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## **New Uses of Haloarchaeal Species in Bioremediation Processes**

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### **Abstract**

The extreme conditions under which haloarchaea survive make them good bioremediation agents in water treatment processes and in saline and hypersaline environments contaminated with toxic compounds such as nitrate, nitrite and ammonia, chlorine compounds such as perchlorate and chlorate, heavy metals, and aromatic compounds. New advances in the understanding of haloarchaea metabolism, biochemistry, and molecular biology suggest that general biochemical pathways related to nitrogen (Nitrogen cycle), metals (iron, mercury), hydrocarbons, or phenols can be used for bioremediation proposals.

The main goal of the chapter is to present a review about the main characteristics of the archaeal species and their possible uses for bioremediation processes paying special attention to the *Halobacteriaceae* family. Several examples about the role of these microorganisms in salty brines or soils with high concentrations of nitrogenous compounds, heavy metals, aliphatic or aromatic hydrocarbons, or oxyanions are also discussed.

**Keywords:** Haloarchaea, wastewater treatments, bioremediation, denitrification, nitrogen, carbon metabolism

## 1. Introduction

The main benefit of using bioremediation is that microorganisms can destroy hazardous contaminants or turn them into less harmful forms. These microorganisms act against the contaminants if there are a variety of compounds aiding them to generate both energy and nutrients in order to grow more cells. In a few cases, the natural condition of the contaminated site provides all the essential material in sufficient quantities so that bioremediation can occur without the need for human intervention, which is known as “intrinsic bioremediation” [1]. Often, bioremediation requires engineered systems to supply microbe-stimulating materials, which is called “engineered bioremediation” and relies on accelerating the desired biodegradation by encouraging growth of further organisms and optimizing the environment where detoxification takes place. Engineered bioremediation may be chosen over intrinsic bioremediation due to the time factor and liability. Where an impending property transfer or potential impact of contamination calls for rapid pollutant removal engineered bioremediation may be more appropriate as it accelerates biodegradation. However, intrinsic bioremediation is an option where the natural occurrence of contaminant biodegradation is faster than contaminant migration. These rates depend on both, the type and concentration of contaminant, the microbial community, and the subsurface hydrogeochemical conditions [1]. Moreover, the lack of a sufficient microbial population can also hinder the cleanup rate.

Terrestrial subsurface ecosystems constitute one of the largest habitats and represent an important resource of microbial diversity. The organisms within provide critical services including mitigation of contaminants. Research in this area has intensified over the last two decades leading to significant discoveries in ecology, physiology, and phylogeny of subsurface microorganisms. Despite considerable progress, the structure–function relationships remain largely uncharacterized. Attempts to correlate microbial abundance and composition with variables likely to control metabolism have for the most part been unsuccessful. New technologies now give us the opportunity to gain further insights [2]

A critical factor as to whether bioremediation is an appropriate remedy depends on if the contaminants are susceptible to biodegradation by the site organisms, or alternatively, if the relevant organisms can be added. While those already present can detoxify a vast array of contaminants, some are more easily degraded than others. On the whole, those most easily degraded are petroleum hydrocarbons; however, technologies which stimulate organisms’ growth to degrade further contaminants are emerging and are being field tested with success [1].

Bioremediation is a branch of environmental biotechnology often used to hasten this process and it guarantees the restoration of damaged ecosystems, using the metabolic capabilities of bacteria, fungi, yeast, algae, and microbial mats to degrade all contaminants harmful to living organisms. Bioremediation follows two main strategies: i) biostimulation, stimulation of indigenous microbial populations; ii) bioaugmentation, the introduction of viable microbial populations. Microorganisms are ideally suited to the task of contaminant destruction because they have enzymes that allow them to use environmental contaminants as food and because they are so small that they are able to contact contaminants easily [1]. Without the activity of

microorganisms, the earth would literally be buried in wastes, and the nutrients necessary for life would be locked up in detritus.

Coastal marine sediments subjected to high anthropogenic inputs can accumulate large amounts of contaminants, which represents a major concern for the potential detrimental consequences on the health of the ecosystem and the subsequent provision of goods and services. In particular, the contamination by metals, due to their persistence and toxicity even at low concentrations, represents a serious and widespread environmental problem. Threats for ecosystem health do not rely only upon the concentration of metals in the sediment, but also upon their oxidation/reduction state and their partitioning in the different geochemical phases [3].

The presence of heavy metals in the environment has been a major concern because of their toxicity. Their elimination from wastewater before being released into the environment is important for the maintenance of the ecosystem and from an economic point of view. Techniques such as ion exchange, precipitation, filtration, electrochemical treatment, or reverse osmosis are used to do away with metals such as Cu, Co, Zn, Hg, etc.; however, these methods are rather costly when the metal concentrations are less than 0.01% [4].

The type and the concentration of carbon source and also the C/N ratio, have had a dramatic effect on the rate of heterotrophic denitrification. Microorganisms, water streams, and environmental conditions vary. In contrast, autotrophic denitrifiers utilize inorganic carbon (carbon dioxide or bicarbonate) as a sole source of carbon. Some advantages of autotrophic over heterotrophic denitrification are: avoiding of the poisoning effect of some organic carbon, low biomass buildup and less sludge production which results in reduction of reactor clogging and easier posttreatment. Since some wastewaters have a very low concentration of biodegradable organic materials, autotrophic denitrification requires the addition of an electron donor substrate. Extensive studies have been carried out on elemental sulfur and H<sub>2</sub> as electron donors for autotrophic denitrification systems [5].

Anaerobic ammonium oxidation (anammox) has received special attention because it is an efficient biological alternative to conventional nitrogen removal from wastewaters [6]. Under anaerobic conditions, ammonium is oxidized to nitrogen gas with nitrite as the electron acceptor:  $\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O}$ , and carbon dioxide is used for growth of the anammox microorganisms involved. Compared with traditional nitrification–denitrification process, anammox consumes 100% less biodegradable organic carbon and at least 50% less oxygen and has, therefore, lower operating cost [5]. If the anammox process is combined with a preceding nitrification step, only part of the ammonium needs to be nitrified to nitrite, while the anammox process combines the remaining ammonium with the nitrite to yield dinitrogen gas.

Many studies have revealed that, while conventional cleanup technologies have prevented the contamination problem from spreading, in most cases they are incapable of restoring the water to meet health-based standards in a reasonable time frame. Soil cleanup procedures have been more successful in meeting regulatory standards. However, conventional soil cleanup methods may transfer contaminants to the air, posing risks that are not always acceptable to residents near the contaminated site. The limitations of conventional groundwater cleanup

technologies and the hazards of conventional soil cleanup methods have spurred investigations into in situ bioremediation, which uses microorganisms to destroy or immobilize contaminants in place.

In developing countries, 90% of untreated wastewater goes into the rivers so the access to safe drinking water is limited. The increasing water demand not only affects surface freshwater like rivers and lakes, but it also degrades groundwater resources [5]. The eutrophication and the presence of excess nitrogen in the environment have caused serious alterations of the natural nutrient cycle between the living world and the soil, water, and atmosphere [5]. The intensification of agricultural production and continuous industrial development have contributed to an increase in the nitrate content in drinking water. This is particularly evident in rural areas, where in private wells the concentration of nitrate nitrogen is often over twenty times above the permissible level. Therefore, it is now necessary to develop a technology which effectively reduces nitrate concentration in drinking water [7].

The engineering of bioremediation processes relies on information about the site and about candidate microorganisms. Process analysis usually begins with fixed waste characteristics but with options for microbial cultures, reactor types, waste pretreatment, and process operating conditions. Laboratory measurements are necessary to explore these options and to design an efficient process. These tests examine degradation rates as functions of critical operating parameters such as pH, oxygen and nutrient concentrations, microbial composition, soil particle size, temperature, and redox potential, shaping the design of a bench-scale process. Mass transfer effects such as agitation and aeration are also explored, although at a small scale. These tests constitute the basis for scale-up to the field scale and for the implementation of process control [8]. A main objective of biological remediation design is to remove the limiting factors in the growth of bacteria [9]. The main objective of site characterization is to identify the contaminants, their concentration, and the extent of contamination.

## **2. General characteristics of archaeal species and their potential availabilities to support bioremediation strategies**

The word “archaea” means “ancient things” (from Greek) and it refers to a group of prokaryotic single-celled microorganisms characterized for the extreme conditions they need to be alive. Archaea, which are single-celled prokaryotic microorganisms, were first classified as a separate group of prokaryotes in 1977 by Woese and Fox [10]. Extreme conditions are necessary for archaea to live [10] and extremophiles such as methanogens, thermoacidophiles, halophiles, or alkalophilic microorganisms are included in the group.

Haloarchaea (salt-loving organisms) can grow in media with high salt concentration in a range of 12% to 30% salt (2-5 M NaCl). The cellular machinery of haloarchaea can work even in such high concentration of salt, because it accumulates potassium ion to counteract high concentration of sodium ion. Whereas biodegradation and bioremediation by non-extremophilic microorganisms have been extensively study, the use of extremophilic microorganisms is less studied and particularly haloarchaea.



Enzymes and metabolic pathways specific in archaea were suggested by the comparison between genome sequences of archaea and others [11]. The metabolic pathways of carbohydrates, carbon and nitrogen assimilation or fixation, and sulfur metabolism were involved in the specific pathways, which are different from classical pathways existing in bacteria and eukarya. The enzymes of archaea may be used for the bioremediation.

## 2.1. Carbohydrate metabolism

In archaea, the modified version of the Embden-Meyerhoff-Parnas and Entner-Doudoroff pathways, and pentose degradation pathway have been described [12]. The Embden-Meyerhoff-Parnas is an optimized pathway of glycolysis for the conversion/oxidation of glucose to two molecules of pyruvate yielding ATP and intermediates for other metabolic pathways. The main modifications in this pathway include the presence of the enzymes as ADP-dependent glucokinase and phosphofructokinase, phosphoenolpyruvate synthase, pyruvate:phosphate dikinase or nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase. This modified pathway is present, for example, in members of *Thermococcales* [13], *Archaeoglobales* [14], and *Desulfurococcales* [15]. The main difference of the classical Entner-Doudoroff pathway is that this pathway is divided into two branches: semi-phosphorylated or nonphosphorylated. The highest enzymes of both branches are phosphoenolpyruvate synthase, pyruvate:phosphate dikinase and nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase, as in the Embden-Meyerhoff-Parnas pathway. The semi-phosphorylated pathway is found in haloarchaea [16], and the nonphosphorylated in the genera *Thermoplasma* [17], *Sulfolobus* [18], and *Thermoproteus* [19].

Pentoses are ubiquitous in nature as part of nucleic acids. The degradation of pentoses has been described in some haloarchaea [16, 20] and species for the genera *Sulfolobus* [21]. This pathway in archaea is similar to that identified in bacteria.

## 2.2. Carbon fixation/assimilation

Regarding the assimilation of carbon, different metabolic pathways have been described in archaea. Acetyl-CoA assimilation was performed by ethylmalonyl-CoA pathway and the glyoxylate cycle. In addition to this glyoxylate cycle, several haloarchaea can assimilate acetate [12]. Recently, Khomyakova et al. [22] have proposed a new acetyl-CoA assimilation pathway in *Haloarcula marismortui*, the methyl aspartate cycle.

Metabolic studies of autotrophic archaea have led to the discovery of several different CO<sub>2</sub>-fixation pathways such as the reductive tricarboxylic acid cycle, the Calvin-Benson-Bassham cycle, the 3-hydroxypropionate cycle, and the reductive acetyl-CoA pathway. In anaerobic or microaerobic *Thermoproteales* and *Desulfurococcales*, the dicarboxylate-4-hydroxybutyrate cycle is present [23,24]. The oxygen sensibility of some of the enzymes of this cycle restricts its use to this kind of microorganism. The hydroxypropionate–hydroxybutyrate cycle was identified in members of the order *Sulfolobales* [25].

### 2.3. Nitrogen metabolism

Some archaea species have reductive pathways of nitrogen such as assimilatory pathway (nitrate assimilation and  $N_2$  fixation) and dissimilatory pathway (nitrate respiration and denitrification). Nitrogen metabolism is much less known in archaea than in bacteria. However, in *Haloferax mediterranei* some of these pathways are well known, making it a good candidate microorganism for bioremediation approaches [26-28].

Many archaea are able to reduce nitrate by assimilatory or respiratory pathways through enzymes such as nitrate and nitrite reductases. These enzymes are found in a variety of halophilic and hyperthermophilic archaea [29,30]. Moreover, denitrification has been described for several halophilic archaea, such as *Haloferax* and *Haloarcula* strains [31, 32], and extreme thermophilic archaea, such as *Ferroglobus placidus* [33] and *Pyrobaculum aerophilum* [34]. However, fixation of  $N_2$  in archaea is exclusive of methanogenic euryarchaeota [35]. Glutamine synthetase, glutamate synthase and glutamate dehydrogenase are the major pathways for ammonium assimilation in archaea. For example, both pathways have been identified in *Hfx. mediterranei* [36-38]. Recently, anammox systems have also been described from haloarchaea [39] and some thermophiles [40].

### 2.4. Sulfur compounds metabolism

Many archaea can utilize sulfur compounds as electron donors or acceptors for energy production [41]. Aerobic sulfur oxidation is common in Crenarchaeota (mainly in the order *Sulfolobales*); while anaerobic reduction of  $S^0$  is a widespread ability in the Crenarchaeota and Euryarchaeota phyla [42]. Dissimilatory sulfate, sulfite, and thiosulfate reduction are present in some thermophile genera of Euryarchaeota and Crenarchaeota [43,44]. Anaerobic DMSO respiration has been found in some haloarchaea that can grow anaerobically using DMSO as electron acceptor, such as *Halobacterium* sp. strain NRC-1 [45].

## 3. Bioremediation processes involving haloarchaea species

The severe environment in which haloarchaea can survive makes this archaea a good agent for bioremediation in water treatment processes and in saline and hypersaline environments contaminated with toxic compounds such as nitrate, nitrite and ammonia, chlorine compounds such as perchlorate and chlorate, hydrocarbons, or heavy metals. New advances in the understanding of the haloarchaea metabolism, biochemistry, and molecular biology suggest that general biochemical pathways related to nitrogen (Nitrogen cycle), metals (iron, mercury), hydrocarbons, or phenols can be used for bioremediation processes. With regard to nitrogen species, it is interesting to note that denitrification and nitrification have been described so far as powerful pathways to remove nitrogenous compounds from wastewater. In this context, several bacterial species are shown to be excellent models for removing nitrogenous compounds contained in wastewater. However, the use of archaea to remove nitrogen from wastewater has been poorly studied. Denitrification and anaerobic ammonium oxidation (anammox) carried out by archaea can be practically used as efficient pathways to remove

nitrate, nitrite, or ammonium from soils or wastewater. The existence of anammox (anaerobic ammonium oxidation, a part of the nitrogen cycle) was at first hypothesized based on thermodynamic calculations, and the hypothesis was subsequently confirmed in a pilot denitrifying wastewater treatment plant. Moreover, recent studies have suggested that anammox species are present not only in wastewater but also in marine- and freshwater with limited oxygenation, including oceans, seas, estuaries, lakes, and rivers. For example, the *amoA* gene coding oxidizing enzyme of ammonia was found from Crenarchaeota isolated from suboxic zones of marine environment by metagenomics. These results indicate that the nitrifying archaea can provide sufficient amount of nitrite by anammox under oxygen-limiting conditions. Thus, application of anammox may offer an attractive bioremediation process to current wastewater treatment systems for the removal of ammonia-nitrogen. In order to design a cheap and efficient process to nitrogen loss, anammox reaction could be coupled to nitrification carried out by archaea.

### 3.1. Halophilic archaea in bioremediation of hydrocarbons

Many hydrocarbon contaminated environments are characterized by low or high temperatures, acidic or alkaline pH, high salt concentrations, or high pressure. The studies on the characteristics and degradation of hydrocarbon under these conditions suggest that the presence of extremophilic microorganisms living in extreme environments play an important role in the biological reaction and they could be useful for bioremediation. Halophilic extremophiles seem to be very important to the bioremediation of oil-polluted salt marshes and treatment of industrial saline wastewaters. However, their full potential has not been sufficiently exploited [46]. Information on hydrocarbon degradation under high salt concentration is limited. Moreover, some authors reported the negative impact of increasing salinity on hydrocarbons biodegradation [47]. Most of these studies were performed using halophilic or halotolerant bacteria or bacterial consortia [48], but the potential alternative microorganisms are probably the extreme halophilic archaea.

Extreme halophilic microorganisms belonging to the Archaea Domain present diverse nutritional demand and metabolic pathways; for example, members of *Haloarcula* and *Haloferax* species use a variety of carbohydrates, organic acids as sole carbon and energy sources [49]. However, little is known about the ability of haloarchaea to grow in the presence of hydrocarbons as sole carbon and energy sources, although there is a lot of information related to physiology of microorganisms belonging to the *Halobacteriaceae* family.

#### 3.1.1. Halophilic archaea in bioremediation of crude oil and aliphatic compounds

Zvyagintseva et al. [50] have reported the ability of halophilic archaea to degrade crude oil in hypersaline environments. Significant amount of isoprenoid and *n*-alkane fractions in crude oil were degraded by halophilic-like isolates from the brines of the Kalamkass oil fields in Kazakhstan. Al-Mailem et al. [51] isolated four extreme *Haloferax* archaea strains from the hypersaline coastal area of the Arabian Gulf. They were identified as two *Haloferax* strains, *Halobacterium* sp. and *Halococcus* sp. These halophilic archaea can use crude oil as sole source of carbon and energy, and the growth was enhanced by increasing the NaCl concentration in



the medium. The optimum salt concentration value for growth was between 3.5 and 4.5 M. They also examined the effect of illumination and casamino-acids enrichment on the bioremediation of crude oil using hypersaline soil and red pond water samples from the supertidal “sabkha” coastal area south of Kuwait [52]. The results indicated that addition of casamino-acids and exposure to light enhanced the oil consumption. Additionally, the antibiotics contained in the medium inhibit the growth of most bacteria except archaeal. These results suggest that degradation of hydrocarbons are mainly performed by haloarchaea species.

Some other researchers have reported archaeal ability to metabolize aliphatic hydrocarbons. Table 1 shows the extreme halophilic archaea able to degrade hydrocarbons and their carbon source. Bertrand et al. [53] isolated a halophilic archaeon, strain EH4, from a salt marsh in France. This strain was originally assigned to *Halobacterium* based on its phenotypic and biochemical characteristics but later analysis of the 16S RNA of strain EH4 indicated that it was closely related (99%) to *Haloarcula vallismortis* [54]. This strain was able to metabolize saturated hydrocarbons (tetradecane, eicosane, hexadecane, heneicosane). The growth of EH4 in the medium containing eicosano was salt dependent, growth and degradation was maximum at 20% salinity and nondetectable below 10% salinity. Kulichevskaya et al. [55] have reported the isolation of an archaeon, *Halobacterium* sp. from hypersaline oil-contaminated wastewater in Russia. This strain grew optimally at 15-32% NaCl and showed a high capacity to degrade C<sub>10</sub>-C<sub>30</sub> *n*-alkanes in a medium containing 30% NaCl. The *Haloferax*, *Halobacterium*, and *Halococcus* strains isolated on the basis of crude oil bioremediation [51] also degraded *n*-alkanes and mono- and polyaromatic compounds.

| MICROORGANISM                                                          | HYDROCARBON                                       | REFERENCE |
|------------------------------------------------------------------------|---------------------------------------------------|-----------|
| Strain EH4*                                                            | Tetradecane                                       | [53]      |
|                                                                        | Hexadecane                                        |           |
|                                                                        | Eicosane                                          |           |
|                                                                        | Heneicosane                                       |           |
| <i>Halobacterium</i> sp.                                               | <i>n</i> -Alkane C <sub>10</sub> -C <sub>30</sub> | [55]      |
| <i>Haloferax</i> sp. <i>Halobacterium</i> sp.<br><i>Halococcus</i> sp. | <i>n</i> -Alkane C <sub>10</sub> -C <sub>34</sub> | [51]      |
| <i>Haloarcula</i> sp. <i>Haloferax</i> sp.                             | Heptadecane                                       | [54]      |

\*Bertrand et al. [53] labeled this strain as EH4 and assigned it to *Halobacterium* sp. based on phenotypic features. Tapilatu et al., [54] reassigned it to *Haloarcula vallismortis* based on 16 rRNA

**Table 1.** Extreme halophilic archaea able to biodegrade or grow on aliphatic hydrocarbons

Recently, the effect of vitamin and organic nitrogen on hydrocarbon removal was assessed by using halophilic bacteria and archaea from the Arabian Gulf. Al-Mailen et al. [56] proved the hydrocarbon remediation potential of five archaea in their natural hypersaline environments and how this potential can be enhanced by the addition of certain vitamins to the cultures. The most effective vitamins were thiamine, pyridoxine, and vitamin B<sub>12</sub>. These results were

obtained not only for individual microorganisms in pure cultures but also for microbial consortia. Therefore, the supplement of vitamins could be an effective practice to enhance bioremediation of oil-contaminated hypersaline environments [56]. Tapilatu et al. [54] have reported the isolation of four alkane-degrading halophilic archaeal strains: one (strain MSNC 2) was closely related to *Haloarcula* and the others (strains MSNC 4, MSNC 14, and MSNC 16) were identified as *Haloferax*. These strains could degrade 32-95% of heptadecane when they were cultured in the medium containing 0.5 g/l of heptadecane and 22.5% NaCl for 30 days at 40°C. The strain MSNC 14 was also able to degrade phenanthrene. Otherwise, surfactants and emulsifiers are used to solubilise and disperse hydrophobic compounds. Halophilic archaea could be employed to this purpose. Post and Al-Harjan reported that the ether-linked phytanyl membrane of *Halobacteriaceae*, which showed emulsification properties, was effective in enhancing the efficiency of oil recovery [57].

### 3.1.2. Halophilic archaea in bioremediation of aromatic hydrocarbons

Studies on aromatic hydrocarbons in earlier times were carried out with microorganisms isolated from samples of diverse hypersaline environments. Table 2 shows the typical halophilic archaea which can degrade aromatic compounds. In 1990, Bertrand et al. [53] isolated from salt-marsh an extremely halophilic archaea able to biodegrade aromatic carbons such as acenaphthene, phenanthrene, and anthracene. Later, Emerson et al. [58] described that *Haloferax* strain D1227 could grow in the medium containing monoaromatic compounds such as benzoate, cinnamate, and phenylpropionate by mineralizing as carbon sources, showing the physiological diversity of this group of archaea.

4-Hydroxybenzoic acid is a contaminant in certain highly saline industrial effluents. Fairley et al. [59] examined the metabolism of 4-hydroxybenzoic acid by *Haloarcula* sp. strain D1. The 4-hydroxybenzoic acid was changed to gentisate in the initial ring-cleavage reaction by the strain, although protocatechuic acid, hydroquinone or catechol is produced in case of the common pathways in aerobic bacteria, fungi, and yeast. In order to isolate new halophilic archaea able to grow in aromatic compounds, Cuadros-Orellana et al. [60] chose five different and unrelated hypersaline sites, the Uyini Salar (Bolivia), solar salterns in Cahuil (Chile), solar salterns in Cabo Rojo (Puerto Rico), *sabkhas* (Saudi Arabia), and the Dead Sea (Jordan). In this study, forty-four new halophilic archaea able to grow in 4-hydroxybenzoic acid as sole carbon and energy sources were isolated (Table 2). Taxonomic characterization of these microorganisms revealed that the isolates represent at least four different groups of haloarchaea. They concluded that the ability to metabolize 4-hydroxybenzoic acid is widespread in the *Halobacteriaceae* family, and thus, these haloarchaea microorganisms are excellent candidates to bioremediate aromatic compounds of hypersaline environments and treatment of saline effluents. These authors also determined biodegradation kinetics of strain L1 isolated from the Dead Sea [61], and suggested that the strain L1 could degrade benzoic acid more efficiently than *Haloferax* sp D1227 [58]. Moreover, features about the benzoic acid catabolism were found in the *Haloarcula* sp. L1. When the strain L1 was grown in the medium containing benzoic acid, gentisic acid was produced, which was not usual in other microorganisms. Therefore, gentisic acid is an intermediate in the degradation of benzoic acid, hydroxybenzoic acid, cinnamate, and

phenylpropionate by the archaea *Haloferax* sp. D1227, and in the degradation of 4-hydroxybenzoic acid by the *Haloarcula* sp. strain D1 [59].

In relation to metabolism of the aromatic compounds in haloarchaea, the gentisate-1,2-dioxygenase genes (*gdoA*), which correspond to the ring-cleavage enzyme of gentisic acid, of *Haloferax* sp. D1227 and *Haloarcula* sp. strain D1 were cloned. Surprisingly, the expression pattern of the genes is different: in *Haloarcula* sp. D1, *gdoA* is expressed in the presence of 4-hydroxybenzoate but not benzoate; however, *gdoA* is expressed in *Haloferax* sp. D1227 during growth on benzoate, 3-hydroxybenzoate, cinnamate, and phenyl-propionate. Moreover, genes of Co-A synthetize (*acdB*) subunit and CoA-thioesterase (*tieA*) also existed at the upstream of the *gdoA* gene. The pattern of these genes expression is also different between the two species, obtaining only expression of *acdB* and *tieA* in *Haloferax* sp. D1227 during growth on benzoate, cinnamate, and phenylpropionate, but not on 3-hydroxybenzoate. This suggests that *acdB* and *tieA* are part of benzoate degradation pathway in *Haloferax* sp. D1227, while the *gdoA* genes encode part of a 4-hydroxybenzoate and 3-hydroxybenzoate pathways in *Haloarcula* sp. D1 and *Haloferax* sp. D1227, respectively [62].

During the last four years, the number of studies which describe the degradation of aromatic compounds by halophilic archaea have increased. As already mentioned, Tapilatu et al. [54] isolated four haloarchaea from hypersaline environment. The MSNC 14, one of the strains (Table 2), was able to degrade 43% of phenanthrene after 30 days of incubation, although the degradation of anthracene and dibenzothiophene was not detected. Al-Mailen et al. [51] isolated four strains (Table 2) from a hypersaline coastal area for the Arabian Gulf on a mineral salt medium containing crude oil vapor as unique source of carbon and energy. The four strains were able to biodegraded not only aliphatic hydrocarbons but also aromatic hydrocarbons after three weeks of incubation. In particular, *Halobacterium* and *Halococcus* could grow in the presence of benzene, toluene, and p-hydroxybenzoic acid, and the two *Haloferax* strains could grow with toluene and phenanthrene, and one of them also with benzene, but both failed to grow on p-hydroxybenzoic acid. This study also revealed that the biodegradation rates increased in proportion to NaCl concentration in the medium, and thus supported the idea that extreme halophilic archaea are suitable biological material to bioremediate oil-polluted hypersaline environments. Bonfá et al. [63] revealed the usefulness of halophilic archaea in the bioremediation of wastewater of petroleum production, which contained high saline concentration and various aromatic acids and hydrocarbons. Aromatic compounds (p-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene) could be also degraded by nine halophilic archaea (Table 2) isolated from Çamalti Saltern, Turkey [64]. This study broadens the understanding of metabolism of aromatic compounds, and the activities of catechol 1,2 dioxygenase and protocatechuate 3,4 dioxygenase were identified as the enzymes involved in *ortho* cleavage pathway. *Ortho* cleavage pathway is widely distributed in soil bacteria and fungi, constituting the major pathway for aromatic compounds catabolism in these organisms. Therefore, these enzymes may be important to remove aromatic hydrocarbons.

These results suggest the following: *Halobacteriaceae* family, which can degrade aromatic compounds, exists widely, and can be used to degrade aromatic compounds in oil-polluted hypersaline environments [60]. Thus, the bioremediation process using those strains is

promising, as the remediation processes using physical and chemical methods are complicated and expensive [65]. However, more precise understanding of the mechanism of carbon-cycling cleavage and their enzymes and genes may be necessary to achieve the bioremediation [66].

| MICROORGANISM                    | AROMATIC COMPOUNDS                                             | REFERENCE |
|----------------------------------|----------------------------------------------------------------|-----------|
| Strain EH4*                      | Acenaphthene<br>Phenanthrene<br>Anthracene                     | [53]      |
| <i>Haloferax</i> strain D1227    | Benzoate<br>Cinnamate<br>Phenylpropionate                      | [58]      |
| <i>Haloarcula</i> sp.            | 4-hydroxybenzoic acid                                          | [59]      |
| <i>Halobacterium</i> species     | 4-hydroxybenzoic acid                                          | [60]      |
| <i>Haloferax</i> species         | 4-hydroxybenzoic acid                                          | [60]      |
| Strain L1                        | Benzoate<br>4- hydroxybenzoic acid                             | [61]      |
| <i>Haloferax</i> strain MSNC14   | Phenanthrene                                                   | [54]      |
| <i>Haloferax</i> sp. strain HA-1 | Benzene<br>Toluene<br>Phenanthrene<br>Naphthalene              | [51]      |
| <i>Haloferax</i> sp. strain HA-2 | Toluene<br>Phenanthrene<br>Naphthalene                         | [51]      |
| <i>Halobacterium</i> sp. HA-3    | Benzene<br>Toluene<br>p-Hydroxybenzoic acid                    | [51]      |
| <i>Halococcus</i> sp. HA-4       | Benzene<br>Toluene<br>Naphthalene<br>p-Hydroxybenzoic acid     | [51]      |
| <i>Halorubrum ezzemoulense</i>   | p-Hydroxybenzoic acid<br>Naphthalene<br>Pyrene<br>Phenanthrene | [64]      |
| <i>Halorubrum</i> sp.            | p-Hydroxybenzoic acid<br>Naphthalene<br>Pyrene<br>Phenanthrene | [64]      |

| MICROORGANISM                   | AROMATIC COMPOUNDS                                             | REFERENCE    |
|---------------------------------|----------------------------------------------------------------|--------------|
| <i>Haloarcula hispanica</i>     | p-Hydroxybenzoic acid<br>Naphthalene<br>Pyrene<br>Phenanthrene | [64]         |
| <i>Halobacterium salinarum</i>  | p-Hydroxybenzoic acid<br>Naphthalene<br>Pyrene<br>Phenanthrene | [64]<br>[56] |
| <i>Halobacterium piscisalsi</i> | p-Hydroxybenzoic acid<br>Naphthalene<br>Pyrene<br>Phenanthrene | [64]<br>[56] |
| <i>Haloferax mucosum</i>        | Phenanthrene                                                   | [56]         |
| <i>Haloferax lucentense</i>     | Phenanthrene                                                   | [56]         |
| <i>Haloferax sulfurifontis</i>  | Phenanthrene                                                   | [56]         |

\*Bertrand et al. [53] labeled this strain as EH4 and assigned it to *Halobacterium sp.* based on phenotypic features. Tapilatu et al. [54] reassigned it to *Haloarcula vallismortis* based on 16 rRNA.

**Table 2.** Extremely halophilic archaea able to biodegrade or grow on aromatic compounds

**3.2. Halophilic archaea in bioremediation of heavy metal ion**

Some heavy metals such as iron, cobalt, copper, manganese, molybdenum, and zinc are trace elements required at a certain level, and thus are necessary for life. However, they are excessively damaging to organisms. Other heavy metals such as mercury, aluminium, cadmium, gold, lead, and arsenic are toxic and are not beneficial to organisms. Both essential and nonessential metals at high concentration, directly or indirectly compromise DNA, protein, and membrane integrity and function [67, 68].

Frequently, heavy metals are found in saline and hypersaline environments due to the evaporation in such environments and also as a result of industrial activities. Therefore, some halophilic archaea have developed tolerance to heavy metals. Wang et al. [69] reported that the *Halbacterium sp.* NRC-1, which had a plasmid carrying genes of arsenite and antimonite extrusion system, showed high resistance to arsenic. Kaur et al. [70] studied the haloarchaeal strategies of adaptation to high metal concentration, using *Halobacterium sp.* NRC-1 as model organism. Sublethal levels of Mn(II), Fe(II), Co(II), Ni(II), Cu(II), and Zn(II) were used to investigate the change on transcriptional level using microarray technology. All growths were inhibited at high concentrations of metals, but the susceptibility was different. Consequently, the effective inhibitory concentrations of Zn (II), Fe (II), Co (II), Cu (II), and Mn (II) were 0.05, 7.5-8.5, 0.6, 1.2, and 2 mM, respectively. Some of the adaptation mechanisms detected include previously known mechanisms such as efflux of metal ions by P1 ATPases, downregulation



of Mn(II) uptake, ion scavenging, protein turnover increase, and minimizing ROS production depletion. Novel discoveries include: control of transcriptional regulation by a TRASH domain, ability of ZntA to confer resistance to several metals, a global control mechanism mediated by GTFs and key metalloregulatory proteins, and simulation of Fe deficiency by Mn(II) [70]. Authors developed a systems-level model to provide an integrated perspective of responses to these metals.

Srivastava et al. [71] have reported the intracellular synthesis of silver nanoparticles by the haloarchaeal isolated *Halococcus salifodinae* BK<sub>3</sub> when the cells were grown in the medium containing silver nitrate. They also described the intracellular synthesis of selenium nanoparticles (SeNPs) by the haloarchaeon *Halococcus salifodinae* BK18, when the cells were grown in the presence of sodium selenite. Also, cadmium tolerance has been reported in haloarchaeal strains from solar salterns of Ribandar and Siridao in India [73].

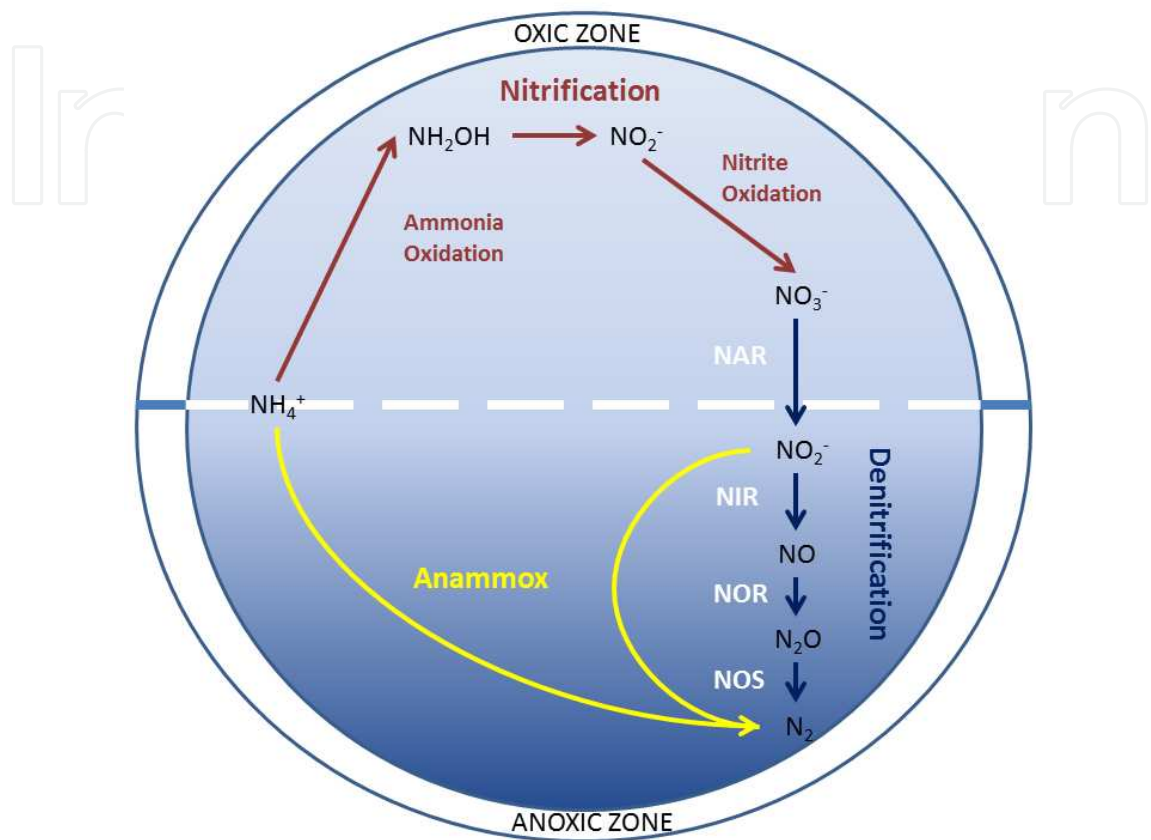
Biosorption of metals by the organisms at the surface or by the exopolysaccharides (EPS) secreted to form the biofilms enables organisms to tolerate metals [74]. The adsorption of heavy metal by EPS has been attributed to interaction between metal cations and negative charges of acidic functional groups of EPS [75]. Kawakami et al. [76] found that *Halobacterium salinarum* CCM 2090 has a Ca(II)-dependent aggregation system. Calcium ion is adsorbed on the surface of the cells and induces ionic cross-bridging between the EPS, resulting in aggregation of the haloarchaeal cells. Mn<sup>2+</sup>, Fe<sup>3+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, and Zn<sup>2+</sup> could replace Ca<sup>2+</sup>. However, Mg<sup>2+</sup>, Sr<sup>2+</sup>, Mo<sup>2+</sup>, Cd<sup>2+</sup>, Sn<sup>2+</sup>, Hg<sup>2+</sup>, and Pb<sup>2+</sup> induced no flocculation of cells of this halophilic archaeon. In addition, Popescu and Dumitru [75] reported the two *Haloferax* strains had the capacity to reduce the concentration of Pb, Cr, Zn, and Ni ions from media with high salinity, by biosorption process. Knowledge regarding molecular mechanisms underlying resistance to metal is cursory. Therefore, more precise understanding of the mechanism is necessary to facilitate the use of haloarchaea for bioremediation of metal-polluted hypersaline environments [74].

### 3.3. Halophilic archaea in bioremediation of wastewater

Wastewater treatments (WWT), such as breakdown of sewage influent, are generally performed by microorganisms. These microorganisms are able to live in the sludge of treatment plants and holding tanks. They obtain nutrients by degrading the solids in WWT to various compounds. Some wastewater treatment systems are efficient and desirable from an economical point of view. However, other systems are not efficient because of the undesirable effects the system itself promote on the microorganisms. Therefore, the establishment of optimal conditions (such as nutrients, pH, temperature, and oxygen availability) for comfortable growth is most important in order to treat WWT effectively.

Modern biological treatment of wastewater involves not only C removal, but also elimination of other nutrients such as P and N. Combined and sequential actions are required for such treatment successively by several groups of microorganisms, such as heterotrophic bacteria, phosphate-accumulating organisms (PAO), or microorganisms able to perform nitrification, denitrification, or anammox [77]. However, it is difficult to design this kind of treatment process, because the system becomes extremely complex to exhibit a satisfactory performance

and it requires expensive costs from economical point of view. Figure 1 shows the nitrogen transformation pathways by archaea. Archaea, which can degrade ammonia, are now one of the main candidates for wastewater treatment.

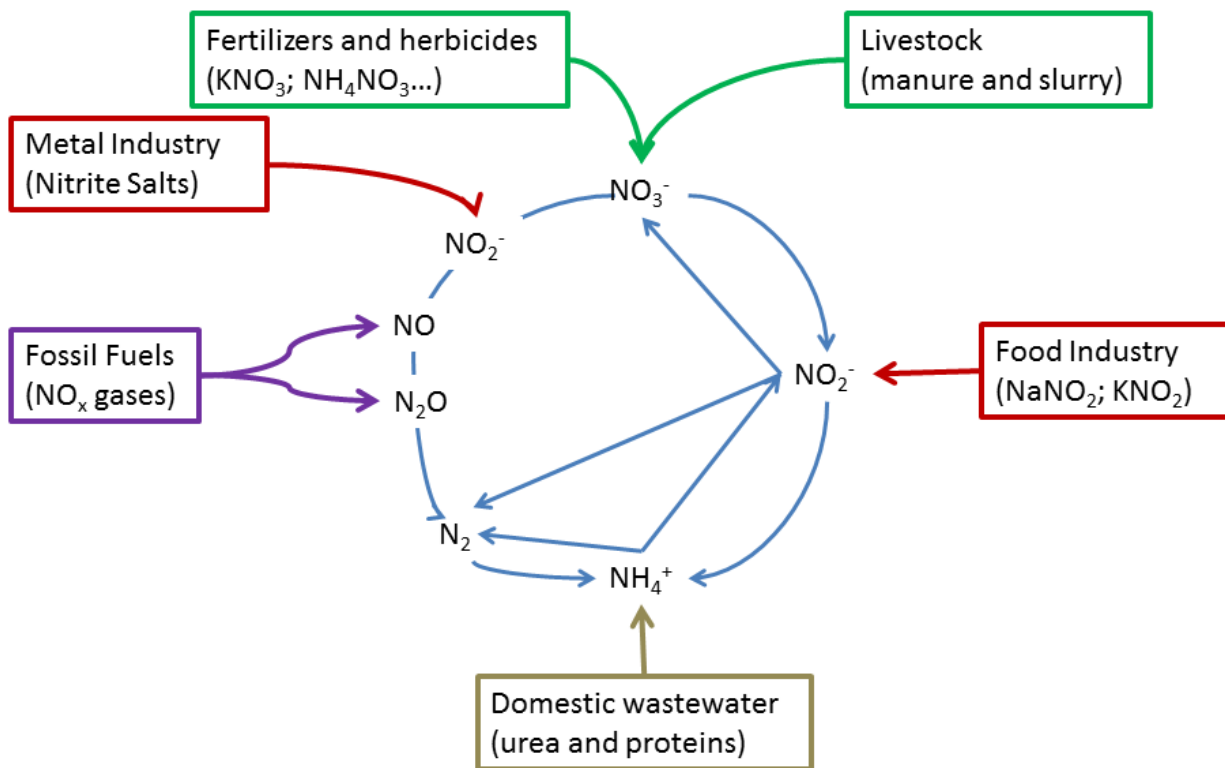


**Figure 1.** Archaeal nitrogen transformations in wastewater treatment environment. The enzymes involved in denitrification process are shown in the figure: NAR, nitrate reductase; NIR, nitrite reductase; NOR, nitric oxide reductase; NOS, nitrous oxide reductase.

Although there are natural microorganisms used in wastewater treatment, the bioremediation process requires further addition of various types of microorganisms known as bioremediators. Since there is an organism available to treat any organic molecule (the microorganism does this by extracting the energy from the molecule bonds), it is a very effective treatment. However, it is essential to distinguish the types of microorganisms present in wastewater as well as the pollutants to be removed and where they are to be located in the wastewater process. When these details are known, it is quite easy to select the best microorganisms to be used along with their best locations in the wastewater treatment processing plant.

Figure 2 shows the nitrogen sources produced by artificial activities and their metabolic cycles. The wastewater containing excess nitrogen compounds is constantly discharged from houses and factories, and overall nitrogen species in wastewater and soil are increased. Due to the accumulation of this nitrogen species ( $\text{NH}_4^+$ ,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$ , etc.), in groundwater or tap water, the wastewater should be removed or reused [81, 82]. Conventionally,

biological nitrogen removal is achieved by nitrification followed by a denitrification process: firstly, aerobic nitrification of  $\text{NH}_4^+$  to  $\text{NO}_2^-$  or  $\text{NO}_3^-$  with  $\text{O}_2$  as the electron acceptor; secondly, anoxic denitrification of  $\text{NO}_2^-$  or  $\text{NO}_3^-$  to gaseous  $\text{N}_2$  using organic matter as carbon and energy source [82].



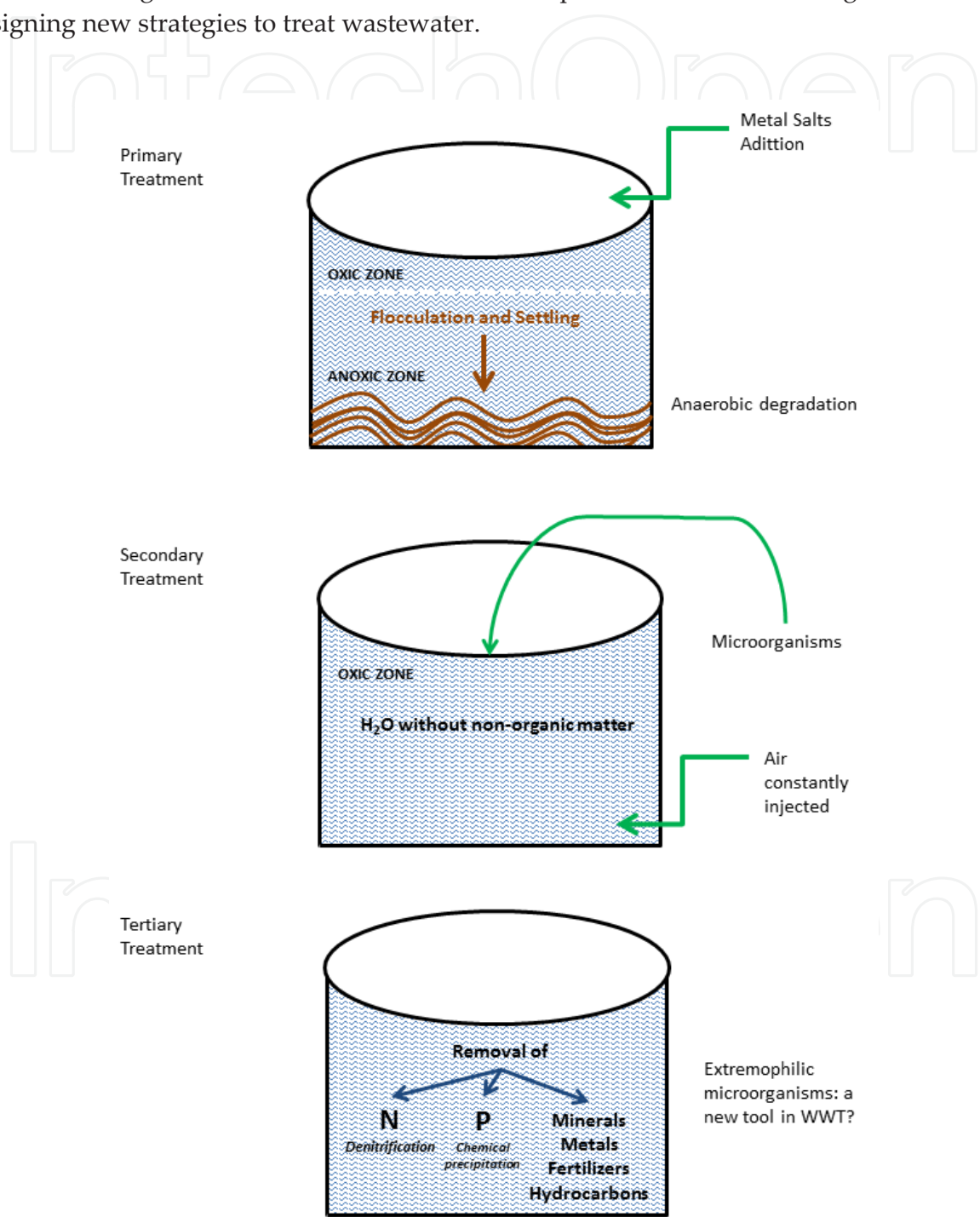
**Figure 2.** N-cycle scheme. The various sources of pollution of nitrogen cycle species are represented.

Figure 3 shows the typical processes in wastewater plant. Treatments were performed by following three processes:

- Primary Treatment:** Flocculation, setting, and anaerobic degradation are carried out in the primary treatment. The solid matter in wastewater containing metal salt is clumped together (flocculated) by storing in the settling tank, and they finally precipitated and composed sludge at the bottom of the tank. The sludge is then subjected to anaerobic degradation. Methane gas, which is used as a fuel, is produced during the degradation, and the efficiency can be enhanced by the addition of suitable anaerobic microorganisms. Flocculation, settling, and anaerobic degradation are essential to remove the nonorganic matter before the secondary treatment.
- Secondary Treatment:** degradation by microorganisms is aerobically carried out in the secondary treatment. The residual effluent is moved to the aeration tank using pumps, and the remaining suspended solids (including fecal matter) are degraded by the addition of microorganisms and the supplement of air. The values of biological organic matter (BOD) can be decreased by this process.

- c. Tertiary Treatment: this treatment removes the nitrogen (mainly through denitrification processing) and phosphates (usually by chemical precipitation from the effluent).

Other compounds such as minerals or metals (such as iron, sulfur, manganese), and runoff pollutants such as fertilizers, hydrocarbons, and tar can also be removed. Therefore, extremophilic microorganisms able to deal with these compounds have become of great interest in designing new strategies to treat wastewater.



**Figure 3.** Primary, secondary (the tank contains the supernatant followed by primary treatment), and tertiary treatments used in wastewater treatment plants.

Activated sludge process is currently the most used for the treatment of both domestic and industrial wastewater. The bioreactor has two chambers: one in which the wastewater is agitated and maintained at constant oxygen, where microorganisms degrade the contaminants; second, where the biologically treated water settles to form a sludge that will be removed. Due to the characteristics of the process, the decantation system limits the efficiency of the treatment as a result of problems such as the production of foam, floatability of the sludge, or the large size required for the installation of the wastewater treatment plants [78].

Alternative treatment technologies are thus of great interest when searching for more efficient strategies regarding nutrient removal and the generation of effluents with enough quality for direct reclamation. One new treatment is based on membrane bioreactors (MBRs) (Figure 4): the MBR system consists of a cylindrical bioreactor equipped with two ultrafiltration membrane units submerged inside the aerated bioreactor, and the extraction of the effluent water takes place by mechanical suction. The membranes are continuously aerated, maintain solids in suspension, and supply oxygen to the process [78].

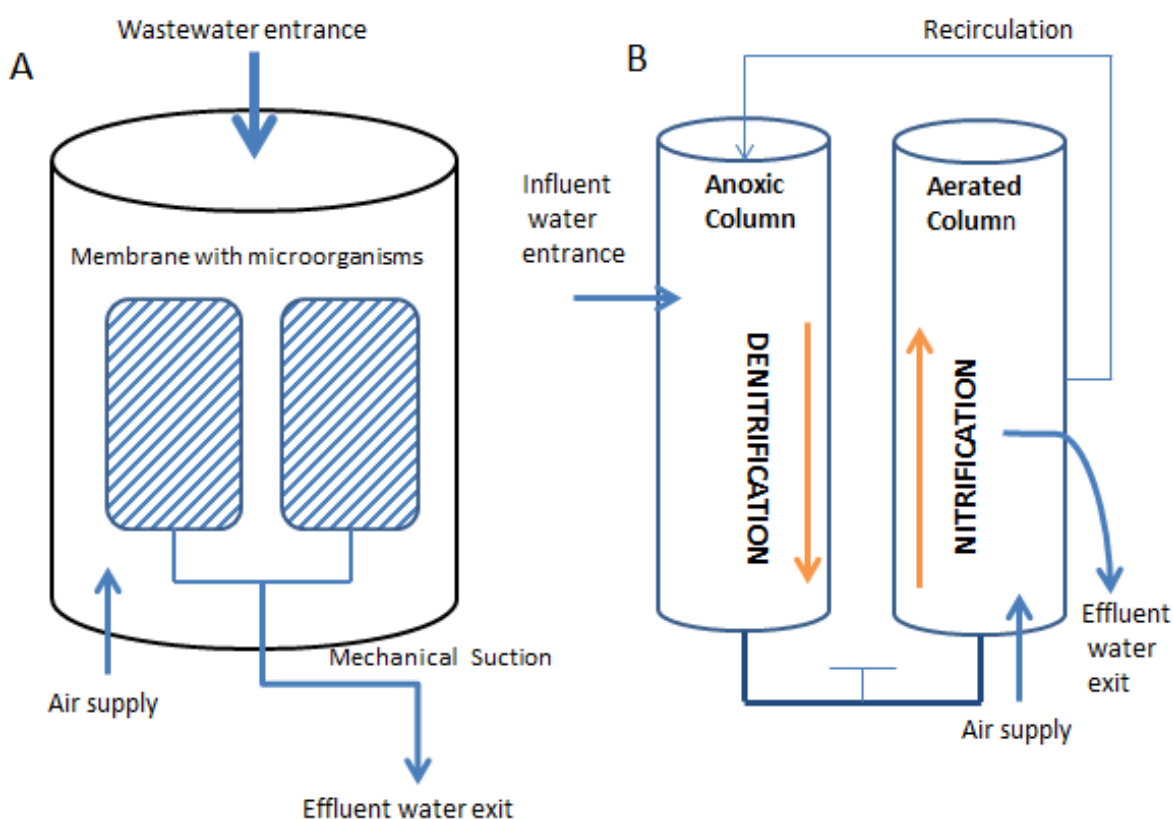
Submerged biofilters (SBs) (Figure 4) are another reduced-size and low-cost alternative of proven efficiencies for the design of WWTPs, which are of simple control and maintenance, and minimize undesirable odors and noise in the vicinity of the installations. SBs consist of two methacrylate cylindrical columns each, packed with clayey schists biofilm support [79, 80]. The columns are connected with a valve that allows a separated cleaning of the biofilters. They operate downflow (denitrifying column, anoxic) and upflow (nitrifying column, aerated) [78].

Another recent process for nitrogen removal has gained importance in the last years: the anaerobic ammonium oxidation (anammox). This reaction is based on energy conversion from anaerobic ammonium oxidation using nitrite as the electron acceptor. It was discovered in 1995 in a pilot plant treating wastewater at Gist-Brocades, Delft, in The Netherlands [7, 83]. This process offers a novel, energy-saving and cost-effective biological nitrogen removal technique.

In the past years, partial nitrification, anammox and denitrification simultaneously in a single reactor (SNAD technology), was developed for the complete removal of nitrogen sources. Under oxygen limitation, ammonium is oxidized to nitrite by aerobic ammonium oxidation; the nitrite in the reactor can be used by anammox microorganisms with ammonium, and finally to dinitrogen gas with small amounts of nitrate produced. Afterward, COD as electron donor can deoxidize nitrate to dinitrogen gas through denitrifying process for the complete nitrogen removal performance. The interaction of aerobic nitrifying, anaerobic ammonium oxidizing, and anaerobic denitrifying microorganisms under oxygen limitation has the potential to make an almost complete conversion of ammonium and organic carbon to dinitrogen gas and carbon dioxide [84].

Although the importance of eukaryotic and bacteria organisms in aerated activated sludge has long been recognized, the role played by archaea in aerobic and anaerobic WWTPs has not been mentioned. However, recent researches suggest that growth and activity of archaea were significant in the treatment of activated sludge and wastewater [85-87]. The roles of methanogenic archaea within a broad range of activated sludge, submerged biofilters, and membrane





**Figure 4.** Schematic diagram of the two new types of WWTPs. (A) Submerged membrane bioreactor (MBR). (B) Submerged biofilter (SB).

bioreactors have been studied in recent research [78, 88-89]. Under oxic conditions, no methanogenesis was detected; however, once oxygen is depleted, methane production ensued. The results suggest that methanogenic archaea can be activated under anoxic conditions [88].

The composition of the wastewater is a key determinant of archaeal community composition in WWTPs [88, 90-91]: the microbial population in industrial wastewater treatments, rich in ammonia, phenol, and with high salinity, are closely related to *Methanobrevibacter smithii*, the predominant methanogen in human intestines [78]. The manufacturing of chemical compounds (pesticides, herbicides, explosives, etc.) usually generates effluents containing complex mixtures of salts and nitrate or nitrite. Also, the increase of salinity in soils and waters in the last few decades has given advantage to some species like *Hfx. mediterranei*. For example, *Hfx. mediterranei* is resistant to very high nitrate (up to 2 M) and nitrite (up to 50 mM) concentrations, which are the highest described from a prokaryotic microorganism [92]. Therefore, it could be useful for bioremediation applications in sewage plants where high salts, nitrate, and nitrite concentrations are detected in wastewaters and brines. In a recent study [93], this haloarchaea was able to eliminate 60% of the nitrate and 75% of nitrite initially present in the brines (initial concentration was 40 mM nitrite). Moreover, it has also been described that nitrate reductase involved in denitrification reduces efficiently other oxyanions such as bromate and (per) chlorate [94]. These results suggest that *Hfx. mediterranei*, and in general, halophilic archaea, are able to carry out denitrification, thus providing excellent models to

explore large-scale bioremediation processes to remove nitrogen compounds from brines and salty water.

## 4. Conclusions

Bioremediation provides a technique for cleaning up pollution by enhancing the same biodegradation processes that occur in nature. Depending on the site and its contaminants, bioremediation may be safer and less expensive than alternative solutions such as incineration or land filling of the contaminated materials. It also has the advantage of treating the contamination in place so that large quantities of soil, sediment, or water do not have to be dug up or pumped out of the ground for treatment. Therefore, bioremediation is considered as of now one of the best options to treat contaminated environments. Taking into account the amazing metabolic features that define haloarchaea metabolism, these microorganisms may become good candidates to improve bioremediation procedures, or even new bioremediation strategies could be defined using them. Thus, aerobic and anaerobic haloarchaea could be considered to design co-metabolic in situ bioremediation to remediate different pollutants in water and soils. Although the potential use of haloarchaea in bioremediation has been extensively demonstrated, different aspects of their metabolism remain poorly known. Therefore, more studies from biochemical and molecular biology points of view are required to properly comprehend haloarchaeal metabolism regulation. Moreover, new niches and extreme microecosystems in terms of temperature, salt concentration, and pH should be explored to identify and locate new microorganisms able to deal with heavy metals, hydrocarbons, chlorinated compounds, and, in general, all pollutants affecting soil and water.

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