

# Microbial degradation of naphthenic acids using constructed wetland treatment systems: metabolic and genomic insights for improved bioremediation of process-affected water

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Editor: [Marcus Horn]

## Abstract

Naphthenic acids (NAs) are a complex mixture of organic compounds released during bitumen extraction from mined oil sands that are important contaminants of oil sands process-affected water (OSPW). NAs can be toxic to aquatic organisms and, therefore, are a main target compound for OSPW. The ability of microorganisms to degrade NAs can be exploited for bioremediation of OSPW using constructed wetland treatment systems (CWTS), which represent a possible low energy and low-cost option for scalable *in situ* NA removal. Recent advances in genomics and analytical chemistry have provided insights into a better understanding of the metabolic pathways and genes involved in NA degradation. Here, we discuss the ecology of microbial NA degradation with a focus on CWTS and summarize the current knowledge related to the metabolic pathways and genes used by microorganisms to degrade NAs. Evidence to date suggests that NAs are mostly degraded aerobically through ring cleavage via the beta-oxidation pathway, which can be combined with other steps such as aromatization, alpha-oxidation, omega-oxidation, or activation as coenzyme A (CoA) thioesters. Anaerobic NA degradation has also been reported via the production of benzoyl-CoA as an intermediate and/or through the involvement of methanogens or nitrate, sulfate, and iron reducers. Furthermore, we discuss how genomic, statistical, and modeling tools can assist in the development of improved bioremediation practices.

**Keywords:** bioremediation; microbial remediation; naphthenic acid; oil sands process-affected water; wetland treatment system

## Glossary

- ACA: 1-adamantanecarboxylic acid.
- AEOs: acid extractable organics (all extractable polar compounds).
- Alpha-oxidation: loss of the alpha-carbon, which is the first carbon atom that attaches to a functional group such as carbonyl (C=O).
- Aromatization: aromatic ring formation from a non-aromatic precursor.
- Beta-oxidation: oxidation of the beta-carbon, which is the second carbon atom that attaches to a functional group, leading to the formation of a carboxylic acid with two carbons less than its precursor.
- CHCA: cyclohexanecarboxylic acid.
- Commercial NAs: naphthenic acids obtained from petroleum distillates that are used in the industry as preservatives, surfactants, paint driers, and other purposes.
- CWTS: constructed wetland treatment systems.
- NA: naphthenic acid.
- NAFCs: naphthenic acid fraction compounds; interchangeable with AEOs.
- Omega-oxidation: when the terminal methyl group (CH<sub>3</sub>) is oxidized to form a carboxyl group (COOH), resulting in carboxylic acids that can then be degraded by beta-oxidation.
- OSPW: oil sands process-affected water.
- OSTWAEOs: oil sands tailings water acid-extractable organics (all extractable polar compounds in OSPW).
- Surrogate NA: an individual model NA that is commercially available.

Received 31 May 2023; revised 27 October 2023; accepted 23 November 2023

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- Tertiary carbon: a carbon atom that is connected to three other carbon atoms. The tertiary carbon blocks beta-oxidation.

## Introduction

Bitumen extraction from oil sands during surface mining operations uses a hot water extraction process that results in the production of large volumes of oil sands process-affected water (OSPW, Nat. Res. Canada 2023). OSPW contains a number of organic and inorganic components that can vary depending on the source. Among the organic compound classes in OSPW are naphthenic acids (NAs), a complex mixture of carboxylic acids that are formed over geological time and are naturally present in crude oils, including bitumen (Clemente and Fedorak 2005). NAs can be toxic toward various organisms (Li et al. 2017 and references therein), are difficult to degrade, and persist in the OSPW environment. NA concentrations range from 20 to 120 mg/L in OSPW (Greuer et al. 2010). In Canada, the oil sands industry is a vital part of the economy and despite interest in investing in renewable energy, international demand and oil sands production are expected to increase for years to come (Natural Resources Canada, 2023).

The chemical characteristics of NAs have been extensively described (e.g. Clemente and Fedorak 2005, Ajaero et al. 2020). According to the classical definition, NAs are alkyl-substituted acyclic and cycloaliphatic carboxylic acids which fit the chemical formula  $C_nH_{2n+z}O_2$ , where  $n$  indicates the number of carbon atoms (which can range from 7 to 30) and  $z$  is a negative even integer that indicates the hydrogen deficiency due to ring cyclization (Clemente and Fedorak 2005). However, molecules containing more than two oxygen atoms ( $C_nH_{2n+z}O_x$ , oxy-NAs) or heteroatoms such as sulfur ( $C_nH_{2n+z}SO_x$ ) or nitrogen ( $C_nH_{2n+z}NO_x$ ) are also common in OSPW (Tomczyk and Winans 2001). In the literature, 'naphthenic acid fraction compounds (NAFCs)' has been used to describe all polar acids, including classical NAs, that can be extracted from OSPW (Ajaero et al. 2020, Vander Meulen et al. 2021). Other studies have used the term 'acid extractable organics (AEOs)' (e.g. Ahad et al. 2020, Hewitt et al. 2020) and 'oil sands tailings water acid-extractable organics (OSTWAEOs)' to refer to all extractable polar compounds in OSPW (Greuer et al. 2010). Furthermore, the term 'commercial NAs' is used to designate the NA preparations obtained from petroleum distillates for use as preservatives, paint driers, emulsifiers, surfactants, and in the manufacture of tires (Scott et al. 2005). The molecular structure of NAs is diverse (Fig. 1). NAs can be linear, branched aliphatic, or can have complex multi-ring structures with  $z$  values reaching -12 (a 6-ring structure) (Clemente and Fedorak 2005). OSPW has also been shown to contain a variety of other types of petroleum acids that do not fall under the classical definition of NAs, such as tricyclic diamondoid acids (Rowland et al. 2011b) and aromatic carboxylic acids (Rowland et al. 2011a).

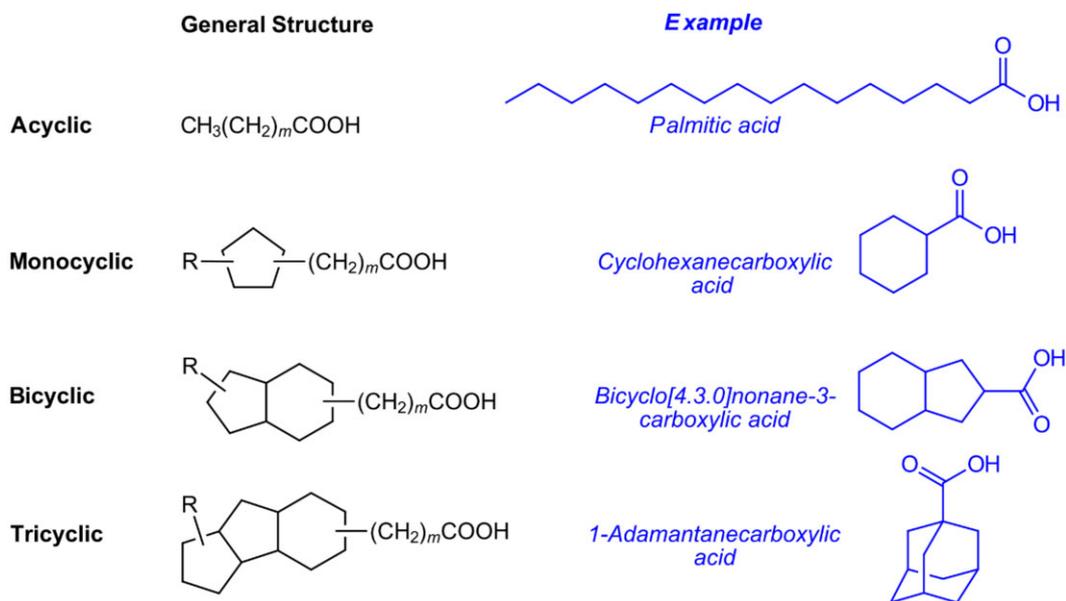
Exposure to NAs can result in physiological changes in aquatic organisms, including narcosis, cellular damage, and impaired embryonic development (Li et al. 2017, Gutierrez-Villagomez et al. 2019). Besides its toxicity towards a variety of organisms, NAs have corrosive properties and can form precipitates that block pipelines (Barrow et al. 2009, Dias et al. 2014). Consequently, government regulations in Canada require that "all fluid tailings should be ready to reclaim within 10 years of the end of a mine's life" (Alberta Energy Regulator 2022). Therefore, there is a major interest by industry to reduce the level of NAs and toxicity of OSPW.

The removal of toxic compounds from OSPW is one of the major challenges of oil sands tailings reclamation. Numerous technologies from wastewater treatment have been proposed and studied for OSPW treatment, including adsorption, advanced oxidation, coagulation/flocculation, filtration, and photodegradation (Quinlan and Tam 2015, Qin et al. 2019). However, questions remain about the scalability, economic viability, and sustainability of these approaches at the level required to be impactful for the oil sands industry (Kannel and Gan 2012, Scott et al. 2020). In this regard, bioremediation represents a low energy and cost-effective strategy for reducing NA concentrations and associated toxicity of OSPW (Kannel and Gan 2012). The degradation of NAs is primarily through metabolic pathways that involve enzymatic degradation, leading to eventual mineralization or transformation of NAs into less toxic compounds (Whitby 2010, Yue et al. 2015). This ability of microorganisms to degrade NAs offers potential as a sustainable bioremediation approach for OSPW and is highly scalable through, for example, constructed wetland treatment systems (CWTS). CWTS are engineered ecosystems designed to mimic the natural processes of wetlands to treat and purify polluted water. A combination of physical, chemical, and biological processes including microbial and plant-mediated transformation are active in CWTS to remove contaminants from the water before it is released or reused (Fig. 2). There are many challenges involved in the development of efficient OSPW remediation through CWTS, such as the presence of recalcitrant NAFCs, low microbial growth, and low oxygen conditions (average of  $2.5 \text{ mg L}^{-1}$  in North American Database (Kadlec 1995),  $1.33\text{--}8.22 \text{ mg L}^{-1}$  (Simair et al. 2021),  $2.65\text{--}6.8 \text{ mg L}^{-1}$  (McQueen et al. 2017)). Region-specific challenges, such as a cooler climate in Canada, also limit efficient OSPW treatment. Therefore, a clear understanding of the ecology, metabolism, and genomic potential of NA-degraders is required to develop solutions for optimized OSPW bioremediation. These solutions could involve biostimulation of microbial growth and metabolism through manipulation of nutrient availability, as well as bioaugmentation with microorganisms capable of degrading recalcitrant NAs or that demonstrate enhanced NA degradation under specific temperature, oxygen concentration, and pH conditions.

Although the NA-degrading potential of microorganisms was reported decades ago (e.g. Rho and Evans 1975, Blakley and Pappish 1982), recent advances in analytical chemistry and in genomics have provided more information on the identity and ecology of NA-degrading microorganisms (e.g. Whitby 2010, Yue et al. 2015, Skeels and Whitby 2019), as well as on metabolic pathways and genes that have potential roles in NA degradation. Here, we summarize current knowledge on the ecology, metabolism, and genomics of microbial NA degradation and CWTS. We also offer suggestions for future research approaches that could be used to provide additional insight and optimization of the biodegradation of NAs.

## Diversity and ecology of microorganisms involved in NA degradation

Understanding the diversity and ecology of NA-degrading microorganisms is critical given the complex nature of environmental NA mixtures and dynamic field conditions, particularly in northern Canada. Skeels and Whitby (2019) summarized the bacterial and archaeal diversity across different NA-impacted environments. They reported a relatively large diversity, with a dominance of microorganisms of the Proteobacteria phylum. Most taxa



**Figure 1.** Examples of classical ( $\text{O}_2$ ) naphthenic acids.  $m$  is the number of  $\text{CH}_2$  units and  $R$  is a small aliphatic group such as a methyl group. Adapted from Whitby (2010).

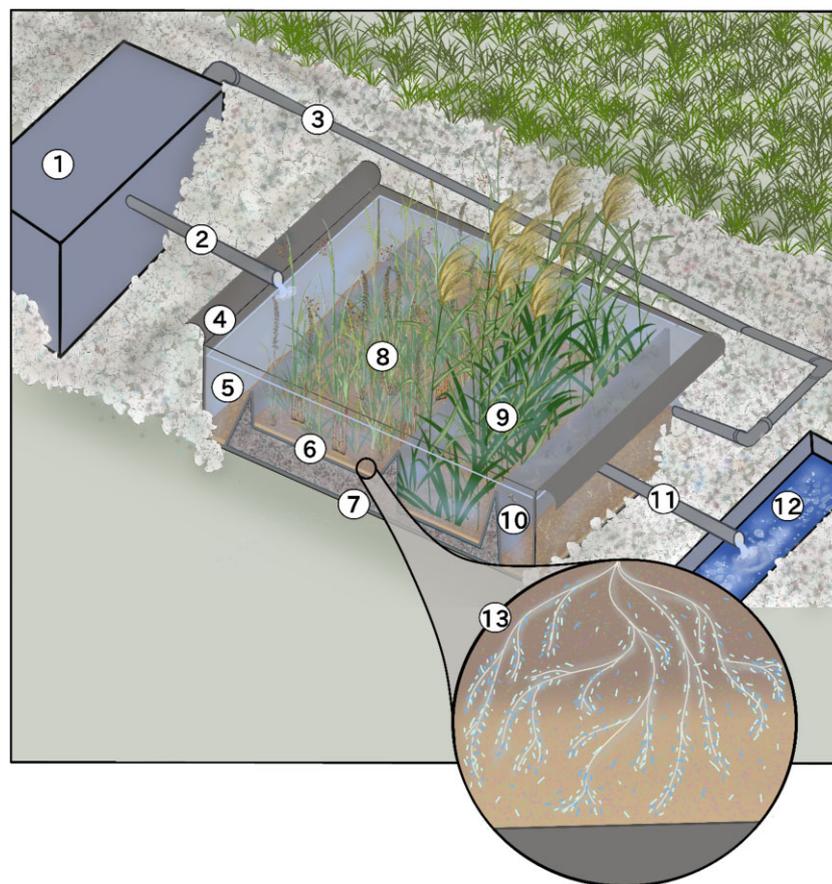
were site specific, but a few were common to more than one environment (e.g. tailings ponds and biofilms/bioreactors). The genus *Pseudomonas* within the Proteobacteria was detected across all environments. Although much less studied than their bacterial counterparts, archaea and fungi have also been reported in oil sands-affected environments. Methanogenic archaea such as *Methanobrevibacter*, *Methanolinea*, and *Methanoregula* have been reported in tailings ponds and are associated with the anoxic conditions of such systems (Skeels and Whitby 2019 and references therein). Some fungal species have demonstrated tolerance to NAs, as evidenced by their high abundance in an oil sands mining lake (Richardson et al. 2019). Furthermore, the ascomycete *Trichoderma harzianum* was shown to improve phytoremediation of petrochemicals and was able to grow on petrochemicals, bitumen, and NAs (NA surrogates cyclohexanecarboxylic acid and 1-adamantanecarboxylic acid, commercial NAs, and OSPW) as sole source of carbon (Repas et al. 2017, Miles et al. 2020).

A few studies have identified environmental drivers of microbial community structure in NA-degrading systems, indicating a key role of electron acceptors, type of NA, and plant species (in plant-associated microbial communities). For instance, in anoxic sediments underlying oil sands tailings ponds, Lv et al. (2020a) found that sediment type (sand or clay), electron acceptor (nitrate or sulfate), and NA source (OSPW or commercial NAs) significantly affected the microbial community composition. In the same system, Lv et al. (2020b) showed that the type of electron acceptor influences the topology of microbial interactome networks and pinpointed the key role of redox state in NA-degrading microbial communities. In microbial communities associated with *Typha* (cattail) roots, the type of NA (extracted from OSPW or a commercial NA mixture) was the critical factor influencing the dominant bacterial species in this system, with high doses of commercial NA favoring the enrichment of potential plant pathogens such as *Dechlorospirillum* sp (Phillips et al. 2010). This study also observed that the effect of NAs on root-associated microbial communities was niche-specific, with the plant itself being the dominant influence on the composition of endophytic microbial communities (Phillips et al. 2010).

Due to the toxicity of NAs, indigenous microbial populations that thrive in the presence of NAs in OSPW and tailings ponds are more likely to degrade NAs (Del Rio et al. 2006, Yu et al. 2018b, Skeels and Whitby 2019). Microorganisms have been successfully isolated from OSPW or other NA-contaminated environments and were capable of degrading NAs. These include the genera *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, *Kurthia*, *Rhodococcus*, *Acidovorax*, and *Rhodoferax* (Herman et al. 1994, Del Rio et al. 2006, Presentato et al. 2018, Yu et al. 2018b). Some of these microorganisms were shown to degrade NAs and use them as a carbon source (e.g. Wyndham and Costerton 1981, Presentato et al. 2018, Yu et al. 2018b). While NA-contaminated environments, such as OSPW (NA concentration: 20–120 mg/L; Grever et al. 2010), typically house microbial communities tolerant to NAs and capable of NA metabolism, the genetic potential to degrade NAs seems to be ubiquitous and not restricted to heavily contaminated environments. For example, NA-degrading microorganisms have been successfully isolated from the rhizosphere of undisturbed forest overlying oil sands deposits (Biryukova et al. 2007) and from uncontaminated clay and sand sediments underlying tailing ponds (Lv et al. 2020a). Similarly, Ahad et al. (2018) reported direct evidence of *in situ* NA degradation of relatively simple NA surrogate compounds (e.g. cyclohexanecarboxylic acid, or CHCA) in groundwater down the hydraulic gradient from an oil sands tailings pond. These findings indicate that natural ecosystems also house the genetic potential for NA degradation, and that NA metabolism is favored and becomes enriched under NA contamination.

## Factors influencing the biodegradability of NAs

A major factor affecting NA biodegradation involves the intrinsic chemical characteristics (i.e. mass and structure) of NAs (Han et al. 2008). NAs with higher molecular weight and increased structural complexity, such as alkyl-substituted aliphatic chains, tertiary substitutions, branches, rings, and diamondoid structures, are more recalcitrant and tend to accumulate in aged OSPW (Smith et al. 2008, Yue et al. 2016).



**Figure 2.** Schematic representation of a horizontal surface flow (HSF) constructed wetland treatment system (CWTS) for oil sands process-affected water (OSPW) treatment. The figure presented is based on the design described in (Cancelli and Gobas 2020). **1. OSPW deposit.** The water deposit usually consists of a tailings pond, where OSPW is temporarily deposited to allow the sedimentation of particles, preventing clogging of the inlet pipes. **2. Inlet pipes.** usually polyvinyl chloride (PVC) pipe, with a variable diameter (at least 100 mm). The inlet pipe can discharge into the wetland directly (as in this figure). Alternatively, it can connect to a distribution pipe running along the width of the wetland with perforated holes evenly through the length of the pipe (~20 mm or more, depending on the flow rates) to help distribute the waterflow. Typically, a constant water flow in and out of the wetland is ensured by automatic pumps, which stop when adequate water levels are reached. **3. Closed circuit pipes.** At a commercial level, there would be no recirculation. However, for research purposes and pilot studies, a recirculation system can be set up (Cancelli and Gobas 2020). If so, these pipes recirculate the treated water back into the OSPW deposit. They are typically made of PVC and can be placed above or under the ground. A closed system with water recirculation allows for subsequent cycles of water filtration through the wetland to further decrease naphthenic acids (NAs) levels, but it is not an essential element of a CWTS. **4. Impermeable lining.** The basin where the wetland is excavated needs to be covered with an impermeable lining to prevent leakage. Usually, a second layer of geotextile material is added to protect the impermeable layer as well as gravel media (Cancelli and Gobas 2020). **5. Forebay area.** This section receives the water from the inlet pipes and ensures a smooth flow towards the subsequent pools. **6. and 7. Wetland basin and Rooting media.** the composition of both wetland basin and rooting media depend on what is naturally abundant in the geographical region and can vary from sandy clay loam to coarse sand and an organic matter layer (i.e. peat mineral mix). **8. Shallow pools.** Wetlands can be divided into a series of pools of different or similar depths. Shallow pools are typically 0.5-1 m deep and allow the establishment of emerging macrophytes. **9. Deep pools.** Deep pools are typically 1.5-2 m deep and allow the establishment of mostly submerged macrophytes, although some emergent species can also be found here (e.g. *Phragmites australis*). **10. Outlet cell.** This collects the water at the lower end of the basin slope. From here, the water will be either recirculated to the initial water deposit or released into a collection basin for treated water. **11. Outlet pipes.** These pipes can collect the water in a single tube (in this figure), or alternatively, the water can run through a collection pipe running parallel to the edge of the wetland that will then be merged into a perpendicular outlet pipe. In that case, the outlet collection pipes are perforated PVC, with a similar diameter than the inlet pipe, and holes with a smaller diameter (5-6 mm). **12. Collection basin.** Water that has been successfully treated is collected at the end of the process and held until analyses confirm the reduction of the targeted pollutants.

Environmental conditions such as oxygen concentration, temperature, nutrients, pH, redox potential, and sunlight may strongly affect the microbial degradation of NAs (Whitby 2010, Wong et al. 2015, Kinley et al. 2016). The effectiveness of biodegradation is dependent on the environmental conditions such as availability of nutrients, temperature, and electron acceptors required for microbial growth. Despite their major importance to *in situ* NA biodegradation in systems such as CWTS, it remains unclear how these factors influence the activity of NA-degraders in NA-contaminated environments. Microbial NA degradation in the presence and absence of oxygen has been reported, with the latter

often being related to the use of sulfate, nitrate, or methanogenesis precursors (e.g. CO<sub>2</sub>, acetate) as electron acceptors (e.g. Rontani and Bonin 1992, Iwaki et al. 2005, Johnson et al. 2012, Clothier and Gieg 2016, Presentato et al. 2018). However, significant degradation of a diamondoid NA compound (1-adamantanecarboxylic acid, or ACA) was not observed under anoxic conditions, suggesting that more recalcitrant NAs may be resistant to anaerobic degradation pathways (Folwell et al. 2016). Also, higher degradation rates were measured for commercial NAs under well-oxygenated conditions (dissolved oxygen concentration >8 mg L<sup>-1</sup>) when compared to less oxygenated treatments (Kinley et al. 2016).

Therefore, the availability of oxygen, sulfate, nitrate, or other putative electron acceptors can be a major factor controlling the type of NA compounds that are microbially degraded and the rate of microbial NA degradation (Skeels and Whitby 2019). In addition, low temperature has been shown to negatively affect NA degradation rates (Lai et al. 1996). In controlled experiments with commercial NAs, Kinley et al. (2016) reported lower NA degradation rates and lower microbial diversity in the low temperature (6–16°C) treatments. Similarly, in the northern region of Alberta, Canada, Wong et al. (2015) detected NA biodegradation only at occasionally high summer temperatures, while no biodegradation was observed at 4°C. This is in agreement with a field study carried out by Ahad et al. (2018), who reported no biodegradation of ACA in late autumn (6–7°C) in groundwater monitoring wells down-gradient from a tailings pond in a low-lying wetland. This poses a particular challenge to the reclamation of oil sands mining sites at higher latitudes with cooler climates. Nutrient availability and pH have also been identified as important factors influencing NA degradation. Kinley et al. (2016) showed that higher pH (between 8 and 9) and nutrient availability (nitrogen (N) and phosphorus (P)) positively affects NA degradation rates. This suggests that biostimulation (i.e. the addition of nutrients such as N, P, K) could potentially be used to improve microbial NA degradation in OSPW. Indeed, previous work showed that indigenous hydrocarbon-degrading microorganisms in contaminated soils can be efficiently stimulated by the addition of nutrients (e.g. Yergeau et al. 2009, 2012, Bell et al. 2013), but the efficacy of such an approach for NA biodegradation remains unexplored.

Microbial community composition also appears to influence NA degradation. A few studies have reported that mixed indigenous microbial communities, rather than a single taxon, are more likely to promote the degradation of recalcitrant compounds due to complementary degradation steps or consumption of intermediate products in syntrophic relationships (communal metabolic efforts). For instance, mixed bacterial communities have been shown to degrade model NAs more rapidly than pure cultures (Demeter et al. 2014), and *Pseudomonas* co-cultures degraded a higher quantity of NAs in OSPW than was observed in pure cultures (Chegounian et al. 2021). Similarly, co-cultures of algae and microorganisms in inocula from an end pit lake degraded cyclohexanecarboxylic acid while the pure algal cultures or pure end pit lake inoculum did not (Yu et al. 2019). Furthermore, bacterial and algal consortia from OSPW degraded the recalcitrant ACA (Paulssen and Gieg 2019), and interactome networks of anaerobic NA-degrading bacteria suggested syntrophic relationships in biodegrading processes (Lv et al. 2020b). In addition, the presence of bacterial biofilms that could form in plant tissues or in the sediment seems to favor the bioremediation of NAs, as higher and particularly more durable degradation activity was detected in biofilms of *Pseudomonas* when compared to its planktonic counterpart (Shimada et al. 2012).

### Plant-microbe interactions in the degradation of NAs and the use of constructed wetland treatment systems (CWTS) for the bioremediation of OSPW

The intimate relationship between plants and microbes could be the key for the treatment of many hydrocarbon-derived compounds (Correa-García et al. 2018) and this interaction is expected to contribute to the successful performance of CWTS for OSPW bioremediation (Fig. 2). Several studies have demonstrated that

wetland plants and their microbes can dissipate NAs in lab-scaled hydroponic systems spiked with the AEO fraction of OSPW or with commercial NA mixtures (Armstrong et al. 2008, 2009, Headley et al. 2009, Toor et al. 2013). These results demonstrated that wetland plants and their associated microbes have the capacity to remove specific classes of NAs from solution and reduce toxicity to invertebrates and fish.

The plant-microbiome relationships are often plant-species specific (Rodríguez et al. 2019), and the dynamics of these interactions depend on the habitat niche (i.e. phyllosphere, rhizosphere, endosphere). For example, root-associated microbial communities appear to be niche-specific under OSPW conditions, as endophytic communities in cattail remained relatively stable after OSPW treatment compared to the rhizoplane and the bulk soil, where exposure to NAs resulted in divergent communities (Phillips et al. 2010). Additionally, community structure in the rhizosphere and the rhizoplane were both influenced by the type of NA added to the water (i.e. commercial mix vs. NAs from OSPW), while NA concentration only had an impact on communities of the rhizoplane (Phillips et al. 2010). These dynamics can be a key factor for the bioremediation effectiveness of NAs. Recent evidence with radiocarbon (<sup>14</sup>C)-labeled NAs has demonstrated the efficient uptake of recalcitrant NAs by plant roots (Alberts et al. 2021). Using five <sup>14</sup>C-labeled NAs representing three NA classes (linear, single ring, and three-ring diamondoid), it was shown that plant roots can directly take-up simple and complex NAs. These results suggest a potential plant-microbe collaboration for the remediation of different classes of NA compounds.

Constructed wetland treatment systems (CWTS) can benefit from the naturally occurring interactions between plants and microbes to enhance the remediation of OSPW, as shown by recent studies. CWTS are excavated basin structures built to mimic the filtration and water depuration effects of natural wetland ecosystems. CWTS consist of several macrofeatures (plant species, water depth, sediment type) that can enhance the treatment success (ITRC 2003, Haakensen et al. 2015, Ajaero et al. 2020). Choosing native wetland plant will ensure a better plant survival rate since they are adapted to the local climate (ITRC 2003, Haakensen et al. 2015, Cancelli et al. 2022). Optimal plant species for CWTS would develop deep and wide root systems and/or strong rhizomes, display rapid growth and provide efficient oxygen transport into the root zone to facilitate the oxidation of NAs. Depending on the geographical location, some of examples of efficient phytoremediation species are *Carex aquatilis* (water sedge), *Phragmites* spp. (common reed), *Typha* spp. (cattails), *Juncus balticus* (Baltic rush), *Calamagrostis canadensis* (bluejoint reedgrass), *Eleocharis palustris* (creeping spike-rush), *Scirpus microcarpus* (small-fruited bulrush) or *Schoenoplectus tabernaemontani* (softstem bulrush) (Cancelli and Gobas 2022, Cancelli et al. 2022). CWTS can be designed to receive a horizontal or a vertical water flow, as well as to be surface or sub-surface flow systems (ITRC 2003, Kuyucak et al. 2006, Haakensen et al. 2015). The size of the basin will depend on the water volume to treat, but it is usually built with a slight slope (0.01–0.1%) to favor water flow through the wetland and towards the collection pipes (Kuyucak et al. 2006, Cancelli and Gobas 2020).

Treatment wetland-based studies for NA remediation have been tested at small scales and have demonstrated that this approach is a viable option for NA remediation in OSPW (Ajaero et al. 2017, 2018, McQueen et al. 2017, Hendrikse et al. 2018, Simair et al. 2021). In a recent landmark study, detailed Orbitrap mass spectrometry analysis demonstrated that the oxidation of classical NAs (i.e. fitting the chemical formula  $C_nH_{2n+z}O_2$ ) is a common outcome of OSPW treatment in a mesocosm-scale CWTS (Ajaero

et al. 2018). Almost half of the O<sub>2</sub>-NAs were degraded in 28 days, with a portion visible as newly accumulated oxidation products. In addition, single- and multiple-ring NAs were removed at similar extents (73%–80%), and NAs with higher carbon numbers (>14) were removed more efficiently than lower molecular weight NAs. Cancelli and Gobas (2022) were the first to report NA remediation results from a full-scale on-site pilot CWTS. In this 1 ha system, NA concentrations reduced by 7.5% to 68.9% in two weeks, and treatment efficiency increased with OSPW turbidity and temperature. This pilot CWTS also reduced polycyclic aromatic hydrocarbons (PAHs) by 54 to 83% (Cancelli and Gobas 2020). Thus, CWTS can remediate NAs and other organic compounds, but further studies are needed to characterize the contribution of plants, microbes, and their interactions on NA remediation.

The characterization of plant-microbe interactions is not typically included in CWTS studies, but they could be similar to the interactions described for hydrocarbon rhizodegradation in soils. In the latter case, microorganisms stimulate plant growth by (i) reducing toxicity in the root environment following degradation, (ii) producing phytohormones that stimulates root growth, or (iii) degrading the precursor of the plant stress hormone ethylene (Correa-Garcia et al. 2018). This microbe-induced stress reduction is potentially important in CWTS since plants can be sensitive to NAs. NAs can negatively affect root cell growth and mitosis, increase reactive oxygen species, and inhibit plant metabolic mechanisms to reduce oxidative stress. For example, *Phragmites* roots exposed to NAs exhibited reduced antioxidant levels (i.e. GSH) and impaired stress-related enzymes (CAT, POD) (Jia et al. 2023). In another study using plant cells that expressed fluorescent proteins to label membrane-bound organelles (mitochondria, endoplasmic reticulum and peroxisomes), NA treatment was shown to disrupt the structure and dynamics of these subcellular organelles (Alberts et al. 2019). This observation is consistent with the narcosis model for NA toxicity, where NAs can partition into and disrupt cellular membranes.

NAs are not the only stressor halting plant productivity in CWTS for OSPW treatment. High salinity is often a characteristic of OSPW constraining the plant species able to colonize CWTS or limiting plant reproduction (Cancelli et al. 2022). Specific endophytes could help improve salt tolerance, as demonstrated by the root endophytic bacterium *Sphingomonas prati* of *Suaeda salsa* plants growing in coastal wetlands (Guo et al. 2021). This type of plant-microbe interaction could indirectly increase plant productivity and NA dissipation, by improving the intracellular osmotic metabolisms and stimulating the production of CAT as antioxidant enzyme (Guo et al. 2021). In turn, plants create an ideal environment for microbial organic acid degradation that can stimulate NA metabolism. Plant roots exude many secondary metabolites that are structurally similar to organic contaminants (Singer et al. 2003), selecting for microbes with the metabolic capacity to degrade these complex organic molecules (Jin et al. 2019). This can be comparable to a priming effect, where the microbial communities under the plant influence are better prepared than microbial communities in bulk soils to degrade NAs. This was shown for various hydrocarbon compounds, but it remains unexplored for NAs in CWTS. For example, the exudation of phenols by *P. australis* stimulated bacterial degraders in its rhizosphere community, such as *Mycobacterium* spp. (degrading benzo[a]pyrene), *Stenotrophomonas* spp., and *Sphingobium* spp. (4-tert-octylphenol) (Toyama et al. 2011). In another example, as compared to bacteria alone, the co-introduction of maize plants and two strains of *Pseudomonas* resulted in a higher degradation rate of phenol, an increase in catechol 2,3-dioxygenase activity, and a reduction in

bacterial ROS levels (Jin et al. 2019). When applied on these *Pseudomonas* isolates, a complex mixture of compounds simulating the maize root exudates could recreate the effect of maize presence. This confirmed the strong plant-microbe synergy on pollutant removal and the essential roles of plant-derived monosaccharides and amino and organic acids in sustaining microbial degradation (Jin et al. 2019). Furthermore, both synthetic and natural maize root exudates increased horizontal gene transfer of the plasmid carrying the phenol degradation genes and stimulated the growth of both the donor and recipient *Pseudomonas* strains (Jin et al. 2019).

Finally, the potential of plant-microbe interactions to remediate NAs in CWTS is expected to be influenced following the succession of plant species, as both the microbial community and environmental characteristics shift over time, as observed in natural wetlands (Ma et al. 2020) and in CWTS (Cancelli et al. 2022). Microbial community succession has been observed in the rhizosphere and endosphere of *Typha orientalis* at different plant developmental stages (Wang et al. 2023), coinciding with seasonal (temperature) and environmental (ammonium, nitrogen, total sulfur, among others) changes in natural wetlands. Seasonal variations of plant-associated microbial community composition in other aquatic macrophytes (*Phragmites australis*) are a common response in natural environments (Zhou et al. 2021). However, the nature and the extent of the influence of seasonal and successional changes on plant-microbe interactions in NA dissipation remains unexplored.

Additional support for an NA-degrading role for rhizospheric microbial communities was observed in experiments demonstrating the effective removal of low molecular weight NAs (with less than 14 carbons) by enriched microbial cultures isolated from roots of native plant species (Biryukova et al. 2007). The organic acids in root exudates (e.g. malic, acetic, and citric acids) can also modify the surrounding pH and improve bacterial NA access by detaching NAs from organic matter in the water and sediments. Importantly, aquatic plants like *P. australis* also contribute to maintaining an aerobic environment in and around their root systems that could favor aerobic NA degradation in waterlogged sediments (Srivastava, Kalra and Naraiyan et al. 2013).

Although underreported in wetland or aquatic environments, bioaugmentation (i.e. the inoculation of microbes to improve biodegradation rate) has been studied extensively in plant remediation in upland soil settings. Typically, pre-established soil organisms dominate over introduced ones, a phenomenon referred to as the priority effect (Vannette and Fukami 2014). However, this priority effect is reduced when the indigenous microbial community is under environmental stress (Calderòn et al. 2017) such as that caused by the presence of contaminants, or when the introduction of non-indigenous microorganisms occurs during the early stages of plant development. Indeed, the most active period for plant microbial recruitment in the soil seems to occur during the seedling phase (Edwards et al. 2018), and seed inoculation can modify the rhizosphere microbiota (Parnell et al. 2016). Inoculation of wetland plants with microorganisms can stimulate hydrocarbon degradation (Syranidou et al. 2016, Pan et al. 2017, Rehman et al. 2018), suggesting it could be used to improve NA degradation in CWTS. Inoculating endophytic bacteria on upland plant seedlings can help remove organics from soils (Khan et al. 2014, Doty et al. 2017). Interestingly, cattail endophytes are stable following long-term exposure to NAs from OSPW (Phillips et al. 2010). Such studies suggest that various bioaugmentation strategies, including early inoculation, and the use of multi-species or endophytic inocula could be used for NA remediation in CWTS.

## Proposed metabolic pathways and genes involved in the microbial degradation of NAs

We performed a literature survey to compile the potential pathways and genes or gene products involved in the biodegradation of NAs. Information was first manually gathered from previously published review papers (e.g. Clemente and Fedorak 2005, Quagraine et al. 2005, Whitby 2010). We then performed a systematic search for more recently published information by searching titles, abstracts, and keywords in the Web of Science and Scopus databases using the following search strings:

Web of Science:

TS=(“naphthenic acid\*” AND (biodegradation OR bioremediation OR “microbial degradation”) AND (“metabolic pathway” OR gene OR transcript\* OR enzyme\*))

Scopus:

TITLE-ABS-KEY (“naphthenic acid\*” AND (biodegradation OR bioremediation OR “microbial degradation”) AND (“metabolic pathway” OR gene OR transcript\* OR enzyme\*))

The final systematic literature search we performed on 24 October 2022 yielded 63 unique research articles published between 2001 and 2022. After reviewing the title and abstracts of each article, 12 articles that were within the frame of this review (i.e. that contained information related to metabolic pathways and/or genes and/or gene products involved in the degradation of NAs) were retained. In total, we identified 28 studies (gathered manually or with a search engine) that described at least the microorganism, metabolic pathway, gene, or gene product involved in the aerobic or anaerobic NA degradation (Table 1).

This literature review indicated that beta-oxidation is a common pathway in aerobic NA degradation. Various transformation steps can occur prior to beta-oxidation, such as alpha-oxidation (Rontani and Bonin 1992), omega-oxidation (Johnson et al. 2012), aromatization (Iwaki et al. 2005), activation as coenzyme A (CoA) thioesters (Zampolli et al. 2020, Zan et al. 2022), or ring cleavage (Wang et al. 2015). Anaerobic NA degradation has also been reported, and was proposed to involve several possible mechanisms, including the production of benzoyl-CoA as an intermediate (Pelletier and Harwood 2000, Elshahed et al. 2001, Peters et al. 2004), beta-oxidation (Arslan and Gamal El-Din 2021, Sanz and Díaz 2022), and/or through the involvement of nitrate, sulfate, iron, and methanogenic reducers (Holowenko et al. 2001, Gunawan et al. 2014, Clothier and Gieg 2016, Cheng et al. 2019).

### Aerobic pathways

Most aerobic microorganisms with known NA-degrading ability seem to ultimately utilize the beta-oxidation pathway. This pathway involves a series of enzymatic reactions (dehydrogenation, hydration, dehydrogenation, thiolysis) leading to the cleavage between the alpha and the beta-carbon, which are the first and second carbons that attach to a functional group (e.g. COOH) (Fig. 3A). This cycle leads to the formation of a carboxylic acid with two carbons less than its precursor and is repeated sequentially, with the formation of a shorter and more readily oxidized carboxylic acid at each round. This ultimately generates a two-carbon acetyl-CoA molecule that can enter central metabolic pathways such as the citric acid cycle for further degradation. The number of beta-oxidation cycles required depends on the length and structural complexity of the compound. Shorter and simpler compounds require fewer cycles, while longer and more complex structures undergo more cycles to be fully metabolized into acetyl-CoA units. However, the presence of a tertiary or quaternary carbon at the

beta position hinders its oxidation, interrupting or delaying the mineralization process (Quagraine et al. 2005). Several bacterial taxa have been shown to degrade NAs via the beta-oxidation pathway, including *Acinetobacter anitratum*, *Pseudomonas putida*, *Alcaligenes sp* PHY 12, *Rhodococcus aetherivorans* BCP1, and *Mycobacterium* (Table 1). Recent genomic research has connected a gene cluster (chcpca) containing *bad-ali* genes to the beta-oxidation of NAs. Those genes encode dehydrogenases, hydrolases and ligases involved in the transformation of benzoate, CHCA and CPCA (Wang et al. 2015, Presentato et al. 2018, Zampolli et al. 2020, Table 1; Fig. 3B).

Aromatization is another potential aerobic NA degradation pathway. In this pathway, alicyclic carboxylic acids are degraded through hydroxylation at the *para* position (across from the functional group), followed by dehydrogenation of the hydroxyl group to form a ketone (Whitby 2010). The next step involves aromatization and ring cleavage by ortho-fission (Quagraine et al. 2005). Taylor and Trudgill (1978) reported that only one strain (*Alcaligenes* strain W1) out of 33 isolated strains performed the aromatization pathway. Strains of *Arthrobacter sp* were also shown to use the aromatization pathway (Iwaki et al. 2005, Fig. 3C). Using mutant strain analysis, Iwaki et al. (2005) linked the *pobA* gene with the aromatization of non-aromatic NAs in *Arthrobacter sp* (Table 1).

Microorganisms can also degrade NAs through combined alpha- and beta-oxidation pathways (Rontani and Bonin 1992, Fig. 3A) and combined omega- and beta-oxidation pathways (Johnson et al. 2012, Table 1). Alpha-oxidation is involved in the degradation of NAs with methyl groups (CH<sub>3</sub>) attached to the alpha-carbon (first carbon next to a functional group). In the alpha-oxidation pathway, the position of the functional group shifts due to the shortening of the carbon chain by one carbon (alpha-oxidation), thereby allowing subsequent oxidation of the beta-carbon. *Alcaligenes sp*, for instance, seems to combine alpha- and beta-oxidation pathways to degrade cyclohexylacetic acid as the presence of a carboxyl group and the cyclohexyl ring precludes direct beta-oxidation (Rontani and Bonin 1992, Fig. 3A). Omega-oxidation is a metabolic pathway involved in the degradation of carboxylic acids with a methyl group attached to the omega-carbon (the carbon farthest from the carboxyl group). Omega-oxidation differs from alpha- and beta-oxidation in that the terminal methyl group is oxidized to a carboxyl group, leading to the breakdown of carboxylic acids from the omega-end. Johnson et al. (2012) reported a strain of *Mycobacterium* isolated from hydrocarbon-contaminated sediments that is capable of omega-oxidation of the tert-butyl side-chain of 4'-t-BPBA (4-t-butylphenyl)-4-butanoic acid) followed by beta-oxidation of an intermediate to produce the final compound (4'-carboxy-t-butylphenyl)ethanoic acid (Fig. 3D). The presence of various metabolic pathways potentially allows microorganisms to efficiently degrade a wide range of NAs with diverse structures, ensuring optimal NA degradation. For example, the capability of microorganisms to use other pathways could help degrade compounds that are recalcitrant to the more common beta-oxidation reactions.

### Anaerobic pathways

Much less is known about anaerobic microbial NA degradation. The benzoyl-CoA degradation pathway seems to have a role in the anaerobic degradation of benzoate and cyclohexane carboxylate in different strains, and variants of the same pathway have been reported in syntrophic organisms (Pelletier and Harwood 2000, Elshahed et al. 2001, Peters et al. 2004) (Table 1). Using mu-

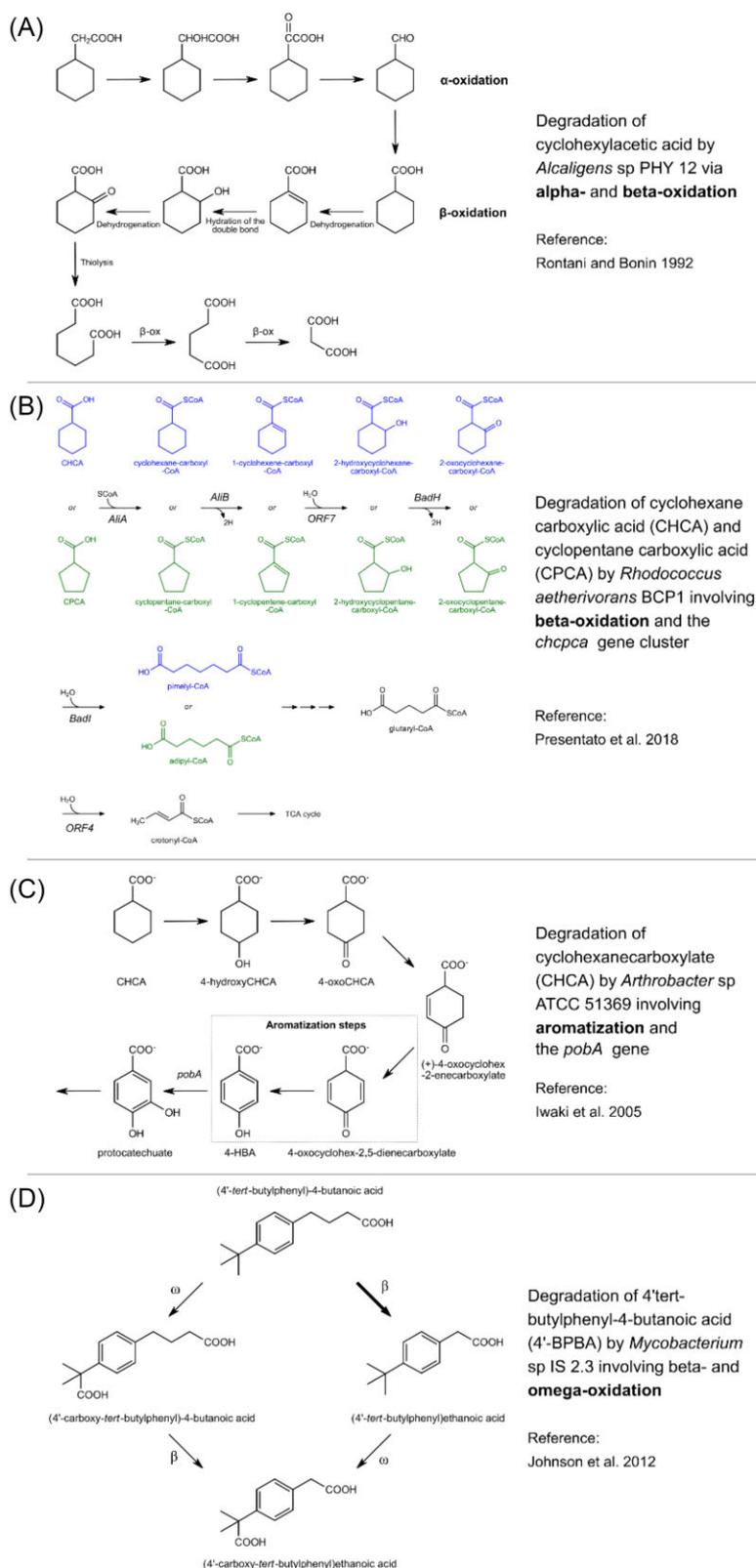
**Table 1.** Microorganisms and the proposed metabolic pathways, genes, or gene products involved in NA degradation.

Microorganism	Condition	Main metabolic pathway	Gene or gene product involved	Reference
<i>Acinetobacter anitratum</i>	Oxic	Beta-oxidation	Not identified	Rho and Evans 1975
<i>Pseudomonas putida</i>	Oxic	Beta-oxidation	Acyl-CoA synthetase and dehydrogenase	Blakley and Papish 1982
<i>Rhodococcus aetherivorans</i> BCP1	Oxic	Beta-oxidation	chcpc cluster: badI, badH, aliA, aliB	Presentato et al. 2018
Soil enrichments with increase in rel. abundance of <i>Pseudomonas</i> , <i>Burkholderia</i> and <i>Sphingomonas</i>	Oxic	Beta-oxidation followed by butylphenyl butanoic acid degradation	Not identified	Johnson et al. 2011
<i>Alcaligenes</i> sp PHY 12	Oxic	Alpha- and beta-oxidation	Not identified	Rontani and Bonin 1992
<i>Mycobacterium Cupriavidus gilardii</i> CR3	Oxic	Omega- and beta-oxidation Ring-cleavage or acyclic-open chain degradation followed by beta-oxidation	Not identified	Johnson et al. 2012
<i>Rhodococcus opacus</i> R7	Oxic	Activation as coenzyme A (CoA) thioester followed by beta-oxidation	chcpc cluster: aliA, aliB, aliA1, badH, badI /DCAA, echA, fadN, fadB, fadA, atoB, putA badI1, badH1, aliA1, aliB	Wang et al. 2015
<i>Alcaligenes</i> sp W1	Oxic	Aromatization	Cyclohexane carboxylate (CHC) hydroxylase	Zampolli et al. 2020
<i>Arthrobacter</i> sp *	Oxic	Aromatization followed by hydroxylation	pobA	Taylor and Trudgill 1978
Mixed microbial enrichment with increase in rel. abundance of <i>Pseudomonas</i> sp	Oxic	Hydroxylation to oxy-NAs	Not identified	Iwaki et al. 2005
<i>Pseudomonas putida protegens</i>	Oxic	Combination of multiple metabolic pathways inferred from metatranscriptomics	Oxidoreductases, hydrolases, and ligases	Folwell et al. 2020
<i>Nocardia</i>	Oxic	Not identified	Not identified	Chegounian et al. 2021
<i>Ochrobactrum Brevundimonas Bacillus</i>	Oxic	Not identified	Not identified	Hasegawa et al. 1982
<i>Pseudomonas fluorescens putida</i>	Oxic	Not identified	PAH-ring hydroxylating dioxygenase (PAH-RHD $\alpha$ )	Yue et al. 2015
<i>Pseudomonas fluorescens</i> Pf-5	Oxic	Not identified	Acyl-CoA dehydrogenase, acyl-CoA thioesterase II, and enoyl-CoA hydratase	Zhang et al. 2015
Fixed-bed biofilm reactor	Oxic/anoxic	Not identified	Functional groups of genes involved in aromatic compounds, organic acids, and benzoate degradation	McKew et al. 2021
<i>Pseudoalteromonas</i> sp	Oxic/anoxic	Activation, dehydrogenation, and hydrolysis through a series of specific coenzyme A (CoA) flowed by beta-oxidation	atoB, fadI, adhP, hmgl-, gabD	Zhang et al. 2020
				Zan et al. 2022

Table 1. Continued

Microorganism	Condition	Main metabolic pathway	Gene or gene product involved	Reference
<i>Aromatoleum</i> sp. C1B (nitrifying bacteria)	Oxic/anoxic	Beta-oxidation via enzymes encoded by bad-ali genes	bad-ali genes	Sanz and Díaz 2022
<i>Rhodopseudomonas palustris</i>	Anoxic	Benzoyl-CoA pathway (activation as coenzyme A (CoA) thioester followed by beta-oxidation)	badH	Pelletier and Harwood 2000
Sewage sludge	Anoxic	Methanogenesis	Not identified	Holowenko et al. 2001
<i>Syntrophus aciditrophicus</i> in syntrophic association with H <sub>2</sub> -using microorganisms. (Fermenting)	Anoxic	Variant of benzoyl-CoA pathway in which cyclohexane carboxylic acid is produced from benzoate	ATP- dependent benzoyl-CoA ligase, cyclohex-1-ene carboxyl-CoA hydratase, 2-hydroxycyclohexane carboxyl-CoA dehydrogenase	Elshahed et al. 2001
<i>Desulfohalobium multivorans</i> (Sulfate reducing)	Anoxic	Benzoyl-CoA pathway	Molybdenum- and selenocysteine-containing enzymes	Peters et al. 2004
<i>Geobacter metallireducens</i> DSMZ 7210	Anoxic	CHdieneCoA intermediate	acdH, barnA, act, acs	Kung et al. 2014
Mixed microbial enrichment	Anoxic	Nitrate reduction	Not identified	Gunawan et al. 2014
Microorganisms indigenous to oil sands tailings ponds	Anoxic	Nitrate, sulfate, and iron reduction; methanogenesis	Not identified	Clothier and Gieg 2016
Methanogenic archaea	Anoxic	Methanogenesis	Not identified	Cheng et al. 2019
<i>Methanosarcina</i> and <i>Methanotherix</i>	Anoxic	Anaerobic digestion likely similar to beta-oxidation, coupled to methanogenesis	Not identified	Arslan and Gamal El-Din 2021

\*formerly *Corynebacterium cyclohexanicum*



**Figure 3.** Examples of proposed metabolic pathways of naphthenic acid (NA) degradation by microorganisms. (A) Degradation of cyclohexylacetic acid by *Alcaligenes* sp PHY 12 via combined alpha- and beta-oxidation pathways postulated by Rontani and Bonin (1992). (B) Degradation of cyclohexane carboxylic acid (CHCA, in blue) and cyclopentane carboxylic acid (CPCA, in green) by *Rhodococcus aetherivorans* BCP1 involving beta-oxidation and the enzymes encoded by the *chcpca* gene cluster, as proposed by Presentato et al. (2018). Genes putatively involved in each step are shown in italics. (C) Degradation of cyclohexanecarboxylic acid (CHCA) by *Arthrobacter* sp ATCC 51369 involving ring aromatization and the enzyme encoded by the *pobA* gene in the transformation of 4-hydroxybenzoate (4-HBA) to protocatechuate, as proposed by Iwaki et al. (2005). (D) Transformation of 4'-tert-butylphenyl-4-butanolic acid (4'-BPBA) by *Mycobacterium* sp IS 2.3 involving beta- and omega-oxidation, postulated by Johnson et al. (2012).

tant analysis, Sanz and Diaz (2022) have shown the importance of the *chcpcA* gene cluster in the degradation of cyclohexane carboxylate in a denitrifying facultative anaerobic bacterium (*Aromatoleum* sp) under oxic or anoxic conditions. Other studies have indicated that the degradation of NAs in the absence of oxygen occurs via the reduction of nitrate or sulfate, or by methanogenesis (Holowenko et al. 2001, Gunawan et al. 2014, Clothier and Gieg 2016, Table 1) but the details of such metabolic pathways have not yet been elucidated.

### Microbial growth, co-metabolism, and fortuitous oxidation of NAs

The biodegradation of NAs may provide a source of carbon and energy for microbial growth, or NA degradation may take place as a consequence of other microbial activity with no utilization of the energy derived from their oxidation. When a contaminant is degraded during microbial utilization of another compound, it is often referred to as co-metabolism. However, when a contaminant is the only carbon source available and its degradation does not generate microbial growth, it is referred to as fortuitous oxidation (Blakley and Papish 1982, Dalton et al. 1982). For instance, Blakley and Papish (1982) reported that *Pseudomonas putida* was capable of oxidizing 3-cyclohexenecarboxylic acid (3-ene-CHCA) via the beta-oxidation pathway but without apparent utilization of the released energy derived from the oxidation. Since the oxidation of 3-ene-CHCA occurred in the absence of other carbon and energy sources, it was suggested to be the result of a fortuitous oxidation, with no benefit for the microbial population. On the other hand, Presentato et al. (2018) reported growth of *Rhodococcus aetherivorans* BCP1 on multiple representative aliphatic and alicyclic NAs as sole source of carbon and energy through the detection of increase in cell abundance and concomitant decrease in NA concentrations in bacterial cultures. Videla et al. (2009) reported <sup>13</sup>C enrichment in the microbial biomass incubated with an oil sands-derived NA extract, suggesting that NAs were used for microbial growth. Similarly, in a mesocosm study, Ahad et al. (2018) reported direct evidence of *in situ* (groundwater) incorporation of <sup>13</sup>C-labeled NA surrogates (CHCA and 1,2-cyclohexanedicarboxylic acid) in bacterial phospholipid fatty acids. In this context, stable isotopes provide a valuable tool to help link NA degradation to microbial metabolism and can potentially allow tracing of NAs into higher trophic levels in the environment such as benthic invertebrates and fish (Farwell et al. 2009).

### Molecular markers for monitoring of the potential for NA biodegradation

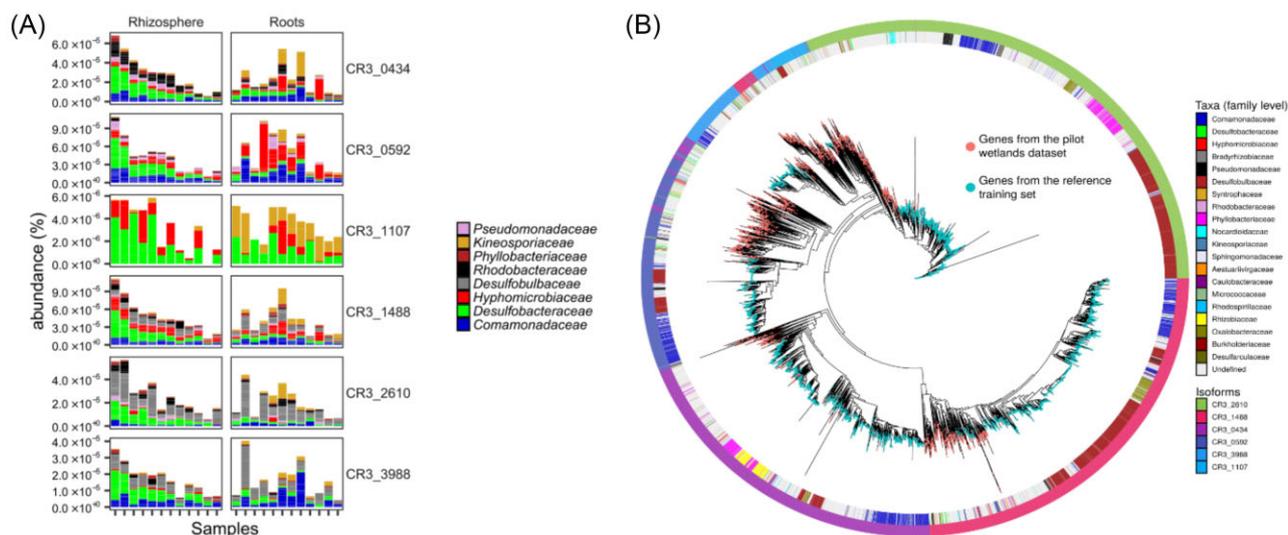
The enzymes and genes presented in Table 1 can be explored for the selection of molecular markers for *in situ* monitoring of NA-degrading potential of CWTS, other bioremediation settings, or in natural environments. For instance, Zampolli et al. (2020) showed that targeting the *aliA1* gene was a useful method for biomonitoring of NA degradation in a model laboratory system. Here we generated a workflow that goes beyond conventional gene sequence identity matching by integrating their isoforms information. Enzyme isoforms perform the same biological function, but differ in their biological activity, temporal/spatial expression, and regulatory activity. Determining which isoforms are prevalent in experimental conditions is important for the thorough understanding of NA degradation dynamics. To demonstrate the po-

tential of such an approach, here a set of Hidden Markov Models (HMM) was created for each of the six isoforms of the *aliA* gene (Wang et al. 2015), encoding a long-chain-fatty-acid-CoA ligase, involved in the aerobic degradation of cyclic carboxylic acids. Briefly, DIAMOND-blastp (v2.0.15) was used to screen for high similarity isoforms present in the NCBI nr database (with parameters -k 1000 and -e 1e-10) followed by filtering hits to keep alignments that were greater or equal to 50% of the reference subject sequence. We then generated multiple alignments (mafft v 7.471; default parameters) followed by HMM generation (hmmer v3.3.2). These six isoforms of the *aliA* gene were then screened (HMM search) against a shotgun metagenomics dataset from a full-scale pilot CWTS in the Alberta Oil Sands region, Canada (Bioproject accession number PRJNA1045646). The CWTS was constructed by Imperial Oil Resources Limited on the Kearn Oil Sands site (near Fort McMurray, Alberta, Canada) in 2015, and consists of a closed, recirculating horizontal surface water flow above sediment containing submerged and emergent vegetation (Cancelli and Gobas 2022).

All six isoforms of the *aliA* gene were detected in the pilot CWTS in roots and rhizosphere of water sedge (*Carex aquatilis*) (Fig. 4). In the context of NA biodegradation, it is crucial to characterize the different isoforms as they may thrive best in different conditions. For instance, in a low oxygen environment, only certain microbial taxa might thrive and the types of NA entering that same environment might have high affinity towards one isoform only. In the pilot CWTS metagenomics dataset, the abundance of the *aliA* gene and its isoforms in the rhizosphere and roots varied largely across the samples taken at different locations and time within the wetland (Fig. 4A). Such information in combination with NA composition and environmental condition characterization can help identify and monitor hotspots of NA degradation within the CWTS, besides providing guidance for future CWTS design and biostimulation and bioaugmentation practices. Additionally, this analysis informed that *aliA* isoforms seem to be enriched in specific taxa (Fig. 4). For instance, a substantial portion of the isoforms CR3\_148 and CR3\_2610 were assigned to the Family *Desulfobulbaceae*, while fewer sequences of the other three isoforms were affiliated with this Family. The isoform CR3\_1107 was the most taxonomically restricted, being affiliated to only three out of eight detected Families. The phylogenetic tree of the *aliA* genes found in the pilot CWTS also revealed that many novel *aliA* gene sequences were present in the shotgun metagenomic dataset. For instance, no satisfying taxa assignment could be achieved for a cluster of genes belonging to isoform CR\_1107. This represents untapped potential of NA biodegradation that can be traced through screening of marker genes and can be further explored. Such an exercise illustrates how marker sequences can be exploited for *in situ* biomonitoring of NA degradation based on a culturing-independent approach and how combining genomic and environmental data can provide unprecedented information to maximize the effectiveness of CWTS for NA degradation and OSPW treatment.

### Future research

Despite significant advances in the field of microbial degradation of NAs, major knowledge gaps remain that need to be addressed to develop efficient strategies for microbial remediation of NAs. For instance, there is much uncertainty around the pathways of microbial NA degradation, and there is limited direct evidence of the involvement of particular genes or gene products. Genomics



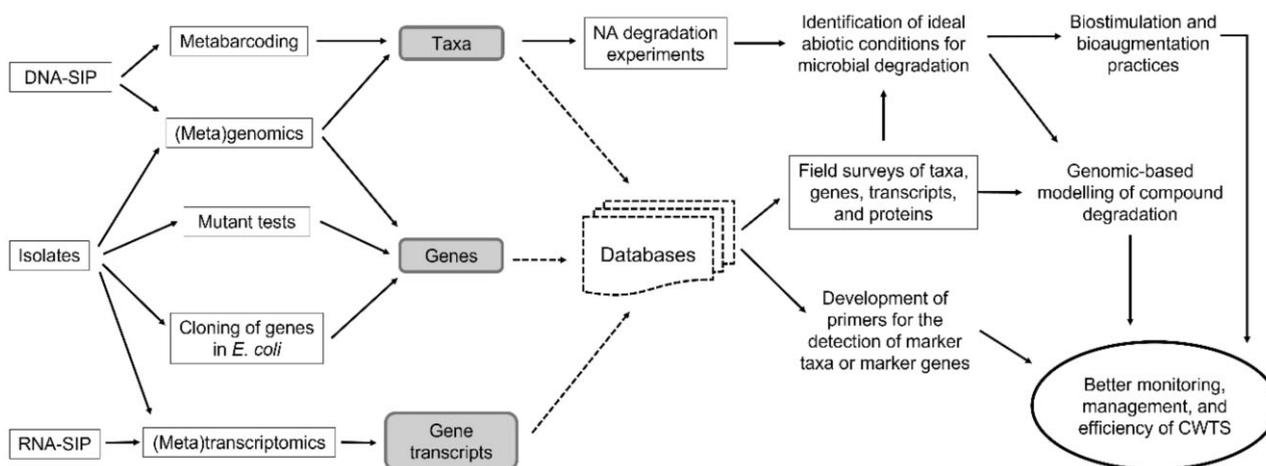
**Figure 4.** A. Abundance (%) of the six isoforms (rows) of the *aliA* gene (long-chain-fatty-acid-CoA ligase) in the rhizosphere and roots of water sedge (*Carex aquatilis*) across samples from different locations within a full-scale constructed wetland treatment system (CWTS) in Alberta (Canada). Abundances were calculated through screening of a shotgun metagenomic dataset for the six gene isoforms. B. Phylogenetic tree of the *aliA* gene (long-chain-fatty-acid-CoA ligase) showing six isoforms (outer circle) and their taxonomic affiliation (Family level; inner circle). Shown are sequences from a pilot CWTS (red; see text for details) and the reference training dataset (blue).

tools can be combined with more classical microbiological methods such as enrichment cultures and mutant analysis to confirm the specific role of taxa and genes in NA degradation. Microbial isolates studies can provide insight into NA degradation pathways and optimal degradation conditions (Fig. 4). At the same time, the discovery of previously uncharacterized taxa and the metabolic machinery involved in NA degradation strongly relies on culture-independent methods. These include stable isotope probing (SIP), as well as wide ‘omic’ surveys (e.g. metagenomics, metatranscriptomics) to scan the degradation potential of unculturable microorganisms. Experiments targeting gene or protein expression analyses (McKew et al. 2021) have so far provided important insight on the diversity of NA-degrading microorganisms, genes, and pathways. This, coupled with the ever-growing accumulation of sequence data, provides the opportunity to explore the NA degradation potential in a range of environments where metagenomic data is available. Conventional alignment tools, such as blastp use position independent scoring matrices to functionally annotate sequences. In contrast, HMMs produce position-specific scores and penalties when searching query sequences, which offer increased sensitivity when identifying homologs of conserved sequence regions. Not surprisingly, databases such as KEGG (Kanehisa and Goto 2000) and Pfam (Finn et al. 2014) have leveraged HMMs to annotate functional genes. KEGG and Pfam provide the capacity to annotate central metabolic pathways using a large number of profile-HMMs. However, they perform poorly when annotating specific secondary metabolic genes, such as the ones involved in hydrocarbon degradation (Khot et al. 2022) and possibly in NA metabolism.

Recent work has demonstrated that various biological processes, including compound degradation, can be predicted from microbiological data, particularly from metabarcoding and metagenomic datasets (Correa-Garcia et al. 2023). For instance, the biodegradation of diesel in Arctic soils could be predicted with

an accuracy of 60% by the relative abundance of three specific betaproteobacterial taxa (Bell et al. 2013). Similarly, the growth of willows in highly contaminated soil after 100 days could be predicted by the initial bacterial and fungal community composition and the initial relative abundance of specific taxa (Yergeau et al. 2015). Modeling of the decrease in the concentration of NAs and in the toxicity for OSPW may be improved by the inclusion of genomic-based data such as the abundance of taxa and functional genes. Such models can be transferred to oil sands operators for making data-driven decisions about treatments. However, genomic-based modeling of NA degradation is currently lacking.

We recommend that research efforts should focus on (i) *in situ* experiments that integrate the natural complexity of environmental mixtures of NAs as well as dynamic biotic (e.g. microbial community composition, plant species and biomass) and abiotic (e.g. temperature, electron acceptors) factors that potentially affect NA biodegradation, (ii) enhancing databases of genes and proteins involved in microbial NA degradation that can be achieved through meta-‘omics’ surveys in NA-exposed sites or direct-evidence techniques such as stable isotope probing (SIP) and gene knockout analysis (Fig. 5), and (iii) modeling approaches of compound degradation that integrate genomic information, such as taxa, marker gene, or transcripts abundance. Such efforts have the potential to provide insight into the optimal conditions for gene expression and NA degradation under ‘real-life’ conditions, the development of molecular primers for marker-assisted *in situ* monitoring of NA-degrading microbial populations, and simulation tests through genomic-based modeling of compound degradation (Fig. 5). *In situ* experiments in pilot wetland treatment systems can be challenging due to dynamic conditions but are key to hypothesis testing and ‘omic’ surveys. Ultimately, such approaches may allow better monitoring, management, and optimization of CWTS and other sustainable methods for water bioremediation.



**Figure 5.** Proposed roadmap for expanding the current knowledge on the microbial degradation of NAs (and other compounds of interest) and for improving microbial bioremediation practices from a genomic perspective. The roadmap depicts examples of complementary methodological approaches (white rectangles) that will lead to the identification of taxa, genes, and gene products (gray rectangles) involved in the degradation of a compound of interest. The identified taxa, genes, and proteins should be compiled in databases (dashed lines) that can be then used in field surveys in contaminated and uncontaminated areas to help (in addition to degradation experiments) identify ideal biotic and abiotic conditions for microbial degradation of the compound and, ultimately, biostimulation and bioaugmentation practices. Databases can also be used for the development of molecular tools such as primers of marker genes for the detection and monitoring of compound degradation *in situ*. Further, 'omics data (taxa, genes, and transcripts abundance) can be incorporated in the modeling of compound degradation to assess the efficiency and explore optimized scenarios of bioremediation settings such as CWTS.

## Author contributions

Paula C. J. Reis (Conceptualization, Formal analysis, Investigation, Writing – original draft), Sara Correa-Garcia (Conceptualization, Investigation, Writing – original draft), Julien Tremblay (Data curation, Formal analysis, Visualization, Writing – review & editing), Aurélie Beaulieu-Laliberté (Conceptualization, Investigation, Writing – review & editing), Douglas G. Muench (Funding acquisition, Investigation, Writing – original draft, Writing – review & editing), Jason M. E. Ahad (Investigation, Visualization, Writing – review & editing), Etienne Yergeau (Conceptualization, Investigation, Writing – review & editing), Jérôme Comte (Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing), and Christine Martineau (Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing)

## Acknowledgements

The authors would like to thank Imperial Oil Resources Limited for providing samples and Marie-Josée Bergeron for performing laboratory work.

*Conflict of interest:* None declared.

## Funding

This work was supported by Genome Canada through a Large Scale Applied Research Project (LSARP) grant (Grant number 18207), in partnership with Genome Alberta and Genome Quebec.

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