follows zero order kinetics, just because the demand for electron acceptors is determined by the rate of mineralization /OTTOW et al., 1985/. The question remains: to what extent is denitrification affected at high rates of mineralization by the presence of both oxygen and a high amount of nitrate in the system. "Aerobic denitrification" had been claimed repeatedly /ROBERTSEN and KUENEN, 1984/ but the ecological prerequisites and conditions of simultaneous respiration and denitrification have never been characterized so far. In the present study the effect of aeration on denitrification under controlled conditions is reported.

Materials and methods

About 500 ml of a separately sterilized /15 min at 120 °C/ synthetic glycerol-mineral salt stock solution /2 l, pH = 7.4, with or without 1 % KNO_3/ were pumped into the sterile reaction vessel /Fig. 1/. Different electrodes for pH, redox potential /Eh/, pO_2, and nitrate were inserted airtight and the whole set-up was sterilized with 0.5 ml ethylene oxide /Merck/ for 2 h. Helium /He/ gas was flushed through the continuously stirred broth until it was completely free of ethylene oxide /gas chromatography/. All parts of the

set up were connected with special air-tight rubber tubes, and anoxic conditions in the vessel were ascertained after several days by gas chromatography before inoculating. Experiments were run anaerobically /He gas/ as well as aerobically /He/O_2 mixture with 5 ml oxygen/min/. Aeration at 5 ml O_2/min corresponds to O_2-saturated conditions with 25-26 mg O_2/l. Each vessel was stabilized for at least 48 h /30 °C/ after which it was inoculated by introducing 5 ml of a 4-h culture together with the remainder of the stock solution /hand pumping/. The broth was homogenized continuously /magnetic stirrer/, kept at 30 °C and covered entirely with aluminium foil. Samples were collected /Fig. 1/ at regular intervals and analyzed for nitrite, nitrate glycerol and populations of denitrifying bacteria using the MPN-technique and a synthetic KNO_3-glycerol-broth /FABIG and OTTOW, 1976/.

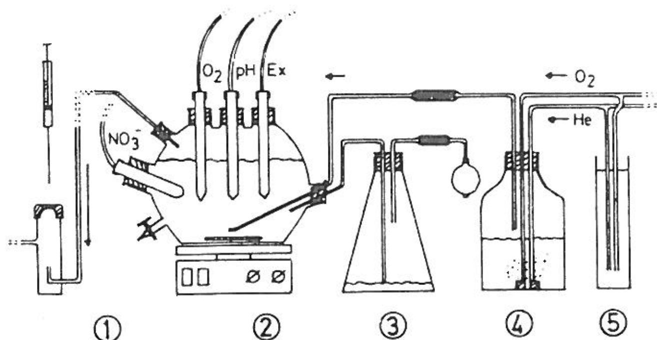

Fig. 1
Experimental set-up to examine the effect of continuous aeration /O_2/ on denitrification and redox level /pH, Eh/ in a synthetic KNO_3-glycerol mineral salt broth /pH 7/ after inoculation with either Acinetobacter sp. 53b or Moraxella sp. 13b. /For the isolation and identification of the test organisms see: FABIG and OTTOW /1976//

Acinetobacter sp. 53b and Moraxella sp. 13b were isolated from columnar denitrification and identified as reported by FABIG and OTTOW /1976/. The strains were subcultured anaerobically in a synthetic mineral salt medium /FABIG and OTTOW, 1976/ containing glycerol /0.3 %/ and KNO_3 /1.0 % = 1400 µg NO_3^--N/ml/. Five-ml samples of a young /48 h, 30 °C/, actively denitrifying culture /ca. 10^8 cells/ml/ were used to inoculate the experimental vessel.

pH and Ex were measured with a glass silver chloride-Pt-electrode /Ingold Type 405/ using a WTW Digi 510 as a recording instrument. The electrodes were adjusted with pH and redox buffer solutions /Ingold 9805, 9807 and 9881/. The Eh was calculated from Ex by the addition of 207 mV /30 °C/ and used to determine the rH values /= negative logarithm of the partial pressure of gaseous hydrogen/ according to the formula

$$rH = Eh\ (mV)/29 + 2pH\ (30\ ^{o}C) \qquad /1/$$

At rH = 42.6 completely oxidized conditions exist; at rH = 0 entirely reduced conditions are established /JAKOB, 1970/. Redox equilibrium /pO_2 = pH_2/ is obtained at rH = 27.5. Reduced conditions prevail at rH < 17. The rH allows the comparison of the redox level in different systems at any time /OTTOW

and GLATHE, 1973/. pO_2 was monitored with a WTW-Oxygen Electrode EO 16 and nitrate using an Orion Electrode model 92-67. All data were continuously monitored and collected with a Philips PM 8235 Recorder.

Samples were withdrawn /Fig. 1/ and analyzed for nitric oxide /NO/, nitrous oxide /N_2O/, dinitrogen /N_2/, CO_2, and O_2 using a Perkin-Elmer gas chromatograph F 22 fitted with a hot wire and operating with He gas as a carrier. Porapak Q and R series were used to separate NO, N_2O and CO_2, while a molecular siever MS 5 Å was used to differentiate N_2 and O_2 /MORETTI et al., 1974/. Results were collected with a servogor S /Metrawatt/ recorder and recorded with a SIP 1 Perkin-Elmer Integrator /FABIG et al., 1978/.

Results

In Fig. 2A the development of the various parameters during the growth of Acinetobacter sp. 53b under complete anaerobic conditions /He gas/ are given. After a lag phase /24 h/ the population increases while glycerol and nitrate decrease. Nitrite accumulates, but decreases slowly as soon as nitrate has been exhausted. Nitrate seems to be the growth-limiting factor. Population development is reflected by CO_2-evolution as well as N_2 and N_2O-production. Both carbon dioxide and denitrification practically cease after 120 h. As soon as metabolism accelerates, the redox level /rH/ drops rapidly and remains at about rH = 18 as long as glycerol and nitrate are available. Gaseous losses of N_2O and N_2 were most intensive at active growth and minimum redox level.

In Fig. 2B the same parameters are presented at continuous aeration /5 ml O_2/min/. After a prolonged lag phase /48 h/ the population of Acinetobacter sp. 53b increases rapidly while glycerol utilization proceeds /up to 168 h/. During the lag phase the pO_2 decreases only slightly, but drops to a complete anaerobic situation within 24 h, as soon as the population enters the lag-growth phase. The redox level is lowered steeply and poises at rH = 13-15 as long as glycerol remains available. Apparently, the redox level of an actively metabolizing Acinetobacter sp. 53b population drops much lower with O_2 than with nitrate as the only electron acceptor.

Fig. 2C represents the changes in the various parameters of the Acinetobacter culture in the presence of both oxygen and nitrate. The following features are obvious. First, glycerol utilization, nitrate dissimilation and respiration are distinctly more intensive than with either nitrate /Fig. 2A/ or oxygen /Fig. 2B/ alone. Secondly, the growth of Acinetobacter sp. 53b as well as CO_2-production is more intensive in the presence of both electron acceptors than with nitrate or O_2. Thirdly, pO_2 and rH level drop considerably if compared to nitrate or O_2 alone. The minimum rH of 14 poises at this redox level only very briefly. During this most active phase of metabolism, both respiration /pO_2 = 0/ and denitrification /$N_2O + N_2$/ occur simultaneously, indicating that both nitrate and molecular oxygen are used at the same time.

In Fig. 3A the behavior of the same parameters in the culture of Moraxella sp. 13b is given. Although the same conditions exist, the metabolism of Moraxella sp. 13b is apparently much less vigorous than that of Acinetobacter sp. 53b. Glycerol is exhausted only after 192 days, and this lower rate of mineralization is clearly reflected by delayed population development and nitrate respiration. Again, denitrification becomes most active as soon as the redox level poises at about rH = 14. This level is significantly lower than that of Acinetobacter /Fig. 2A/.

Fig 3B shows glycerol mineralization and redox changes during respiration of Moraxella sp. 13b at an aeration rate of 5 ml O_2/min. As with

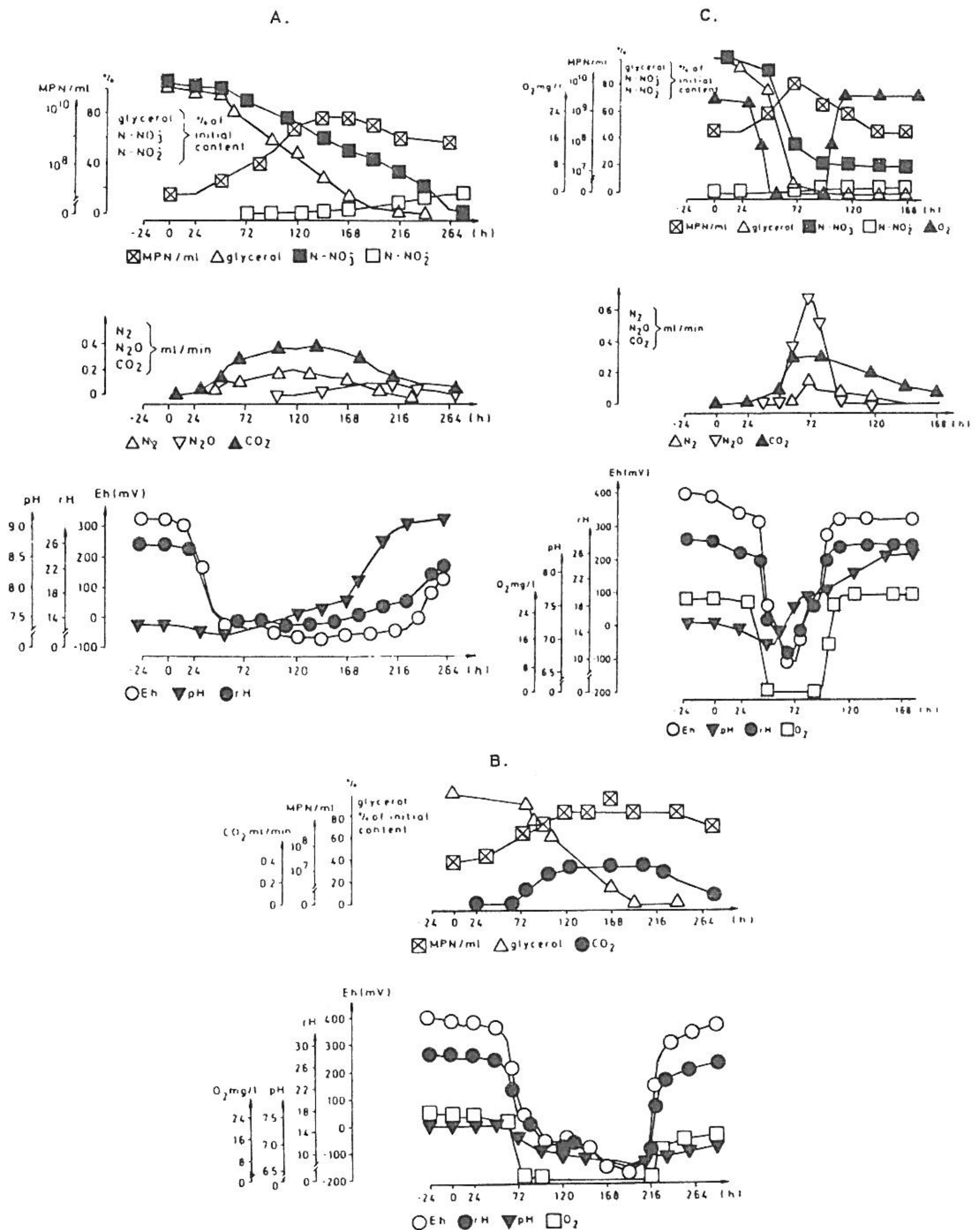

Fig. 2

Development of Acinetobacter sp. 53b population, glycerol mineralization, carbon dioxide evolution, denitrification /H_2, N_2O/ and ecological conditions /pH, Eh, rH/. A.: at anaerobic conditions /He-atmosphere, batch culture, 30 °C/; B.: at continuous aeration /5 ml O_2/min, batch culture, 30 °C/; C.: in the presence of nitrate and O_2 /5 ml/min, batch culture, 30 °C/

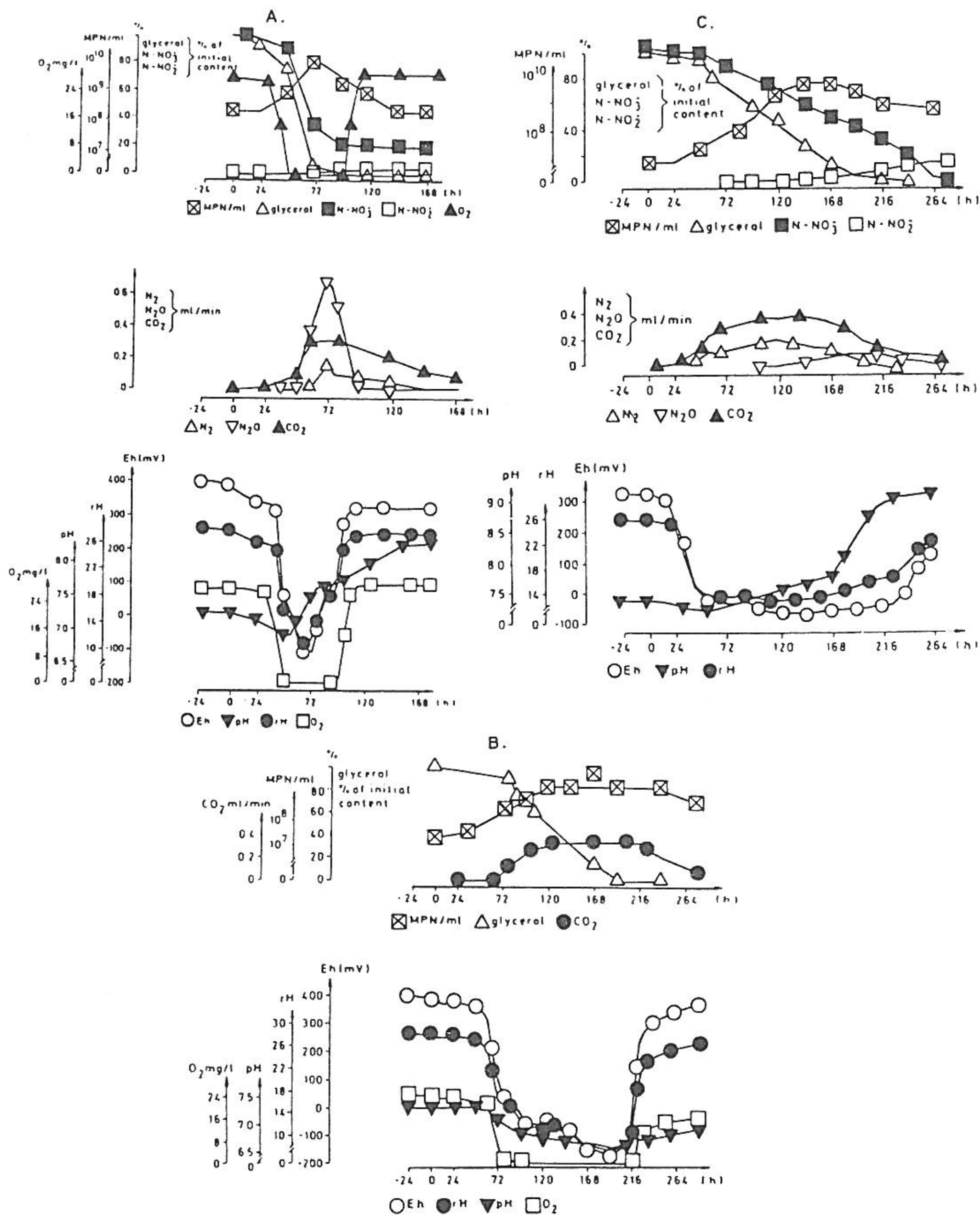

Fig. 3

Development of Moraxella sp. 13b population, glycerol mineralization, carbon dioxide evolution, denitrification /N_2, N_2O/ and ecological conditions /pH, Eh, rH/: A. at anaerobic conditions /He-atmosphere, batch culture, 30 °C/; B. at continuous aeration /5 ml O_2/min batch culture, 30 °C/; C. in the presence of nitrate and O_2 /5 ml/min, batch culture, 30 °C/

Acinetobacter sp. 53b, anaerobic conditions /pO_2 = 0/ are established rapidly and remain until glycerol has been exhausted. The rH level drops and stabilizes at approximately 14 to 10 which is again significantly lower than with nitrate as the only electron acceptor /Fig. 3A/.

In Fig. 3C the changes in the various parameters in the presence of both molecular oxygen and nitrate are presented. The results obtained with Moraxella sp. 13b are essentially the same as those observed with Acinetobacter sp. 53b /Fig. 2B/. Again, metabolism is much more intensive in the presence of both electron acceptors with either oxygen or nitrate. Since glycerol is utilized more rapidly, the redox level poises only briefly at rH = 12 which is in between the redox conditions reached with nitrate /Fig. 3A/ and oxygen /Fig. 3B/ alone. Maximum denitrification /N_2O + N_2-production/ is most active as soon as the rH minimum has been established.

From the results obtained, two significant conclusions may be drawn.

First, there is no doubt that oxygen and nitrate can be respired simultaneously by an actively metabolizing population of bacteria such as Acinetobacter sp. and Moraxella sp. This synchronous activity of respiration and denitrification in the same batch culture may be explained by the presence of both respiring and denitrifying bacteria within the same population. This is quite likely, the more so since molecular oxygen could not be detected /pO_2 = 0/ during the most active phase of metabolism. Consumption of the continuously introduced molecular oxygen must have been fast enough to warrant the induction of denitrification within a part of the flora. On the other hand, energy conservation by respiration and denitrification in the same organism cannot be excluded, since these processes may have occurred in different compartments /inner cell membranes/ of one cell. From an ecological viewpoint it is important to stress that respiration and denitrification may, indeed, occur simultaneously even at a relatively high rate of oxygen, if a sufficient amount of easily decomposable organic matter is available. Among the ecological factors that determine denitrification in natural systems, the amount of available organic matter rather than the oxygen diffusion rate /caused by water saturation or small pore size and volume/ should be considered as the triggering factor /OTTOW et al., 1985/. The higher the demand for electron acceptors, the greater the chances are for denitrification even under aerobic conditions.

Secondly, it seems as that respiration will lower the redox level of a system much more than denitrification. This is contrary to the sequence predicted by the half reactions involved /MUNCH and OTTOW, 1983/. In natural mixed systems, such as soils and sediments, the redox conditions /pH-Eh-dependent/ will poise at the level required to activate the electron acceptor in question /OTTOW and MUNCH, 1978; MUNCH and OTTOW, 1982; OTTOW, 1982/. Stabilization at a certain level will continue until all oxidants concerned have been transformed into the reduced state. However, if the rate of electron acceptor availability becomes lower than the rate at which electrons are provided by mineralization, the redox level may drop beyond the expected level. This is particularly true if only one electron acceptor is available in the system at a restricted rate. At an aeration rate of 5 mg O_2/min, the demand for O_2 by either Acinetobacter sp. or Moraxella sp. apparently exceeded the supply /pO_2 = 0/. This was opposite to nitrate which remained in excess even after glycerol had been utilized. The lack of O_2 during aeration was confirmed indirectly by the rise in rH level when nitrate was added. In general, redox potentials provide little diagnostic information on the type and intensity of a process as long as detailed

information is lacking on the type and amount of available organic matter, on the composition of the redox systems, and on the ecological conditions /moisture tension, temperature, etc./. In a given situation, the measured Eh depends on the rate of organic matter turnover /mineralization/, the activation energy barrier of the redox system involved, and the proton- and electron-buffering capacity of the whole system, as well as on the oxygen diffusion rate /OTTOW and GLATHE, 1973; OTTOW, 1982/. Consequently, the redox level does not provide reliable information on the type of reductive processes that occurred in the past nor at the moment of measuring /OTTOW, 1982/. Moreover, most relevant redox systems in flooded soils and sediments /nitrate, ferric oxides and hydroxides, sulfate/ are electromotively extremely sluggish /their equilibria are shifted to the left/. Thus, they do not respond, or respond only weakly, to an increased electron activity /= low Eh/ if specific catalysts /enzymes/ required to lower the activation energy barrier are missing /OTTOW and MUNCH, 1978; MUNCH and OTTOW, 1977, 1980, 1982, 1983; OTTOW, 1982/. From an ecophysiological viewpoint, respiration and denitrification may occur simultaneously but temporarily at all microsites /hot spots/ of intensive mineralization processes, even in apparently aerobic soils if the demand for electron acceptors is high /ABOU SEADA and OTTOW, 1985; GÖK and OTTOW, 1987; OTTOW et al., 1990/. This is an important aspect for field studies. Final proof, however, must await further experiments.

Summary

"Aerobic denitrification" has been questioned repeatedly. In general, it has been accepted that denitrification is most active under anaerobic conditions in compacted soils or under flooded conditions. In continuous batch cultures with two different bacteria /Acinetobacter sp. and Moraxella sp./ the influence of oxygen on denitrification /N_2 and N_2O/ and physico-chemical characteristics /pH, Eh, pO_2/ were examined using a synthetic glycerol-KNO_3-broth at pH 7. Denitrification and changes in physico-chemical features were compared with complete anaerobic conditions /He gas/. Under aerobic conditions, mineralization and nitrate consumption were more rapid than in the absence of oxygen. Although O_2 was introduced continuously while stirring the broth, complete anaerobic conditions /pO_2 = 0/ and N_2 + N_2O production were recorded. Generally speaking, the redox level /rH-values/ dropped more intensively under aerobic than anaerobic conditions. In the presence of O_2 and nitrate, respiration and denitrification occurred simultaneously. Oxygen did not inhibit the onset of denitrification and the release of N_2O and N_2. It seems as if respiration and denitrification may occur in one culture and even in one and the same population. In general, intensive denitrification may be expected if the demand for electron acceptors exceeds the supply. This prerequisite is determined by the rate of mineralization rather than by the presence of anaerobic conditions per se. "Aerobic denitrification" is discussed from a physiological viewpoint.

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