Microbiology and application of the anaerobic ammonium oxidation ('anammox') process

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Ten years ago, an anaerobic ammonium oxidation ('anammox') process was discovered in a denitrifying pilot plant reactor. From this system, a highly enriched microbial community was obtained, dominated by a single deep-branching planctomycete, *Candidatus* Brocadia anammoxidans. Phylogenetic inventories of different wastewater treatment plants with anammox activity have suggested that at least two genera in Planctomycetales can catalyse the anammox process. Electron microscopy of the ultrastructure of *B. anammoxidans* has shown that several membrane-bounded compartments are present inside the cytoplasm. Hydroxylamine oxidoreductase, a key anammox enzyme, is found exclusively inside one of these compartments, tentatively named the 'anammoxosome'.

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Abbreviations

CANON	completely autotrophic nitrogen removal over nitrite
FISH	fluorescence *in situ* hybridisation
HAO	hydroxylamine oxidoreductase
SBR	sequencing batch reactor
SHARON	single reactor system for high-rate ammonium removal over nitrate

Introduction

For a long time the oxidation of ammonia was believed to be restricted to oxic environments [1•]. Apparently, many microbiologists did not know that anaerobic ammonia oxidation (anammox; Equation 1) is actually energetically more favorable than 'normal' oxic nitritification (Table 1). It was on the basis of such thermodynamic calculations that Broda [2] predicted the existence of chemolithoautotrophic anammox bacteria two decades ago:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O \qquad (1)$$

Broda's prediction has been experimentally confirmed and patented only recently [P1]. The biological nature of the process has been verified, and nitrite was found to be the preferred electron acceptor [1•]. Hydroxylamine and hydrazine are formed as the intermediates [1•]. In the process, bacteria are enriched on a mineral medium containing ammonia, nitrite and bicarbonate as the only carbon source.

Table 1

Parameters of aerobic and anaerobic ammonia oxidation.

Parameter	Nitrification $NH_4^+ + O_2 \rightarrow NO_2^-$	Anammox $NH_4^+ + NO_2^- \rightarrow N_2$	Unit
Free energy	–275	–357	kJ/mol
Biomass yield	0.08	0.07	mol/mol C
Aerobic rate	200–600	0	nmol/min/mg protein
Anaerobic rate	2	60	nmol/min/mg protein
Growth rate	0.04	0.003	/h
Doubling time	0.73	10.6	days
K_s NH_4^+	5–2600	5	μM
K_s NO_2^-	N/A	<5	μM
K_s O_2	10–50	N/A	μM

N/A, not applicable; K_s, affinity constant.

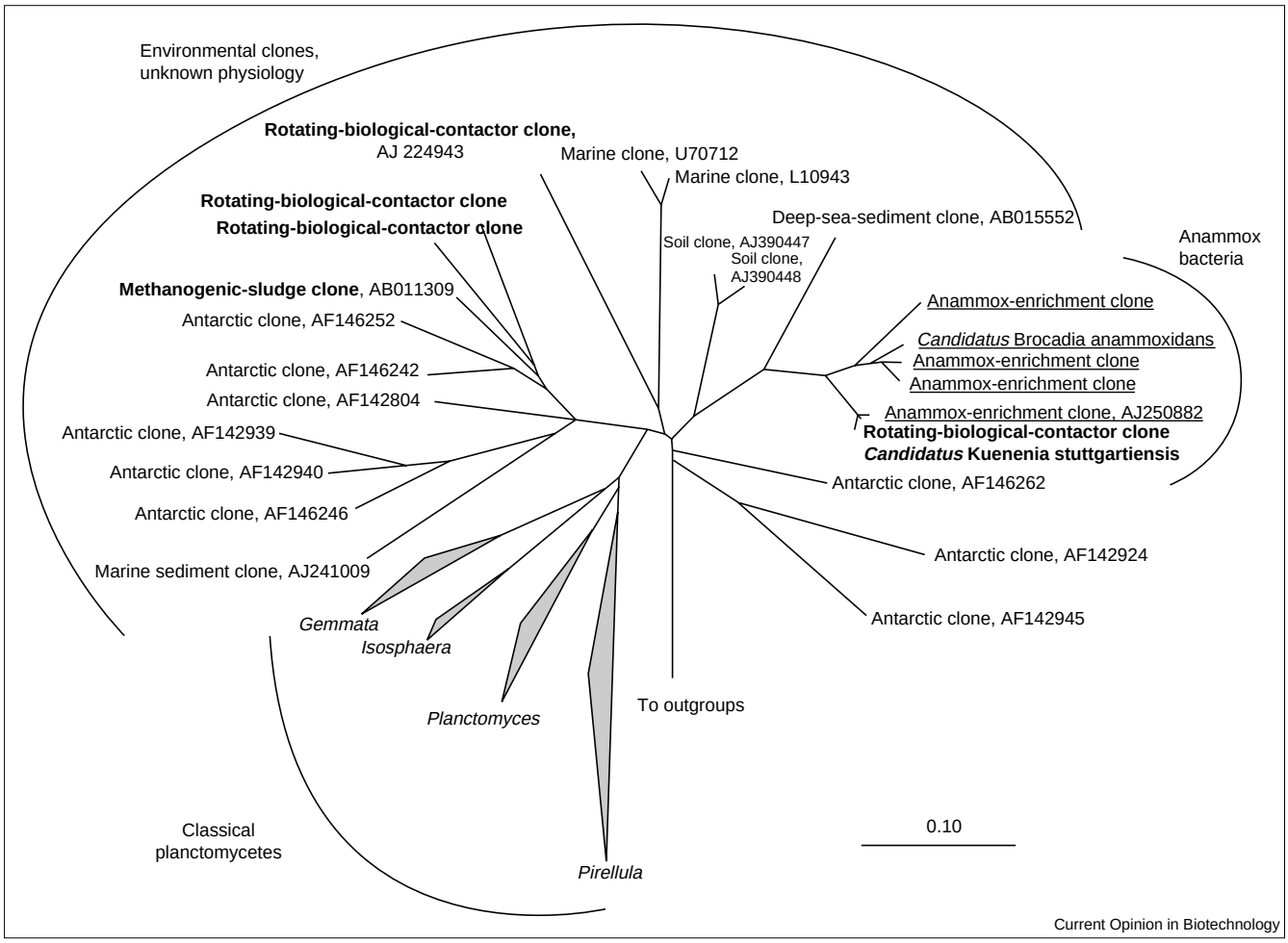
Because the growth rate of the enrichment cultures is extremely low — the doubling time is three weeks — the process needs reactor systems with very efficient biomass retention. For this reason, a sequencing batch reactor (SBR) was applied [3] and optimised to study quantitatively the microbial community. By using an SBR, several important physiological parameters [3,4] have been determined, such as the affinity constants and biomass yield of the system (Table 1). In addition, the persisting stable and strongly selective conditions of SBRs leads to a high degree of enrichment of the dominant bacterium: a bacterium that has a conspicuous morphology and that seems to be responsible for the anammox process.

Here we review the biodiversity, biochemistry and cell biology of the bacteria responsible for anaerobic ammonium oxidation. We also discuss the feasibility of wastewater treatment with these bacteria.

Identification and survey of the responsible bacteria

We tried all classical isolation methods but failed to obtain the enriched bacterium in pure culture. After that initial failure, we applied and optimised an alternative isolation strategy that was based on density gradient centrifugation [5••]. This method produced cell suspensions that contained only one contaminating bacterium for every 200–800 target bacteria. Such suspensions had high anammox activity and fixed CO_2. From these cell suspensions, DNA or RNA was

Figure 1



Diversity of deep-branching planctomycetes. A maximum-likelihood 16S rDNA tree is shown. The tree was calculated with 16S rDNA sequences longer than 1400 nucleotides. Subsequently, shorter 16S rDNA sequences were added using the ARB parsimony method without changing the overall tree topology [20•]. Triangles indicate phylogenetic groups. Environmental clones obtained from wastewater treatment plants and anammox enrichments are labelled in bold and underlined, respectively. The scale bar represents 10% estimated sequence divergence.

extracted and then amplified using a universal 16S ribosomal DNA (rDNA) primer set, and a clone library of 16S rDNA genes was generated. The dominant 16S rDNA sequence in the library was found to be planctomycete-like, branching very deep within the planctomycete lineage of descent.

The Planctomycetales are a separate division within the bacterial domain. They all display distinctive phenotypic properties, such as an absence of any peptidoglycan in their cell walls, pits on the cell surface called 'crateriform structures', budding reproduction, and internal cell compartmentalisation [6•]. We have named the anaerobic ammonium oxidising planctomycete *Candidatus* Brocadia anammoxidans. '*Brocadia*' refers to the place of discovery (a pilot plant at Gist-brocades) [P1] and '*anammoxidans*' describes the metabolism of the bacterium.

The 16S rDNA sequence information was used to design ten specific oligonucleotide probes for application in fluorescence *in situ* hybridisation (FISH). The probes were tested using cell suspensions of *B. anammoxidans* in the almost complete absence of other bacteria. All probes hybridised specifically with the target organism. Next, the probes were used to survey the presence of *B. anammoxidans* and related bacteria in several wastewater treatment systems with a very high nitrogen load and limited air supply [7,8•]. The observed probe-binding patterns indicated, however, that bacteria other than *B. anammoxidans* were present in these environments.

To investigate the diversity of deep-branching planctomycetes in wastewater systems with anammox activity, 16S rDNA-based phylogenetic inventories were established for two biofilm wastewater treatment reactors [8•] (see Update) (M Schmid, M Wagner, unpublished data). Phylogenetic analysis of the retrieved 16S rDNA sequences revealed a diversity in the Planctomycetales that had not been recognised previously (Figure 1).

Interestingly, none of the clones analysed was closely related to *B. anammoxidans*. However, several environmentally retrieved sequences formed a clearly separated monophyletic cluster (with about 90% 16S rDNA sequence similarity to *B. anammoxidans*) within the *B. anammoxidans* line of descent. FISH with specific probes for this cluster showed that these bacteria dominated the microbial biofilm communities of the investigated trickling filter [8•] and rotating biological contactor (M Schmid, M Wagner, unpublished data), whereas *B. anammoxidans* could not be detected. Consequently, these bacteria, provisionally named *Candidatus* Kuenenia stuttgartiensis [8•], represent a novel genus of anammox bacteria.

As this genus is genetically only distantly related to *B. anammoxidans*, we expect that there will be physiological differences between these two anammox genera that should be characterised in future studies (see Update). Furthermore, we are currently investigating, by using a combination of FISH and microautoradiography [9], whether the anammox phenotype is restricted to these two genera or is more widespread within the bacterial domain.

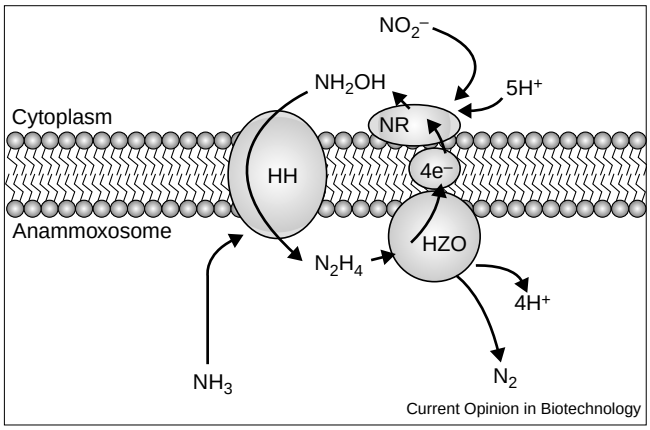
Mechanisms of anaerobic ammonium oxidation in *Candidatus* Brocadia anammoxidans

The possible metabolic pathway for anaerobic ammonium oxidation was investigated using ^{15}N-labelling experiments. These experiments showed that the electron acceptor nitrite is reduced to hydroxylamine and that hydroxylamine somehow reacts with the electron donor ammonium, leading to the ultimate production of dinitrogen gas [1•]. In batch experiments with excess hydroxylamine and ammonium, we observed a transient accumulation of hydrazine, indicating that hydrazine is the intermediate of this final step. We postulated that the oxidation of hydrazine to dinitrogen gas generates the electrons for the initial reduction of nitrite to hydroxylamine (Figure 2). As far as we know, the occurrence of free hydrazine in microbial nitrogen metabolism is rare, if not unique [10].

Cell-free extracts of anammox cultures showed a strong absorption at 468 nm in reduced cytochrome spectra. We purified the enzyme associated with this peak [11•]. The enzyme appeared to have some similarity to the hydroxylamine oxidoreductase (HAO) of *Nitrosomonas europaea*. HAO of *N. europaea* has a similar peak at 460 nm. Both enzymes are capable of oxidising both hydroxylamine and hydrazine.

Apart from the different absorption maxima the enzymes also differed in size, as shown by polyacrylamide gel electrophoresis, HAO from *B. anammoxidans* being the smaller enzyme. The amino acid sequence of several polypeptides obtained from *B. anammoxidans* HAO digests showed no homology to any other sequences in the databases. The two enzymes did, however, share the presence of eight *c*-type cytochromes per enzyme subunit (and three identical subunits per enzyme).

Figure 2



Mechanism of anaerobic ammonium oxidation. NR is a nitrite-reducing enzyme (NH_2OH is the assumed product); HH (hydrazine hydrolase) condenses hydrazine out of ammonia and hydroxylamine; HZO is a hydrazine-oxidising enzyme (which might be equivalent to hydroxylamine oxidoreductase).

Using polyclonal antibodies raised against the purified enzyme, HAO has been detected with immunogold labelling in cells of *B. anammoxidans* [12]. HAO was found to be present only inside a cytoplasmic membrane-bounded region, which makes up about 30–60% of the cell volume. As this 'organelle' seems to have an important role in the catabolism of *B. anammoxidans*, we have named it the 'anammoxosome'.

The anammoxosome is completely surrounded by a compartment containing the nucleoid and ribosomes, and so resembles structurally the pirellulosome in other planctomycetes [12]. The pirellulosome is a major internal cell compartment containing the nucleoid and surrounded by a single membrane. It was first described in chemoheterotrophic planctomycetes in the genus *Pirellula*, but later found to be a structural feature shared by all planctomycetes examined. *Brocadia* cells also have an outer compartment containing cytoplasm termed the 'paryphoplasm', which is separated from more interior compartments of the cell by an intracytoplasmic membrane. They share this feature with other planctomycetes [12].

The exact function of the compartments in *B. anammoxidans* is currently under investigation. One possible function would relate to membrane potential generation internally across the anammoxosome membrane during ammonium oxidation. Some features of the compartmentalisation of the cell in *B. anammoxidans* are exactly analogous to the organisation of compartments in cultured heterotrophic planctomycete genera, such as *Gemmata obscuriglobus*, *Planctomyces maris*, *Isosphaera pallida*, *Pirellula marina* and *Pi. staleyi*. These features include the nucleoid- and ribosome-containing compartment that is equivalent to the pirellulosome, the outer paryphoplasm compartment, and the intracytoplasmic membrane that separates the

paryphoplasm from the pirellulosome [12]. The double-membrane-bounded nucleoid-containing compartment found in *Gemmata obscuriglobus* — the nuclear body — does not occur in *B. anammoxidans*, however. FISH with rRNA-based probes applied to *B. anammoxidans* yielded ring-shaped staining of the cells, consistent with the internal compartmentalisation and presence of the anammoxosome revealed by electron microscopy; *K. stuttgartiensisis* is expected to reveal similar internal organisation to that found in *B. anammoxidans*, as a similar FISH staining pattern is observed [8•].

In addition to HAO, the anammoxosome of *B. anammoxidans* may also contain some of the cell's DNA, although most of the DNA seems to reside in a fibrillar nucleoid in the pirellulosome compartment, external to the anammoxosome [12]. The anammoxosome also possesses tubule structures of unknown function [12]. On the basis of these other features, the anammoxosome may prove to be multifunctional and concerned with activities such as cell division and chromosome replication, as well as physiology.

Combined anoxic and oxic ammonium oxidation

For both application and microbial ecology, it is important to know how anaerobic ammonium oxidisers cope with oxygen. Experiments with *B. anammoxidans* enrichments have shown that oxygen as low as 2 μM completely, but reversibly, inhibits anammox activity [1•].

The obligate anaerobic nature of anammox contrasts with the more versatile aerobic ammonium oxidisers. At least some of these aerobic bacteria are known to be facultative anaerobes: both mixed and pure cultures of *Nitrosomonas eutropha* are capable of denitrification under oxygen limitation [13]. When the gas phase of such experiments was supplemented with 25 p.p.m. nitrogen dioxide, the anaerobic activity of the nitrifiers was boosted to 2.2 nmol NH_4^+/min/mg protein. But even this 'boosted' *N. eutropha* is about 50-fold slower than the dedicated anaerobic ammonium oxidiser *B. anammoxidans* (Table 1), and more than 200 times slower than the aerobic activity of *N. eutropha* itself. The consistent presence of oxic nitrifiers in our anammox reactors [1•] and anammox biofilms [8•] confirmed that although nitrifiers are not enriched for under anoxic conditions, they can at least survive.

Do anaerobic ammonium oxidisers and aerobic nitrifiers share the same microhabitat in nature? Theoretically, nitrifiers and anammox would be able to coexist under oxygen-limiting conditions. The nitrifiers oxidise ammonium to nitrite and keep the oxygen concentration low, while anammox converts the toxic nitrite and the remaining ammonium to nitrogen gas. Indeed, it has been possible to establish such a system by gradually supplying more and more air into an anammox SBR reactor [12,14•].

In this reactor, *N. eutropha* or related bacteria consumed the oxygen effectively so that the actual oxygen concentration remained below the detection limit of 2 μM. Nitrite concentrations never exceeded 1 mM, indicating that *B. anammoxidans* was active as well. After five months, 30 mM ammonium was converted into dinitrogen gas and some nitrate, according to Equation 2:

$$2.5\ NH_4^+ + 2.1\ O_2 \rightarrow 0.2\ NO_3^- + 1.15\ N_2 + 3.6\ H_2O + 2.8\ H^+ \quad (2)$$

In anoxic batch experiments, biomass from this reactor converted ammonium and nitrite simultaneously at a rate of 35 nmol NH_4^+/min/mg protein according to Equation 1, with some nitrate production. In oxic batch experiments, ammonium was converted to nitrite at a rate of 30 nmol NH_4^+/min/mg protein, with no nitrate production.

The microbial composition of the biomass was analysed with FISH using probes specific for anammox and nitrifiers. Initially, *B. anammoxidans* dominated (70%), but over time more and more *N. eutropha* or related bacteria were detected. Aerobic nitrite oxidisers (such as *Nitrobacter winogradskii* or *Nitrospira moscoviensis*) were never detected, consistent with the absence of nitrite-oxidising activity in oxic batch tests.

This symbiosis of aerobic and anaerobic ammonium-oxidising bacteria is relevant for ecology and particularly for wastewater treatment. We have shown that ammonium can be removed in a simple, single oxygen-limited step, which we have patented [P2] and named CANON ('completely autotrophic nitrogen removal over nitrite'). CANON also refers to the way in which the two groups of microorganisms interact — that is, by performing two sequential reactions simultaneously.

Two other oxygen-limited processes for one-step ammonium removal, 'Oland' and 'deammonification', have been described [15,16]. The main difference between these and CANON is that Oland and deammonification make use of the denitrification activity of conventional aerobic nitrifiers, whereas CANON incorporates the anammox process. Finally, stable conversion of ammonium into dinitrogen gas has also been achieved in anammox and nitrifying cells that have been co-immobilised on alginate in air-loop reactors [17].

Application of the anammox process

The application of anammox to nitrogen removal would lead to a reduction of operational costs of up to 90%. The process targets wastewaters that contain much ammonium and little organic material, such as sludge digestor effluents. Anammox would replace the conventional denitrification step completely and would also save half of the nitrification aeration costs. Before anammox can be applied, however, two important questions need to be answered. First, is the CANON process the way to go or are the nitrification and anammox steps best engineered separately? Second, can anammox cope with the variable and harsh conditions of wastewater treatment, compared with the optimal laboratory conditions in which it has been studied?

We have targeted both questions in feasibility studies with sludge digestor effluents on laboratory scale. The composition of such effluents did not negatively affect the anammox activity. The optimum pH (7.0–8.5) and temperature (30–37°C) for the process were well within the range expected for digester effluents. In a 2 L fluidised bed reactor, the anammox biomass removed ammonium efficiently when the wastewater was supplied with nitrite. Over one year, the nitrogen load of the anammox fluidised bed reactor was increased from 0.2 kg N_{tot}/m^3/day to 2.6 kg N_{tot}/m^3/day.

In a separate study, we investigated the possibility of using the SHARON (single reactor system for high-rate ammonium removal over nitrite) process in combination with anammox. SHARON was developed recently [18] for the removal of ammonium through the so-called 'nitrite route'. It was tested for two years in the laboratory and successfully scaled-up from 2 L to 1800 m^3 (full-scale) [19]. SHARON is essentially a chemostat (no biomass retention) with a dilution rate higher than the maximum growth rate of nitrite-oxidising bacteria but lower than the growth rate of ammonium oxidisers. Under these conditions, nitrite is the stable end product of nitrification.

Anammox needs ammonium and nitrite in a ratio of roughly one to one. For sludge digestor effluents, this ratio can be achieved without control, because these effluents contain bicarbonate as the counter ion for ammonium. When half of the ammonium is converted, the alkalinity of the water is depleted leading to a drop in pH and preventing further nitrification (Equation 3):

$$NH_4^+ + HCO_3^- + 0.75\ O_2 \rightarrow 0.5\ NH_4^+ + 0.5\ NO_2^- + CO_2 + 1.5\ H_2O \quad (3)$$

We have shown the feasibility of using SHARON for the production of ammonium and nitrite (1:1) in a 0.02 m^3 laboratory system with sludge liquor from a Rotterdam wastewater treatment plant [7]. Fifty-three percent of the ammonium was oxidised to nitrite at 1.2 kg N/m^3/day, without pH control. The ammonium : nitrite ratio in the effluent of the SHARON process could be fine-tuned by adjusting the pH between 6.5 and 7.5. The effluent of this SHARON reactor was fed to an anammox SBR. This reactor removed all nitrite and left some ammonium (Table 2). During the test period the nitrogen load was 0.75 kg N/m^3/day. The specific activity of the anammox biomass was very high: 0.8 kg N/kg dry weight/day.

The SHARON–anammox process has been patented [P3] and its feasibility for full-scale implementation has been demonstrated [7]. On the basis of the combined SHARON–anammox process, we made a cost estimate of 0.75 Euro/kg N. This is very low compared to the 2–5 Euro/kg N calculated for other processes that have been tested on a pilot plant scale for nitrogen removal from sludge digestion liquors [7].

Table 2

Conversion in a granular sludge SBR anammox reactor fed with a nitrified effluent from a SHARON reactor.

Parameter	Steady-state operation
Test period	110 days
Influent NH_4–N	0.55 ± 0.1 kg/m^3
Influent NO_2–N	0.60 ± 0.1 kg/m^3
NH_4–N conversion	0.35 ± 0.1 kg/m^3/day
NO_2–N conversion	0.36 ± 0.01 kg/m^3/day
Effluent NO_2–N	0 kg/m^3
Volumetric conversion	0.75 ± 0.2 kg N_{tot}/m^3/day

Conclusions

The anammox process is catalysed by a group of deep-branching planctomycetes, including *Candidatus* B. anammoxidans and *Candidatus* K. stuttgartiensis. Co-cultures of oxic and anoxic ammonia-oxidising bacteria convert ammonia directly to dinitrogen gas under oxygen limitation.

The introduction of partial nitrification/anammox to the treatment of high-strength wastewaters will lead to substantial savings of energy and resources. Such systems have been tested over prolonged periods and demonstrated stable effluent quality and compact ammonium removal without the need for process control. Given the low costs of our system, a full-scale implementation is to be expected in the near future.

Update

Recent work has indicated that *K. stuttgartiensis* is in many ways similar to *B. anammoxidans*. Both the ultrastructure (including the presence of an anammoxosome) and the production of hydrazine from hydroxylamine were confirmed for this second genus of anaerobic ammonium oxidisers. Some differences were also reported: *K. stuttgartiensis* has a lower specific activity and is more tolerant to high nitrite [21].

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