Brazilian Journal of Microbiology (2012): 865-872
ISSN 1517-8382

BIODEGRADATION OF PETROLEUM HYDROCARBONS IN HYPERSALINE ENVIRONMENTS

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Submitted: September 27, 2011; Returned to authors for corrections: December 07, 2011; Approved: June 07, 2012.

ABSTRACT

Literature on hydrocarbon degradation in extreme hypersaline media presents studies that point to a negative effect of salinity increase on hydrocarbonoclastic activity, while several others report an opposite tendency. Based on information available in the literature, we present a discussion on the reasons that justify these contrary results. Despite the fact that microbial ability to metabolize hydrocarbons is found in extreme hypersaline media, indeed some factors are critical for the occurrence of hydrocarbon degradation in such environments. How these factors affect hydrocarbon degradation and their implications for the assessment of hydrocarbon biodegradation in hypersaline environments are presented in this review.

Key words: hydrocarbon biodegradation; halophile; hypersaline environment; halophilic/halotolerant hydrocarbon-degraders.

INTRODUCTION

Hypersaline environments characteristically present salt concentrations higher than 35 $g.L^{-1}$ (seawater salinity) (17) and can be established by either natural or anthropic forces that favor salt deposition and accumulation. Therefore, this definition includes a broad range of aquatic and terrestrial environments, with salinities up to saturation (~350 $g.L^{-1}$) and different salt compositions.

Although high salt concentrations constitute a stressful agent for most of the known living organisms (16, 45), hypersaline environments can harbor functional and taxonomical diversified biological communities (34, 35). The organisms that grow best in these environments are called halophiles, while the ones whose optimal growth occur in non-saline media but are able to grow under hypersaline conditions are indicated as halotolerant organisms (19).

Algae of the genus *Dunaliella* are the producers in saturated or closed to saturation media (salinities higher than 200 – 250 $g.L^{-1}$), where they support heterotrophic communities composed mainly by archaea (Family: Halobacteriaceae) and *Salinibacter ruber* (Bacteria, Order Bacteroidetes) (35, 36). Cyanobacteria respond for most of the primary production in aquatic environments with salinities up to 25% (36) and support essentially bacterial communities (30, 31, 32). Biological productivity can be quite high in aquatic hypersaline environments and, therefore, hypersaline sediments tend to accumulate high amounts of organic matter, including

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hydrocarbons (14).

Scientific and technical knowledge on halophilic and halotolerant microorganisms have been improved because of increasing perspectives of their application in industrial production bioprocesses, hypersaline wastewater treatments and also basic evolutionary studies, since the oldest fossil microorganisms are found in stromatolites, sedimentary structures similar to contemporary microbial mats found in hypersaline environments (17). On the other hand, literature about hydrocarbon degradation by halophilic and halotolerant microorganisms is still scarce, notwithstanding the importance of this subject for several issues related to petroleum industry: reclamation of oil and brine impacted soils, remediation of oil polluted hypersaline lakes, treatment of oily hypersaline wastewater and hydrocarbon degradation processes in hypersaline petroleum reservoirs.

In a recent review on organic pollutants degradation by halophilic prokaryotes LeBorgne *et al.* (17) demonstrated that the results about hydrocarbon degradation under high salt concentrations can differ: while some reports point to a negative influence of salinity on hydrocarbonoclastic activity, others show an opposite tendency. In the present work we propose a discussion about the reasons that could justify such differences and indicate the main factors affecting petroleum hydrocarbons biodegradation in hypersaline environments.

EFFECT OF SALINITY ON HYDROCARBONS BIODEGRADATION

Hydrocarbonoclastic activity in non-saline soils (25, 26, 38) and groundwater (43) is impaired when salinity increases, since microbial communities in such environments are not expected to be previously adapted to high salt concentrations (20). Minai-Tehrani *et al.* (25) observed 41% crude oil degradation in soil samples with no NaCl added, while only 12% was obtained in samples from the same soil subject to 50 $g.L^{-1}$ NaCl after 120 days (Table 1).

The negative impact of increasing salinity on hydrocarbons biodegradation is also observed in environments where halotolerant and/or slightly halophilic microorganisms tend to be dominant, as the case of mangroves (11, 39) and intertidal microbial mats (1). Abed *et al.* (1) investigated microbial mats from an Arabian Gulf area chronically exposed to oil spills and subject to high daily salinity and temperature fluctuations: 50 $g.L^{-1}$ salts and 25 ºC (high tide) to 150 $g.L^{-1}$ and 40 ºC (low tide). The authors (1) evaluated the degradation rates of several hydrocarbons under a range of salinities: 0; 35; 50; 80; 120 and 160 $g.L^{-1}$. They reported that almost 100% of initial phenanthrene and dibenzothiophene were degraded at 35 $g.L^{-1}$, while the best degradation results for pristane (approximately 75%) and n-octadecane (around 85%) occurred between salinities of 35 and 80 $g.L^{-1}$ (Table 1). Díaz *et al.* (11) assessed hydrocarbon biodegradation capability of a mangrove microbial consortium immobilized onto polypropylene fibers. They verified that alkanes biodegradation was lower than 40% in medium with 0 $g.L^{-1}$ NaCl, around 50% at 20 $g.L^{-1}$, reaching 65% (highest biodegradation obtained) at 40 $g.L^{-1}$. At salinities ranging from 60 to 140 $g.L^{-1}$ alkanes biodegradation rates were 50 - 60%, falling to less than 30% at 180 $g.L^{-1}$ (Table 1).

Even in typical hypersaline environments a negative impact on hydrocarbon biodegradation induced by increasing salinity has been reported. Ward and Brock (44) observed that the negative effect of salinity increase was pronounced on hexadecane biodegradation, more than on glutamate biodegradation. These authors collected samples (salinity range: 33 – 284 $g.L^{-1}$) from the water column at Great Salt Lake and at shores of various evaporation ponds nearby. It was observed that hexadecane mineralization (CO_2 production) decreased from 50% at 33 $g.L^{-1}$ salts (< 150 h) to negligible values at salinities higher than 250 $g.L^{-1}$ (Table 1), while glutamate mineralization was 68% at salinity of 33 $g.L^{-1}$ (143 h) and 54% at 284 $g.L^{-1}$ salts (450 h). These mineralization results were very consistent with data about microbial growth in enrichment media for hydrocarbonoclastic aerobic bacteria: no growth was observed at salinities higher than 250 $g.L^{-1}$.

Nevertheless, Bertrand *et al.* (6) isolated from interface water sediment with salinity of 310 $g.L^{-1}$ (31%) a strain of a halophilic archaeon, recently classified as *Haloarcula vallismortis* (41) (labeled EH4 by Bertrand *et al.* (6)), that degraded hydrocarbons more efficiently at the highest salinities tested. Eicosane biodegradation percentage increased from ~10% in medium with

146 g.L^{-1} NaCl to a 64 % at 204 g.L^{-1} NaCl, 30 days incubation (Table 1). Such haloarchaeon presented optimal growth at 45 ºC and capability to degrade a few aliphatic and aromatic hydrocarbons (Table 2), especially tetradecane, whose initial concentration decreased 88% after 30 days (6).

Indeed, several hydrocarbonoclastic halophilic and halotolerant microorganisms have been isolated from chronically hydrocarbon impacted sites and petroleum industry facilities, most of them affiliated to the class Gammaproteobacteria (10, 13, 17, 27, 28, 40, 46) (Table 2). *Marinobacter hydrocarbonoclasticus* is an extreme halotolerant (4,7 to 204 g.L^{-1} NaCl, optimal growth at 35 g.L^{-1} NaCl) able to use a lot of hydrocarbons as only carbon and energy sources, especially intermediate chain length aliphatics (5, 13). Mnif *et al.* (27) obtained from saline wastewater samples collected in a petroleum rig a *Halomonas* strain adapted to grow in media with crude oil, diesel, lubricant oil or hexadecane as the only carbon sources. Although the optimal growth range for this strain was between 50 and 80 g.L^{-1}, it was shown its ability in maintaining a basal hydrocarbonoclastic metabolism in media with up to 140 g.L^{-1} salts. Other Gammaproteobacteria's representatives isolated from sites impacted by petroleum industry activities were capable to completely degrade monoaromatic compounds in one to two weeks at salinities of 140 to 230 g.L^{-1} (28, 40), or up to five weeks at 290 g.L^{-1} (40).

Table 1. Summary of reports regarding salinity influence on hydrocarbon biodegradation cited throughout this review.

Refer.	Sample features		Cultivation conditions						Notes
Rhykerd *et al.* (38)	Matrix	Soil	50 g.Kg^{-1} Motor oil	Salinity [A]	~0,2	~24	~72	~120	Authors evaluated hydrocarbon degradation based on CO_2 production
	Salinity[A]	~0,2 g.L^{-1}		H.D. [C]	43,3	42,5	37,7	36	
	Poll. backg.[B]?	No		Time/ Temp.[D]	80/ N.R.[E]				
Minai-Tehrani *et al.* (25)	Matrix	Soil	20 g.Kg^{-1} Crude oil	Salinity	0	10	30	50	
	Salinity	N.R.		H.D.	41	32	16,5	12	
	Poll. backg.?	N.R.		Time/ Temp.	120/ 25				
Minai-Tehrani *et al.* (26)	Matrix	Soil	20 g.Kg^{-1} Crude oil	Salinity	0	10	30	50	Authors cited that sampling area is nearby a desert place which contains a big saline lake
	Salinity	N.R.		H.D.	40	32	16	13	
	Poll. backg.?	No		Time/ Temp.	120/ 25-28				
Ulrich *et al.* (43)	Matrix	Groundwater + subsurface soil	0,1% (v/v) Hexadecane	Salinity	0	5	10	25	50	Authors evaluated hydrocarbon degradation based on CO_2 production
	Salinity	N.R.		H.D.	55	44	35	32	29	
	Poll. backg.?	Yes		Time/ Temp.	15/ 25				
Díaz *et al.* (11)	Matrix	Microbial consortium from a mangrove	11 g.Kg^{-1} Crude oil	Salinity	0	20	40	60	Consortium immobilized in polypropilene fibers
				H.D.	32	50	65	60	
	Salinity	It grows at 0 - 220 g.L^{-1}		Salinity	80	100	140	180	
				H.D.	50	58	49	27	
	Poll. backg.?	N. R.		Time/ Temp.	20/ 28				
Abed *et al.* (1)	Matrix	Microbial mat collected from tidal sand sediments	33 mg.L^{-1} Octadecane	Salinity	0	35-120	160		Hydrocarbons were provided altogether as a mixture adsorbed to a hydrophobic clay
				H.D.	0	85	60		
			33 mg.L^{-1} Pristane	Salinity	0	35-80	120	160	
				H.D.	0	75	50	negl.[F]	
	Salinity	50 g.L^{-1} at sampling moment. It varies daily from 50 (high tide) to 150 g.L^{-1} (low tide). It can reach 250 g.L^{-1} on Summer	33 mg.L^{-1} Phenanthrene	Salinity	0	35	50	80	>120	
				H.D.	0	100	negl.	50	negl.	
			33 mg.L^{-1} Dibenzothiophene	Salinity	0	35	50	80	>120	
				H.D.	0	100	10	60	≤ 10	
	Poll. backg.?	Yes		Time/ Temp.	26/ 28					
Ward and Brock (44)	Matrix	Surface water	0,02 mM Hexadecane	Salinity	33	112	122	134	Authors evaluated hydrocarbon degradation based on CO_2 production
				H.D.	50	12,5	34	30	
				Time	6		12		
	Salinity	33 to 284 g.L^{-1}		Salinity	172	204	258 - 284		
				H.D.	19	10	< 3		
	Poll. backg.?	Yes		Time		19			
				Temp.	Ambient temperature				
Bertrand *et al.* (6)	Matrix	Interstitial water	500 mg.L^{-1} Eicosane	Salinity	50	105	146	176	205	
	Salinity	310 g. L^{-1}		H.D.	0	0	10	33	64	
	Poll. backg.?	Yes		Time/ Temp.	30/ 32					

[A] Salinity values expressed in g.L^{-1} NaCl; some original papers used different units, which were converted to be reported in this review.; [B] Poll. backg.=Pollution background; [C] H.D. = hydrocarbon concentration decrease, expressed in %; [D] Time expressed in days; temperature (Temp.) in °C; [E] N.R. = not reported; [F] negl. = negligible

More examples of hydrocarbon-degrading haloarchaea have been reported recently (Table 2). Al-Mailem *et al.* (4) isolated from a sabkha on the coast of Arabian Gulf four strains of haloarchaea (*Haloferax* (two), *Halobacterium* and *Halococcus*) able to degrade several aliphatic and aromatic hydrocarbons, with biodegradation rates increasing as salinities increased through 58 to 175 $g.L^{-1}$ NaCl.

Zvyagintseva *et al.* (47) evaluated hydrocarbonoclastic activity by *Dietzia maris*, an actinobacterium, at salinities of 5 to 100 $g.L^{-1}$ NaCl. Optimal biodegradation occurred at 50 to 100 $g.L^{-1}$ NaCl: paraffins ($C_{14} - C_{18}$) were degraded at 70 and 100 $g.L^{-1}$ NaCl slower than at 50 $g.L^{-1}$, however, all these media presented equivalent amounts of CO_2 after seven days. Among the halophilic fungi, *Fusarium lateritium* and *Drechslera* sp. were shown to metabolize crude oil, with degradation efficiency improved more than thrice if petroleum was an additional source of carbon and energy instead of being the exclusive one (29).

The works on the influence of salinity on petroleum hydrocarbons degradation cited in this review are summarized in Table 1. Table 2 presents a summary of the reports on halophilic/halotolerant hydrocarbon-degraders cited herein. It has been evidenced that impairment to hydrocarbonoclastic activity at high salinities (44) can not be attributed only to osmotic stress on metabolic activity of native microorganisms or to absence of hydrocarbon catabolism among halophiles. So, how to explain the divergence of conclusions observed in literature on hydrocarbon biodegradation at high salinities?

Table 2. Halophilic and halotolerant hydrocarbon-degraders and the hydrocarbons these microorganisms proved to degrade.

Microorganism	Hydrocarbon pollution background?	Salinity ($g.L^{-1}$) applied in biodegradation assay	Hydrocarbons shown to be metabolized	Reference
Haloferax sp. HA-1 *Haloferax* sp. HA-2 *Halobacterium* sp. *Halococcus* sp.	Yes, despite low hydrocarbon concentration measured	175	Crude oil, Octadecane, Phenanthrene	4
Marinobacter hydrocarbonoclasticus SP.17	Yes	35	Tetradecane, Hexadecane, Heneicosane, Pristane Phenanthrene, Phenyldecane	5 and 13
*Haloarcula vallismortis**	Presumably yes	310	Tetradecane, Hexadecane, Eicosane, Heneicosane, Pristane Acenaphthene, Phenanthrene, Anthracene, Methyl anthracene	6
Ten strains of Haloarchaea	Presumably not	200	Naphthalene, Anthracene, Phenanthrene, Pyrene, Benzo(a)anthracene	7
Fundibacter jadensis T9T	Not informed	35	Tetradecane, Hexadecane, Pristane	10
Halomonas sp.	Produced water	100	Crude oil (C_{11}-C_{22}), Hexadecane	27
Consortium of *Marinobacter* sp. strains	Yes	146	Benzene, Toluene, Ethybenzene, Xylenes	28
Fusarium lateritium *Drechslera* sp.	Presumably yes	100	Crude oil	29
Consortium Gammaproteobacteria + Bacteroidetes	Yes	292	Benzene, Toluene	40
Haloarcula argentinensis *Haloferax volcanii* *Haloferax alexandrinus* *Haloferax volcanii*	No	225	Heptadecane Heptadecane Heptadecane, Phenanthrene Heptadecane, Eicosane	41
Alcanivorax borkumensis	Presumably yes	35	Hexadecane	46
Dietzia maris	Not informed	5 - 100	Mixture of n-alkanes: C_{14} - C_{18}	47

* Bertrand *et al.* (6) labeled this strain as EH4 and assigned it to *Halobacterium* sp. based on phenotypic features. Recently, Tapilatu *et al.* (41) re-assigned it to *Haloarcula vallismortis* based on 16S rRNA.

CONSIDERATIONS ABOUT HYDROCARBON BIODEGRADATION AT HIGH SALINITIES

The effect of salinity on microbial hydrocarbonoclastic activity depends on the amplitude variation of salt concentration imposed to and on the salinity of the habitat of the microorganisms under investigation. In Abed *et al.* (1) and Díaz *et al.* (11) studies, it is clear that very low salt concentrations reduce hydrocarbonoclastic activity and the optimal biodegradation results are reached within moderate salinity ranges. In the case of Bertrand *et al.* (6) it was necessary to use extreme salinity conditions because the strain under investigation was a halophilic archaeon isolated from an extreme hypersaline environment. However, Ward and Brock (44) worked with samples from hypersaline environments as well. Besides, these authors mentioned that their study site (Great Salt Lake) had been affected by petroleum and it can be supposed that the sampling site of Bertrand *et al.*'s report (6) also presented oil pollution background, since it was located in the vicinity of an urban area (Aigues-Mortes, France) and because eicosane, heneicosane and pristane were detected in water samples collected from there. Why the tendencies about salinity effect on hydrocarbon biodegradation observed by Ward and Brock (44) were opposite to the ones presented by Bertrand
et al. (6)?

First of all, it is necessary to consider that hydrocarbons are less bioavailable in hypersaline environments than in non-saline ones. This is a consequence of the "salting out" effect: because soluble salts reduce hydrophobic organic compounds solubility in water, the higher the salts concentration in aqueous phase the higher the tendency of organic compounds to be adsorbed to the solid matrix (sediment or soil) (18, 24, 42).

This effect of salinity on hydrocarbons solubility is mentioned by Margesin and Schinner in a review about biodegradation of hydrocarbons by extremophiles (20) and is appointed by McGenity (23) as a reason for the low number of known halophilic hydrocarbon degraders. Means (24) suggests that polyaromatic hydrocarbons sorption to the sediment particles increases in the presence of salts, not only because the salting out effect on such hydrocarbons but also because the salting out effect on the organic phase of the sediment, turning it into a better solvent for the polyaromatics. This explanation is reinforced by Turner & Rawling's conclusions (42), which point to an enhancement of hydrophobicity or solvency of the sediment organic matter through interactions between organic matter and seawater cations.

Salting out effect on hydrocarbons could justify the low rates of hexadecane biodegradation observed by Ward and Brock (44) at the highest salty samples. Since these authors sampled from the water column they probably dealt with microbial populations that had not been submitted to hydrocarbons selection pressure, since the hydrocarbons were expected to be adsorbed to the sediments at their study site.

Bertrand *et al.* (6), on the other hand, collected interstitial water from the sediment and probably obtained samples with microorganisms which had been directly exposed to the hydrocarbon polluted sediment and, consequently, were adapted to metabolize hydrocarbons.

Other important aspect that could be taken into account to comprehend the opposite conclusions between these studies is the laboratory growth conditions. In extreme hypersaline environments (204 to 286 $g.L^{-1}$ in Ward and Brock (44); 310 $g.L^{-1}$ in Bertrand *et al.* (6)) halophilic archaea constitute the dominant populations (33, 34, 36), whose optimal temperature range is generally higher than 35 ºC (9). We can suppose that temperatures used by Ward and Brock (44) had probably impaired archaea growth since they reported applying ambient temperature.

Despite the fact that several studies have been reporting the presence of hydrocarbon-degraders in hypersaline environments, we do not know in which extent the halophilic biodegradation of petroleum hydrocarbons does occur *in situ*. High salinities restrict not only microbial access to hydrocarbons but also the availability of oxygen, since its

solubility decreases as salt concentration increases (23, 37). This fact makes studies on anaerobic biodegradation of hydrocarbons by halophiles critical for the comprehension of hydrocarbonoclastic activity in hypersaline environments, but there is a lack of such studies (23). It is important to bring to attention that all the reports summoned in this review refer to results obtained under laboratory conditions, in which hydrocarbons and oxygen can be easily provided.

On the other hand, aquatic hypersaline environments usually present high biological productivity and, therefore, high amount of organic matter, including hydrocarbons, is deposited in evaporitic sediments (14). This fact has even led to the proposal that many of the world's oil reservoirs are formed from hypersaline environments (3). So, it can be inferred that halophiles have been exposed to high hydrocarbons concentrations very long before the advent of petroleum industry era and, consequently, the ability to metabolize hydrocarbons could be an evolutionary acquisition widespread among halophiles more than it was considered by some authors (23, 33). This hypothesis is supported by recent studies that report the existence of halophilic prokaryotic populations adapted to degrade petroleum hydrocarbons even in pristine environments, i.e., without oil pollution background (7, 12, 41). Tapilatu *et al.* (41), for example, obtained from water samples of a salt crystallizer pond (268 $g.L^{-1}$) with no known oil pollution history four strains of halophilic archaea (*Haloarcula* and *Haloferax*) able to degrade 32 to 95% of initial heptadecane concentration in 30 days, growing in complex media with salinities of 225 $g.L^{-1}$. Yet D'Ippólito *et al.* (12) observed growth and chemotactic response towards hydrocarbons by two strains of *Halomonas*, one of them from a non-polluted brine. Bonfá *et al.* (7) evaluated ten strains of haloarchaea isolated from hypersaline habitats presumably with no oil pollution background to degrade a mixture of polyaromatic hydrocarbons and observed that all strains were capable of degrading these compounds at 200 $g.L^{-1}$ NaCl. These strains were shown to reduce organic content in produced waters more efficiently than hydrogen peroxide treatment (7).

McGenity (23) highlights the hydrophilic nature of halophiles' cell surface as an obstacle for the metabolism of hydrophobic compounds, but, regarding this scenario, the ability to produce emulsifying agents observed in several hydrocarbon-degrading halophiles (5, 21, 22, 46) might be considered an adaptation to circumvent the problem of access to carbon sources, since emulsifiers increase hydrocarbon availability (8, 15).

CONCLUSIONS AND FINAL CONSIDERATIONS

For assessing the effects of salinity variation on hydrocarbonoclastic activity it is necessary to take into account the sampling sites background regarding petroleum hydrocarbons occurrence (although this last one is not always mandatory) and salt concentrations, as well as sampling strategies and laboratory microbial growth conditions applied, since microbial growth requirements vary according to the dominant halophilic populations.

Increase in salt concentration *per se* is not necessarily an impediment to petroleum hydrocarbons biodegradation. Instead, microbial hydrocarbonoclastic activity is impacted by fluctuations in salt concentrations either below or beyond the salinity range for optimal growth of the microorganism under investigation. We have to consider that anthropic salinization impairs the hydrocarbonoclastic activity of native non-halotolerant microorganisms, as well as artificial desalinization is an obstacle for bioremediation of petroleum polluted hypersaline environments.

Hypersalinity is required for hydrocarbon catabolism to be performed in hypersaline environments, but at extreme salinities the salting out effect on hydrocarbons imposes a critical barrier to hydrocarbon biodegradation. Not only the accessibility to carbon sources becomes restricted, but so is the availability of oxygen. In this sense, the presence of halophilic photoautotrophs can be a critical factor for the achievement of hydrocarbon biodegradation (2), since their photosynthetic

activity could compensate the lack of oxygen imposed by hypersalinity. Higher accessibility to and, consequently, improvement in biodegradation of hydrocarbons in hypersaline conditions were reached through immobilization of microbial cells in polypropilene fibers (11). On the other hand, several halophiles are emulsifier-producers, characteristic that is expected to make possible for the halophiles to access the hydrocarbons instead of the effects of the salts on the hydrocarbons' solubility. In this sense, application of emulsifiers or halophilic emulsifier-producers could be part of a bioremediation strategy.

Hydrocarbon catabolism might be a widespread ability among halophiles because of the typical prolific production and accumulation of biomass in their habitats, but strong support for such hypothesis will come from the efforts in screening for genes encoding hydrocarbonoclastic enzymes within halophilic genomes.

Notwithstanding the expressive biodegradation results presented in this review, attempts to assess petroleum hydrocarbons catabolism by halophilic and halotolerant microorganisms *in situ* are required to evaluate the extent of such microbial activity in nature, which necessarily implies to fulfill the gap on anaerobic biodegradation of hydrocarbons at high salinities. Comprehension about the aerobic and anaerobic hydrocarbon biodegradation processes *in situ* will support research on microbial enhanced oil recovery and petroleum degradation in reservoirs.

ACKNOWLEDGEMENTS

The authors thank J. Medeiros for her grammatical revision of this manuscript. L.F. Martins thanks A. U. Soriano, G. V. Sebastián and J. Medeiros for encouraging the development of this review and PETROBRAS for the support to the research. The authors also thank the reviewer for the suggestions.

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