A microbial solution to oil sand pollution: Understanding the microbiomes, metabolic pathways and mechanisms involved in naphthenic acid (NA) biodegradation

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A microbial solution to oil sand pollution: understanding the microbiomes, metabolic pathways and mechanisms involved in naphthenic acid (NA) biodegradation.

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Contents:

Abbreviations used in this chapter

Box 1: Abbreviations used

AEOs	Acid extractable organics
AOSR	Athabasca Oil Sands Region
BML	Base Mine Lake
CT	Composite (consolidated) tailings
CWTSs	Constructed wetland treatment systems
DGGE	Denaturing gradient gel electrophoresis
EPL	End pit lakes
ETF	External tailings facilities
ETA	External tailings area
FFT	Fluid fine tailings, having low solids content (e.g. ≤8 wt%)
MLSB	Mildred Lake Settling Basin
MFT	Mature fine tailings, having solids content ≥30 wt%
NAs	Naphthenic acids
NAFCs	Naphthenic Acid Fraction Compounds (NAFCs)
OSPW	Oil sands process-affected water
OSTPs	Oil sands tailings ponds
PAHs	Polycyclic aromatic hydrocarbons
SAGD	Steam-Assisted Gravity Drainage
TAN	Total acid number
TT	Thickened tailings
WSO	Water-soluble organics

Abstract

Bituminous oil sands arise from the microbial oxidation of petroleum hydrocarbons over geological time, with one of the largest deposits found in northern Alberta, Canada. Associated with bitumen are mixtures of toxic and persistent organic acids, known as Naphthenic Acids (NAs). NAs can be found in various natural environments that are in contact with the bitumen, such as river and wetland sediments. However, NAs may also accumulate through anthropogenic activities that follow oil sand mining operations. During bitumen extraction, vast quantities of toxic oil sands process-affected water (OSPW), are generated that are stored in large oil sands tailings ponds (OSTPs), often for decades and cause serious impacts to the local environment. Bioremediation of NAs using microorganisms, has clear cost and environmental advantages, yet there is a critical lack of fundamental knowledge of the functional microbiome in these NA associated ecosystems. This chapter seeks to unravel the complex microbiomes present in natural and anthropogenic bitumen-rich environments, including the genes, enzymes and mechanisms involved in NA biodegradation.

1. Introduction.

With increasing global demand for energy and the rapid depletion of conventional light crude-oil resources, unconventional oil resources, such as heavy oils, oil sands and bitumen are receiving growing interest as future energy sources (Zhang et al 2020b). Bituminous oil sands are formed as a result of the partial oxidation of petroleum hydrocarbons by microorganisms over geological time (Head et al 2013). It is currently estimated that there are >6 trillion barrels of heavy and super-heavy crude oils, bitumen and oil/tar sands worldwide, which together account for ~70% of total global oil reserves (**Fig 1**) (Zhang et al 2020b). The largest known oil sand deposits are found in North and South America, with the largest in Venezuela, followed closely by the Athabasca Oil Sands Region (AOSR) in Northern Alberta, Canada (**Fig 2A**) (Head et al 2003). It has been estimated that >169 billion barrels of recoverable bitumen is present in the AOSR (Brown and Ulrich, 2015; Chalaturnyk et al 2002).

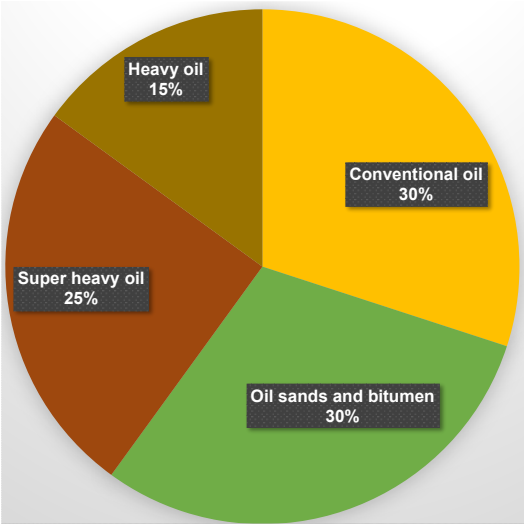

Fig 1 Percentage of global oil reserves (conventional and unconventional) (modified from Fargestad 2016; Zhang et al 2020b).

Compared to conventional crude oils, bitumen has fewer saturates, but contains more resins and asphaltenes (Woods et al 2008). Bitumen-rich ores co-occur with clays, sand and silt (~10% bitumen, ~5% water and ~85% quartz sand, silt and clays) (Chalaturnyk et al 2002). To extract the bitumen from surface-mined ores, the Clark hot water process is generally used, which involves mixing crushed ore with hot, alkaline water (historically ~70-80ºC; but now ~50 ºC; pH ~8.5) (Foght et al 2017). The process produces vast quantities of toxic wastewaters, known as oil sands process-affected water (OSPW), which have to be stored in large ponds, called oil sands tailings ponds (OSTPs) (**Fig 2B**). These OSTPs are often in operation for decades, until approved strategies are available to effectively treat and safely release the OSPW back into the environment (Whitby 2010; Bauer 2015). It is currently estimated that ~1 billion m^3 of tailings have accumulated in the AOSR, and this volume is set to increase as new operations are being developed, and until reclamation or their removal occurs (Foght et al 2017).

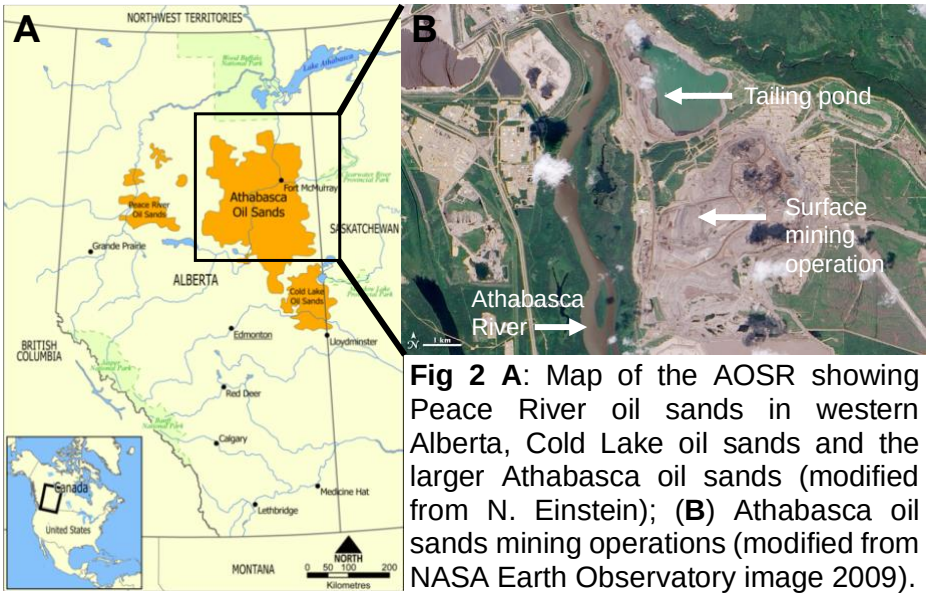

Fig 2 A: Map of the AOSR showing Peace River oil sands in western Alberta, Cold Lake oil sands and the larger Athabasca oil sands (modified from N. Einstein); (**B**) Athabasca oil sands mining operations (modified from NASA Earth Observatory image 2009).

Microorganisms play a fundamental role in both natural, and anthropogenic ecosystems that are associated with NAs. The *in situ* activities of microorganisms are not only important in the reclamation, removal and detoxification of NA-contaminated environments, but their metabolic processes also shape the very nature of the oil sand deposits themselves. Yet their importance and functional role in these environments is often overlooked, and there remains a lack of knowledge on the NA biodegradation mechanisms and the microorganisms involved. Recent advances in analytical and molecular techniques, however, has started to unravel the functional microbiomes found in these natural and anthropogenic ecosystems that are associated with NAs.

1.1. The challenges with Naphthenic Acids (NAs).

OSPW is known to contain sand, clay, unrecoverable bitumen, inorganic and a complex mixture of toxic, organic compounds termed '*Naphthenic Acids (NAs)*' (Mikula et al 1996, Whitby, 2010). NAs were traditionally defined as mixtures of acyclic and cyclic, saturated and (to a lesser extent) aromatic carboxylic acids with the empirical formula $C_nH_{2n+z}O_2$, where *n* represents the number of carbons and *Z* is either zero or a negative even integer representing "hydrogen deficiency" lost due to double-bond or ring formation (**Fig 3 A,B**) (Barros et al 2022; Whitby 2010). OSPW is also known to contain bi-, tri-, tetra-, penta-cyclic, mono-carboxylic and diamondoid carboxylic acids (**Fig 3C**) (Rowland et al 2011a, b, c; Wang et al 2013; Wilde and Rowland, 2015; Wilde et al 2015). Indeed, tricyclic (and bicyclic) diamondoid carboxylic acids are major components of OSPW, and unrefined oil sands (Rowland et al 2011b; Bowman et al 2014). In addition, oxy-NAs (Barrow et al 2009; Wang et al 2013), and structures with heteroatoms such as nitrogen and sulfur have been found (Bauer et al 2015; Grewer et al 2010; Hewitt et al 2020). NAs that do not fit the 'classical' formula are referred to as Naphthenic Acid Fraction Compounds (NAFCs) (Vander Meulen et al 2021), which includes unsaturated and aromatic NA derivatives, acids with increased oxygen content, and compounds containing nitrogen and/or sulfur (Yang et al 2019; Grewer et al 2010). Current terminology also refer to the acid extractable organics (AEOs); water-soluble organic (WSO) and oil sands tailings water acid-extractable organics (OSTWAEO) (Yang et al 2019; Grewer et al 2010).

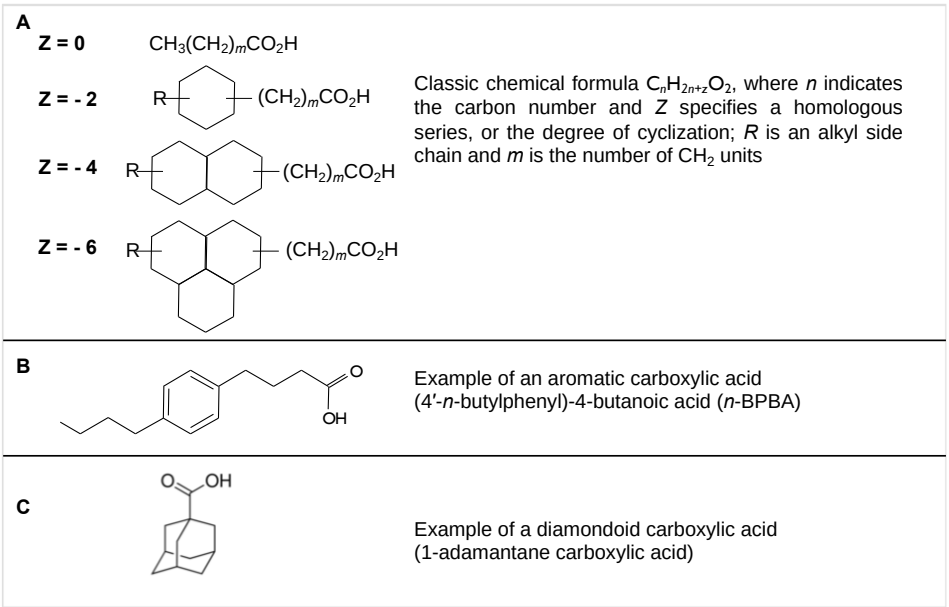

Fig 3. Example structures of naphthenic acids (NAs) A: NAs following the classical chemical formula; B: example of an aromatic NA; C: example of a diamondoid carboxylic acid (Modified from Whitby, 2010, Folwell et al 2020; McKew et al 2021).

NAs pose several environmental challenges. For example, they demonstrate both acute and chronic toxicity to a variety of different organisms (Li et al 2017; Morandi et al 2015; Headley and McMartin 2004; Frank et al 2009; Beddow et al 2016). NAs are highly toxic to fish at concentrations >2.5–5 mg L^{-1} (Dorn, 1992), and concentrations up to 2.8 mg kg^{-1} have been detected in rainbow trout (*Oncorhynchus mykiss*) (Young et al 2007). Toxicity effects in fish include reduced hatch success, a decrease in embryonic heart rates and cardiovascular abnormalities (Marentette et al 2015). They are also known to act as endocrine disruptors (Rowland et al 2011b). The degree of toxicity is generally related to molecular weight with higher molecular weight acids often demonstrating greater acute toxicity, as their increased acid content decreases hydrophobicity making them more bioavailable and facilitating bioaccumulation in cells (Holowenko et al 2002). Acute toxicity may also be related to the surfactant properties of NAs, whereby NAs penetrate the cell wall, disrupting the membrane lipid bilayer, or change membrane properties (Frank et al 2009; Schramm et al 2000). Aromatic NAs, which comprise >30% of the NAs in OSPW relative to the acyclic acids (Jones et al 2012), contribute disproportionally to the overall toxicity of NAs (Johnson et al 2011; Scarlett et al 2013). Other toxic substances such as aromatic, olefinic, hydroxyl and dibasic acids (which by strict definition are not NAs) may also influence NA toxicity (Whitby 2010).

NAs also act as oxidizing agents and are highly corrosive, leading to equipment failures, safety and reliability issues (Whitby, 2010). In particular, heterocyclic acids with multiple different heteroatoms are one of the main causes of corrosion during heavy oil processing (Tomczyk et al 2001). The rate of corrosion depends on several factors, including temperature, the availability of the carboxylic acid group to form metal complexes, molecular size and chemical structure (Clemente and Fedorak 200). NAs can also form metal naphthenate precipitates (e.g. calcium naphthenates), which block pipelines (Whitby 2010). The main fraction of calcium naphthenate deposits are a family of C_{80} isoprenoid tetracids (known as ARN acids) (Baugh et al 2004) with structures closely resembling glycerol diphytanylglycerol tetraethers (GDGTs), which are core lipids found in some hyperthermophilic methanogens and Crenarchaeota (Albaiges et al 1985; Chappe et al 1982; Lutnaes et al 2006). NAs also increase the total acid number (TAN), which may devalue the oil (Whitby 2010).

A wide variety of physical and chemical methods have been applied to remove and detoxify NAs, including ozonation and UV irradiation treatments (Scott et al 2005). However, such methods are expensive, often difficult to implement on a large scale, and may yield little results (MacKinnon and Boerger, 1986). Biologically based treatments, which exploit the degradative ability of microorganisms, have clear cost and environmental advantages to remove and detoxify the NAs (Folwell et al 2020; Mahdavi et al 2015; Yue et al 2016). However, in order to better understand the mechanisms involved in NA biodegradation and the microorganisms involved, we need to further understand the functional role of the complex microbiomes found in the various natural and anthropogenic bitumen-rich environments.

2. Natural versus anthropogenic Naphthenic Acid (NA) exposed microbiomes

In northern Alberta, Canada, the oil sands deposits underlay a vast stretch of boreal forest with many lakes, streams, rivers, wetlands and groundwaters (Brandt et al 2013). These natural ecosystems may be in direct contact with the natural bitumen deposits and consequently their biota may become exposed to varying levels of NAs (Del Rio et al 2006; Clemente and Fedorak, 2005). NAs may also enter aquatic ecosystems via natural processes such as surface run-off, erosion of the riverbank oil deposits and precipitation (Richardson and Dacks 2019; Ross et al 2012). However, NAs may also enter natural systems (e.g. groundwaters) as a result of seepage of OSPW from nearby tailings ponds and this is an ongoing environmental threat in the AOSR (Ahad et al 2013; Fennell and

Arciszewski 2019). Whilst infiltration of OSPW from tailings ponds into groundwaters does occur, seepage into surface waters is less common (Fennell and Arciszewski 2019) (**Fig 4**). However, any groundwater seepage reaching the rivers is rapidly diluted (Ferguson et al. 2009). Currently, the release of contaminants from anthropogenic activities to aquatic ecosystems is principally via atmospheric release such as dust from open areas (e.g. open pits) (Culp et al 2021; Brook et al. 2019). Specifically, emissions of oxidized sulfur and nitrogen associated with mining activities have been reported which acidify the surrounding boreal lake ecosystems via acid precipitation (Hazewinkel et al., 2008). This is exacerbated due to increased run-off as a result of land clearing destabilizing the soil matrix and reducing water retention (Culp et al 2018; Connor et al 2018). This higher run-off, in-turn, increases the sediment and nutrient loading of receiving waters leading to an increase in total suspended solids and the potential for eutrophication (Connor et al 2018). Extensive upstream water extraction and river engineering in the oil sands region also reduces the volume of receiving waters in rivers and downstream wetlands, further exacerbating eutrophication as nutrient concentrations increase in the water column (Connor et al 2018). In addition, the impacts from oil sands development are intensified by reduced water levels as a result of warmer and drier conditions associated with climate change in Canada's northern region (Rouse et al., 1997). Thus, the physicochemical conditions of aquatic ecosystems in the AOSR are driven by both natural processes (e.g., atmospheric deposition, runoff, erosion, groundwater flow) and human activities (e.g., deposition of contaminants, alterations to natural landscapes and water flow) (Culp et al 2021).

Research into cause-and-effect pathways related to contaminant, sediment and nutrient inputs from oil sands development suggested that the impacts identified on the surrounding ecology appear to be associated with contaminant exposure (Culp et al 2021). However, more research is required, especially as the source of this exposure is confounded by co-location of, and inability to differentiate between, oil sands mining operations (e.g. atmospheric emissions, seepage) and inputs from the natural bitumen outcrops (e.g. erosional material transported by surface and groundwater) (Culp et al 2021) (See Chapter 3). Assessment of exposure source is further complicated by the complex interplay between the natural environmental conditions, oil sands operations and other anthropogenic activities (e.g. nutrients and contaminants from municipal sewage effluent) (Culp et al 2021). Critically, there is an urgent need for improved environmental monitoring to assess the potential ecological impacts of current oil sands developments especially any cumulative effects on freshwater resources (Brook et al. 2019). This is even more critical if the direct release of treated OSPW into the surrounding environment occurs in the future (Culp et al 2021).

Fig 3. Example structures of naphthenic acids (NAs) A: NAs following the classical chemical formula; B: example of an aromatic NA; C: example of a diamondoid carboxylic acid (Modified from Whitby, 2010, Folwell et al 2020; McKew et al 2021).

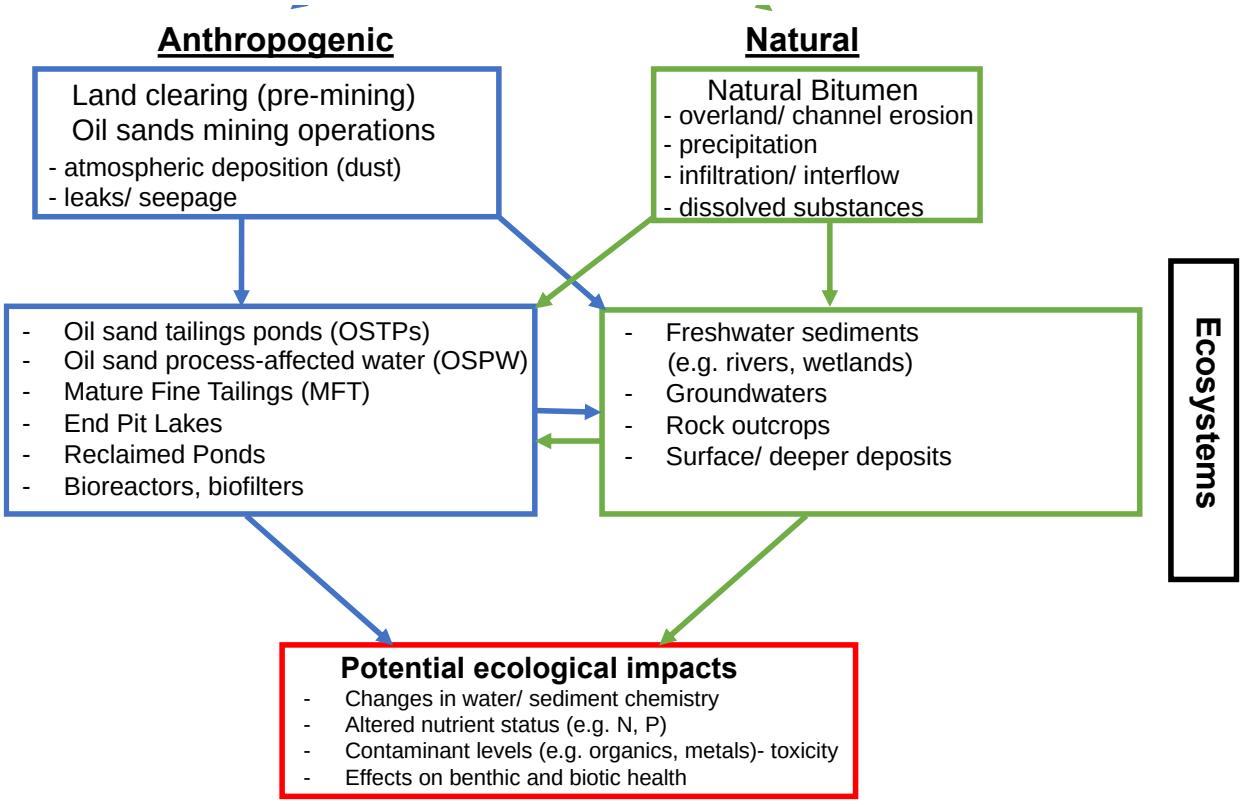

Fig 4 Anthropogenic versus natural sources of naphthenic acids (NAs) and potential ecological impacts on ecosystems.

2.1 Natural exposed microbiomes: Freshwater sediments

In the area surrounding the Athabasca River and its tributaries, there are natural bituminous sediments that are composed of oil (10-12%), sands (83-85%) and water (4-5%) (Wang et al 2014). In 2011, the Governments of Canada and Alberta established a Joint Oil Sands Monitoring Plan (JOSM) (now rebranded as the Oil Sands Monitoring Program (OSM) (Arciszewski et al 2021) for the lower Athabasca River between Fort McMurray and its confluence with Lake Athabasca to monitor several parameters including water quality in the main stem of the Athabasca River, its tributaries, and the deltaic and wetland ecosystems at the river mouth) (Chambers et al 2018). Tributary waters of the lower Athabasca watershed were characterized as moderately alkaline and moderately hard to hard water, due to the presence of Mg^{2+}, Ca^{2+} and bicarbonate, arising from the underlying layers of shale, sandstone and limestone in the region (Chambers et al 2018). In the Athabasca River mainstem and its surrounding tributaries variable concentrations of NAs are found (e.g. between 0.1–0.9 mg L^{-1} in the Athabasca River (Schramm et al 2000), whilst in other locations NA concentrations may be as high as that found in natural bitumen (i.e. 7994 µg g^{-1} with C_6 to C_{21} NAs (α-group) making up ~44.2% of the total NAs in Alberta oil sands bitumen) (Sun et al 2017; Yang et al 2019). High concentrations of other contaminants have also been found to exceed guidelines downstream of oil sands operations such as metals (e.g. aluminium, copper, iron, lead), and other organic compounds (e.g. pyrene) (Kelly et al 2009; 2010). In addition, parts of the Athabasca River near the oil sands mining operations are considered nitrogen and phosphorus limited (Chambers et al 2006; Herman

et al 1994; Lai et al 1996). More research is required however, to determine whether it is the oil sands activities or whether it is the changing climatic conditions (or both), that are influencing the nutrient status of these aquatic ecosystems in the region (Laird et al 2017).

Microorganisms such as those found in sediments overlying bitumen deposits are not only are under additional nutrient stresses, but are continuously exposed to NAs, and must be able to thrive in these complex and toxic NA-rich environments (Brient et al 1995). Indeed, water quality has been suggested as an important factor in shaping the microbial community (Bergsveinson et al 2021). As a result, these ecosystems can harbour diverse microbiomes, and includes microorganisms with the ability to degrade NAs (Del Rio et al 2006). Although, microorganisms of the Athabasca watershed are well-known to degrade NAs (Del Rio et al 2006); microorganisms without any prior exposure to NAs may also metabolize NAs; including estuarine sediments (Smith et al 2008) and soils (Johnson et al 2011). Thus, more research is needed into the distribution, ecology, and mechanisms of NA-degrading microorganisms found in these various ecosystems.

2.1.1 Lotic systems (River sediments)

Freshwater (river) sediment microbiomes are crucial components of these ecosystems; yet there remains a lack understanding of the effects of NA exposure on the microbiomes present. Specifically, the microbiome of the Athabasca watershed is in continuous contact with NAs through oil sands erosion and oil-upgrading activities (Kelly et al 2009, 2010; Kurek et al 2013; Del Rio et al 2006). Under the zero discharge policy in the region, only small releases have been approved by the regulator (i.e. <1% of receiving river flows) (Culp et al 2021). Although there is no deliberate discharge of contaminated tailings ponds into the nearby rivers and streams, leaching and seepage of tailings water may occur into nearby aquatic ecosystems, which influence their *in situ* microbiomes (Yergeau et al 2012). Indeed, recent data suggests that OSPW infiltration may be present in the McLean Creek and Lower Beaver River, which are tributaries of the Athabasca River that flows through the AOSR and is adjacent to many tailings ponds (Fennell and Arciszewski 2019).

Previous work has shown that proximity to tailings ponds is an important factor in shaping the bacterial and archaeal communities present in the Athabasca River sediments (Yergeau et al 2012). Indeed, the river sediment microbiomes located close to the ponds were found to be more similar to each other (and to those from the tailings ponds), compared to the sediment microbiomes located further away (Yergeau et al 2012). Generally, bacterial diversity was lower in sediments heavily disturbed by mining operations, and significant differences between bacterial communities of bitumen-associated and non-bitumen-associated streams from the Athabasca River and its tributaries (e.g. Firebag Creek, Steepbank Creek, and Ells River) have also been reported (Yergeau et al 2012). Yet, diverse bacterial and eukaryotic communities have still been found in the Athabasca River, regardless of the degree of NA contamination (Bergsveinson et al 2021).

Taxa found in sediments of the Athabasca River and its tributaries included *Pseudomonadota* (e.g. *Pseudomonas* sp., *Xanthomonas* sp.), with Alphaproteobacteria and Betaproteobacteria dominating, except in Steepbank Creek and the upper Athabasca tributary where Deltaproteobacteria dominated (Yergeau et al 2012). *Bacteroidota*, *Bacillota*, and *Chloroflexota* have also been found in relatively high abundances (Yergeau et al 2012). Moreover, all members of the *Actinomycetota*, Betaproteobacteria, and *Bacteroidota* correlated with the highest concentrations of PAHs and NAs in the river sediments, suggesting that these taxa could be used as putative bio-indicators of contamination in the region (Yergeau et al 2012). *Mycobacterium* sp., *Nocardia* sp., and the yeast *Rhodotorula* sp. were also found in the Athabasca River region (Wyndham and Costerton 1981a, b). High abundances of Chitinophagaceae, *Rhizobiales*, Rhodocyclaceae, Comamonadaceae, and Sphingomonadaceae were found in river and creek bed sediments from along the Horse River and Saline Creek (Wong et al 2015).

Indeed significant differences in the abundance of specific genera have been found among the different habitats across the Athabasca River region (Bergsveinson et al 2021). Moreover, analysis of NA concentration and profiles with water quality variables showed two tributaries to the Athabasca River- Beaver River and McLean Creek as possibly receiving OSPW seepage, demonstrating the importance of monitoring ambient water quality (Ross et al 2012). There is also evidence to suggest that the river sediment community had adapted to the presence of hydrocarbons even before the influence of oil sands mining (Reid et al 2018). Moreover, several processes have been found in these hydrocarbon-rich freshwater sediments; including sulfate- and nitrate- reduction, methanogenic and methanotrophic activities, suggesting an ecologically active microbial community is present in these river sediments (Reid et al 2018).

2.1.2 Lentic systems (Wetland sediments)

Natural wetlands provide many ecosystem services, such as water and carbon storage, climate regulation and ecological protection (Liu et al 2022). In the AOSR, wetlands dominate the landscape, covering over 50,500 km^2 and equating to around 54% of total land cover (Volik et al 2020). Generally, these wetlands have a low buffering capacity and are particularly vulnerable to acidification (Hazewinkel et al., 2008). Underlying these wetlands are poorly buffered soils where organic carbon is typically high (e.g. 25.05 ± 1.00 mg DOC L^{-1} and 28.74 ± 1.61 mg TOC L^{-1}) (Connor et al 2018). The elevated levels of organic carbon may be attributed to the oil sands as a result of atmospheric deposition or surface water contributions from the upstream oil sands development (Connor et al 2018) Thus, the rapid development of oil sands within the region is challenging ecosystem functionality. NA concentrations of <45-80 mg L^{-1} in OSPW affected wetlands have been measured, compared to <2 mg L^{-1} for non-OPSW affected wetlands (Del Rio et al 2006; Pollet & Bendell-Young, 2000). Moreover, wetlands are generally established on land that has been significantly disturbed following mining operations, that consequently may include little or no vegetation (Foght et al 2017). Yet despite their ecological importance, little is currently known about the microbiomes found in wetlands associated with NAs. Previous studies which were largely based on obtaining isolates from enrichment cultures derived from wetland sediments, have made some headway and identified microorganisms such as *Pseudomonas putida* and *Pseudomonas fluorescens* (Del Rio et al 2006). Although both of these two microbes are very cosmopolitan species and often found in culture based studies (e.g. Johnson et al 2013) and so may not be indicative of the composition of wetland microbiomes. However, there currently remains a lack of information on the *in situ* microbiomes of wetlands.

Many of the wetlands in the vicinity of the Athabasca oil sands are naturally exposed to low levels of bitumen and therefore have a history of long-term exposure to the associated NAs therein (Headley et al 2000). Distinct microbial communities have been found in wetlands that receive process-affected water, compared to more heterogenous communities that are present in pristine wetlands with no previous exposure to NAs (Hadwin et al 2006). Once wetlands are exposed to even low levels of OSPW (i.e. low-impact sites), the microbial communities may degrade certain NAs such as bicyclic NAs, (i.e. those belonging to the Z = -4 family) (Del Rio et al 2006). Yet significant degradation of the bicyclic NA decahydronaphthoic acid (DHNA) was found to only occur when the microbial communities were exposed to process-affected water (Hadwin et al 2006). With monocyclic NAs, such as cyclohexane carboxylic acid (CCA), wetland microbiomes were equally capable of their degradation, regardless of prior exposure to OSPW (Del Rio et al 2006).

In general, the microorganisms found in NA exposed wetlands degrade NAs much faster than communities from non-exposed wetlands (Del Rio et al 2006; Hadwin et al 2006; Headley et al 2000). Despite wetland communities being able to reduce the total NAs in OSPW, in some cases long residence times (i.e., 400 days) may be required before

significant NA reduction occurs (Toor et al 2013a). Furthermore, the more persistent NAs that are associated with residual chronic toxicity can often remain (Toor et al 2013b). In some wetland microbiomes, nitrification may also inhibit NA mineralization as NA-degrading communities may be outcompeted by nitrifiers for available oxygen and nutrients, such as nitrogen and phosphorus (Lai et al 1996). NA concentration has also been suggested as a major factor influencing wetland sediment microbiomes (Hadwin et al 2006). For example, one study reported a shift towards *Actinomycetes* in wetlands associated with a decrease in NA metabolism (Hadwin et al 2006). Although exposure to above ambient levels of NAs may shift the community structure to one that is capable of degrading NAs, the time required for this to alter the community is currently unknown and such information is crucial for wetland reclamation strategies. Indeed, bioaugmentation with NA-degrading sediment communities may be advantageous for wetland remediation (Hadwin et al 2006). Currently there is limited information on the diversity of communities in these wetland ecosystems and more research is required to better understand the community dynamics of wetland microbiomes in relation to NA exposure and biodegradation.

2.2 Groundwaters

Bitumen reservoirs are often naturally associated with groundwater and/or aquifers which may be freshwater or brackish (Foght et al 2017). However, a major environmental concern associated with the Athabasca oil sands mining operations is seepage of NAs from OSPW and tailings ponds into surface and/or groundwaters (Ahad et al 2018; Sun et al 2017; Fennell and Arciszewski 2019). Ferguson et al (2009) showed that leakage from the tailings pond and dkye system into underlying aquifers was possible. Indeed, infiltration of OSPW into groundwaters near some ponds has been reported (Fennell and Arciszewski 2019) and high concentrations of NAs have been found in some groundwater aquifers (>55 mg L^{-1}; Kilgour et al 2019). Specifically, the tricyclic diamondoid acids have been found to persist in groundwaters following seepage from a low lying wetland site (Ahad et al 2018). Recently however, it has been suggested that the clay and sand in the sediments underlying ponds may provide a protective mechanism reducing the infiltration of NAs into the sand-sediment layer, where groundwater channels occur (Lv et al 2020). Leakage may also be restricted via the development of fine sediment layers at the bottom of these ponds as the tailings settle (Ferguson et al 2009). Lv et al (2020) analysed the microbial community of the clay sediment layer beneath an OSTP and showed that different electron acceptors, sediment types, and NAs sources were associated with specific microbial taxa and these factors explained the variation of microbial community structures observed. In addition, the importance of groundwater labile carbons in NA biodegradation was also reported (Lv et al 2020).

To assess whether infiltration by OSPW into groundwaters has occurred is challenging, as groundwaters unaffected by OSPW can contain similar mixtures of potentially toxic compounds (i.e. metals, salts, organics) to those found in OSPW (Roy et al 2016). These pollutants may enter groundwater ecosystems from atmospheric inputs (e.g. upgrader emissions, open-pit mine erosion) or acquired from the groundwater flow path from the geological formations in the area, such as the McMurray formation which contains natural oil sands (Roy et al 2016). Biological-based strategies, such as using bioindicator species, may offer a potential approach for source differentiation. However, there is currently a paucity of information regarding the *in situ* biodegradation of NAs in groundwater systems and the microorganisms involved, making baseline comparisons impossible.

One study which used ^{13}C-labelled stable isotopes of specific NAs, investigated a shallow glacio-fluvial aquifer and followed NA biodegradation that was occurring along the groundwater flow-path from the OSTPs to the Athabasca River (Ahad et al 2018). Biodegradation of cyclohexanecarboxylic acid (CHCA) and 1,2-cyclohexanedicarboxylic acid (CHDCA) was reported and it was suggested that the indigenous microbial communities

in the shallow subsurface groundwaters near to the tailings ponds could readily break down these compounds prior to surface water discharge (Ahad et al 2018). There is currently a lack of information on the microbial diversity found in groundwater ecosystems contaminated with NAs. One study that tackled this knowledge gap by analysing water samples from a basal aquifer (up to 20 m in thickness underlying a 50-80 m thick layer of oil-saturated sands reservoir) found a dominance of *Epsilonproteobacteria* affiliated with *Sulfuricurvum*, *Arcobacter* and *Sulfurospirillum* sp. with lesser proportions of *Methanomicrobiales* (Hubert et al 2012). Methane-oxidizing bacteria have also been found in contaminated groundwaters from a low lying wetland (Ahad et al 2018). Although some headway has been made into unravelling the microbiomes of NA-contaminated groundwaters, more research is still required in this area, especially in relation to their functional role in these ecosystems.

2.3 Marine ecosystems

NAs may enter the marine environment either directly or indirectly from multiple sources, including surface runoff, natural leakages from oil sands tailing ponds, and accidental crude oil spills (Kannel and Gan, 2012). For example, NAs were detected in the surrounding waters and sediments following the Deepwater Horizon (Gulf of Mexico) and Hebei Spirit (Taean, South Korea) oil spills (Reddy et al 2012; Wan et al 2014). Concentrations of NAs have been found to be 50-100 times greater than total polycyclic aromatic hydrocarbons (PAHs) found in the sediments from the Taean spill (Wan et al 2014). Furthermore, whilst acyclic $NAs_{n=5–20}$ were found to be easily degraded, the cyclic $NAs_{n=21–41}$ persisted (Wan et al 2014). NAs were also found to be 10-30 times higher than that of PAHs in the sediment of Dalian bay, China after an oil spill accident (Zan et al 2019). These findings highlight the potential ecological impact of NAs on marine environments. Yet the transportation, NA biodegradation and risk of NAs to the marine microbiome is largely unknown. More research is needed on NA biodegradation and the microbial communities involved in marine ecosystems.

2.4 Bitumen saturated outcrop deposits

The AOSR contain shallow bitumen deposits found at depths ranging from ≤50–75m below the surface that can be surface mined (Foght et al 2017). In addition, along many of the riverbanks in the area, natural bitumen-containing surface outcrops of the McMurray Formation are found (Wyndham and Costerton 1981a; Wang et al 2014; Wong et al 2015). This includes exposed surface outcrops such as those located along the Athabasca-Clearwater River network (Wong et al 2015). These bitumen-rich ores cooccur with sand, fine silt, and clay minerals (‘fines’) with an average of ∼10 wt% bitumen, ∼5% water and ∼85% quartz sand, silt and clays (Chalaturnyk et al 2002; Foght et al 2017), and glycocalyx-mediated adhesion of bacteria to the surface of the bitumen may occur (Wyndham and Costerton, 1981b).

The *in situ* microbiota found in rocky outcrops must be able to survive the extreme temperature variations that occur in the region, which range from summer highs of +30ºC to winter lows between −40 to -50ºC (Richardson and Dacks 2019; Wong et al 2015). In these bitumen outcrops, there is some evidence for aerobic rather than anaerobic bitumen biotransformation, despite some known anaerobic phylotypes (e.g. methanogens) being detected (Wong et al 2015). Metagenome analysis has revealed an abundance of genes encoding for known bacterial aerobic hydrocarbon biodegradation enzymes (such as mono- and di-oxygenases and fungal cytochrome P_{450} oxidases) but, not those involved in anaerobic hydrocarbon-degradation (Wong et al 2015). Thus, in these exposed bitumen outcrops, bitumen degradation occurs and is primarily via aerobic processes (Wong et al 2015), although these findings are only based on two metagenome samples, and that with further sampling contrasting results may be found.

Examination of the bitumen-saturated sandstone outcrops sampled from northeastern Alberta found diverse taxa including fungi, hydrocarbon-, methane-, and acetate-oxidizing heterotrophic bacteria (Wong et al 2015). Specifically, two distinct networks were found in the outcrops with Network I comprising largely of aerobic putative hydrocarbon degraders (e.g. *Burkholderiales, Flavobacteriales, Pseudomonadales, Sphingomonadales,* anaerobic methanogens (e.g. *Methanomicrobiales, Methanosarcinales*, and *Methanobacteriales*), *Clostridiales*, and syntrophs (e.g. *Syntrophobacterales* and *Anaerolineales*), and Network II comprising aerobic bacteria and fungi (e.g. Protomyces and Coniosporium) as well as the putative hydrocarbon-, C_2-, or C_1-oxidizing bacteria (Wong et al 2015). It was suggested that hydrocarbon degradation in these outcrops was initiated by fungi and the products of which were subsequently metabolized predominantly by the aerobic bacterial community (Wong et al 2015). In addition, thermophilic microorganisms (e.g. *Methanothermobacter, Thermotaga, Thermomonas, Caldanaerobacter, Caldimicrobium*, and *Thermanaeromonas*) were also found, and likely due to the higher summer temperatures (i.e. 55-60 ºC) experienced on the outcrop slopes although the air temperatures immediately above the outcrop surface were between 33-38°C, indicating that the bitumen-containing outcrop surface has effective heat-absorbing properties not unlike those of asphalt roads (Wong et al 2015). Thus, these bitumen-containing outcrops are a unique ecosystem that harbors a diverse microbiome that may be important for NA biodegradation (Wong et al 2015).

2.5 Deep oil sand deposits

Whilst surface-mining is generally used to extract the shallow bitumen deposits that are found tens of meters below the surface (i.e. ≤50–75 m depth), much deeper deposits (>75 m depth) are also found that require alternative methods of extraction, such as Steam-Assisted Gravity Drainage (SAGD), an enhanced oil recovery technology involving an advanced form of steam stimulation (Foght et al 2017; Gates and Larter 2014). There is currently limited information on the microbiome of these deeper bitumen deposits, which is largely due to the difficulty in obtaining intact samples. However, one metagenomics/ metabarcoding study, which analysed the microbiome of deep oil sand deposits from SAGD core drillings, found that both aerobic and anaerobic communities were in close proximity (An et al 2013). Specifically, a network of aerobes were found which included the genera/ (orders): *Rhizobium (Rhizobiales)*, *Cupriavidus (Burkholderiales)*, *Brevundimonas (Caulobacterales/)*, *Delftia (Burkholderiales)*, and *Methylobacterium (Rhizobiales)* (An et al 2013). In addition, metagenome analysis identified several aerobic related oxygenase enzymes in the deep oil sand samples (An et al 2013). These communities also contained hydrocarbon-degrading bacteria and methanogens such as *Methanosarcina*, which suggested limited oxygen ingress in these cores (An et al 2013). Despite these findings, the microbiomes of deep oil sand deposits and their functional role in NA biodegradation remains understudied and further research is required in this area.

3. Anthropogenic Naphthenic Acid (NA) contaminated microbiomes

The oil sands mining operations in northern Alberta, Canada, started in 1967 and undertook a rapid expansion between 2000 and 2010 (Fennell and Arciszewski 2019). Such a rapid development in the Athabasca watershed has raised considerable concerns regarding their effects on the surrounding ecosystems. Bitumen extraction disturbs the land, causing multiple environmental issues, including habitat fragmentation; freshwater abstraction; emission of biogenic greenhouse gases, and emissions of other pollutants, such as mercury and volatile hydrocarbons (Foght et al 2017; Siddique et al 2008; Simpson et al 2010; Small et al 2015). As a result of bitumen extraction, upgrading, storage and export operations, large volumes of OSPW are stored in vast tailings ponds, where NAs accumulate. However, the long-term storage of OSPW poses potential environmental risks

to local and downstream ecosystems, where organic (residual hydrocarbons, NAs) and inorganic (heavy metals) contaminants from tailings ponds may enter surface and groundwaters as a result of effluent discharge, pond breaches, seepage and spills (Ahad et al 2020; Fennell and Arciszewski 2019). Indeed, seepage of OSPW (especially NAs) into surface and groundwaters from tailings ponds is a major environmental concern (Fennell and Arciszewski 2019).

Differentiation of the source of NAs in the local environment (i.e. identifying whether from natural or anthropogenic origin) would help to determine whether any accidental NA incursions had occurred. Yet the differentiation of NA source is complicated by the diverse and complex nature of the mixtures of organic compounds found within all bitumen ecosystems (Hewitt et al 2020). Moreover, the structural composition, concentration, toxicity, and chemistry of NAs may differ depending on the source of NAs, which in turn influences the *in situ* microbiomes found (Whitby 2010). Several methods have been reported to differentiate between anthropogenic water soluble organics (WSOs) and naturally occurring bitumen impacted WSOs, including high resolution mass spectrometry techniques, ultraperformance liquid chromatography time-of-flight (ToF) mass spectrometry, and multidimensional gas chromatography with ToF mass spectrometry, among others (Hewitt et al 2020). Recently, the use of orbitrap mass spectrometry with carbon isotopes (e.g. $\delta^{13}C_{pyr}$ and $\delta^{34}S$) has made some headway into distinguishing between naturally occurring and anthropogenic NA sources (Ahad et al 2020). In addition to these analytical techniques, biologically based approaches to track specific NAs in aquatic environments could also prove useful. For example, the marine coccolithophore *Emiliania huxleyi* as well as other organisms such as cyanobacteria have been found to be highly sensitive to NAs and could be used as potential bioindicators of NAs in the environment (Beddow et al 2016; Yergeau et al 2013). However, more research is required into their sensitivities towards the range of concentrations and types of NA compounds found in the various bitumen-associated ecosystems. Thus, having a better understanding of the source and transport of NAs in the environment will allow us to better understand the factors shaping the *in situ* microbiomes in these impacted ecosystems.

3.1. Oil sand tailings ponds (OSTPs)

In the AOSR, various different OSTP structures may be found. These include in-pit tailings ponds, which are modified from former mine pits, external tailings facilities (ETF) or external tailings area (ETA), which are dyked areas built aboveground and external to the active mining area (Fennell and Arciszewski 2019; Jeeravipoolvarn et al 2017). In general, OSTPs are constructed of coarse tailings (largely sand) that form a containment dyke (**Fig 5**) (Roy et al 2016). Within the dyke, there are collection systems to capture internal drainage, which may contain OSPW or any pond leakage (Roy et al 2016). Any water that is captured is subsequently returned to the tailings ponds (Roy et al 2016). In general, the ponds are underlain with a glacial till or clay, which has a low permeability, but where permeable materials do occur, the ponds are overlain with other lower permeability materials (Fennell and Arciszewski 2019). Despite these measures, there remains a concern surrounding OSTPs and potential leakage of OSPW into the surrounding environments.

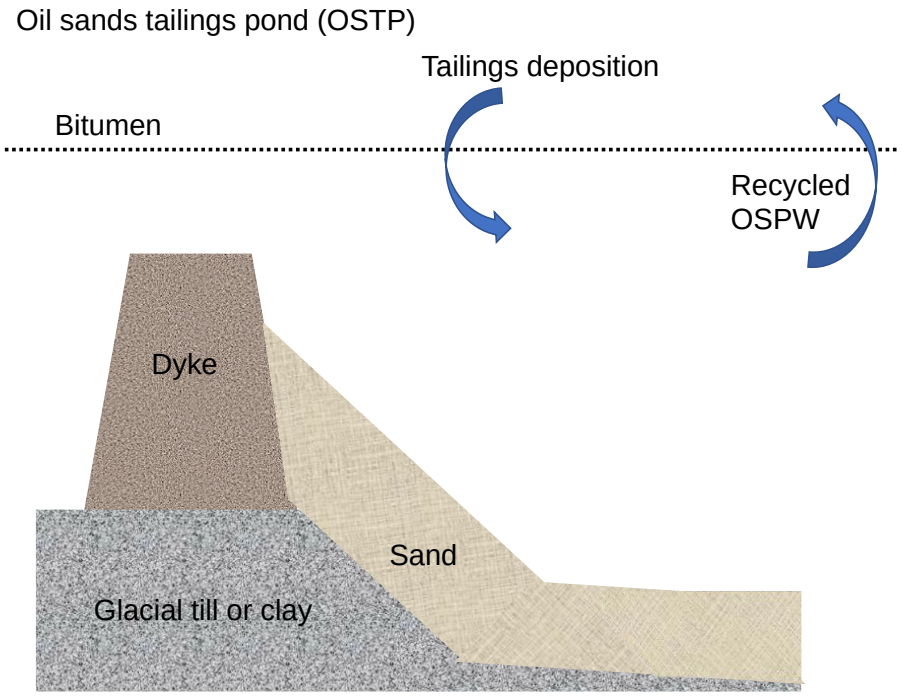

Fig 5. Simplified schematic of the structure of an oil sands tailing pond (OSTP).

Tailings ponds are heterogenous ecosystems where unique microbiomes are found (Foght et al 2017). For each pond, different environmental parameters and management practices exist which result in various physicochemical gradients, and in turn influence the microbiomes present (An et al 2013; Foght et al 2017; Penner and Foght 2010; Siddique et al 2018). For example, the addition of fresh tailings to ponds generate temperature gradients as they are still warm from processing when added (historically this was up to 60ºC) (MacKinnon 1989). Generally, ponds are also nitrogen limited, as low concentrations of ammonium, nitrate and nitrite occur (Foght et al 2017). Ponds are also typically stratified comprising of both oxic and anoxic zones. In general, oxygen ingress is limited to the upper surface layers (up to 1 m depth), and below this a sharp oxycline occurs, as the OSPW rapidly becomes anoxic (Ramos-Padrón et al 2011; Stasik and Wendt-Pethoff 2014). This anoxic zone is largely a result of aerobic and/or facultative microbial metabolism and the oxidation of microbially produced sulfides at depth (Foght et al 2017). In some ponds, sulfate becomes depleted as a result of sulfate reduction generating hydrogen sulfide which is subsequently removed and removing the inhibitory effect on methanogens facilitating a concomitant production of methane (Clemente and Fedorak, 2005; Fedorak et al 2002). Consequently, deposited tailings can emit significant quantities of methane (CH_4) and carbon dioxide (CO_2), where residual hydrocarbons is being used in methanogenesis (Siddique et al 2007). Indeed, (one of the largest and oldest tailings ponds, Mildred Lake Settling Basin (MLSB), has been estimated to emit ~43 million L CH_4 day^{-1} to the atmosphere (Siddique et al 2008).

Within tailings ponds, the solid content increases with depth, and density increases from <0.1 wt% in the upper layers to >10% solids content in the lower layers (Lv et al 2020; Siddique et al 2018). At the pond surface, there is an upper water layer of OSPW, which may range in depth from 2 to 10m (Foght et al 2017). In general, OSPW makes up only a small volumetric proportion of the ponds, largely due to re-use and recycling of the OSPW (which varies depending on the operator and/or seasonal changes) (Foght et al 2017). Below the water layer is the solids enriched layers of fine silt, sand and clay minerals (known as ‘fines’). These ‘fines’ include fluid fine tailings (FFT), which has a low solids content (Foght et al 2017). Below this layer is the mature fine tailings (MFT) layer, which has a higher solids content (≥30 wt.%), and is anoxic (Lv et al 2020; Siddique et al 2018; Foght et al

2017). The MFT layer can often remain undisturbed for decades and may reach depths of ≥40 m, depending on natural consolidation, dewatering, depth of the basin, age of the pond and management practice (Foght et al 2017).

Some operators mix gypsum (calcium sulfate dihydrate) with fine tailings and coarse sand to produce composite tailings (CT) as a type of dry reclamation strategy. The idea is to neutralize the net negativity of clay minerals to act as a densifying agent and recover interstitial pore water, resulting in an increase in the solids content and flocculation (Ramos-Padrón et al 2011; Mikula et al 1996; Warren et al 2016). It has been estimated that around 9.6 x 10^8 m^3 of CT are either in, or awaiting impoundment in surface pits within the AOSR (Warren et al 2016). These chemically engineered CT tailings are slightly alkaline (pH 8), moderately saline and consist of a mixture of FFT (i.e. saline water, suspended Fe^{3+} rich clay minerals, and residual bitumen) and post-processed sand amended with gypsum (Matthews et al 2002). However, the process causes an increase in the levels of Ca^{2+}, sulfate and NAs compared to MFT (Fedorak et al 2002; Foght et al 2017). The microbial diversity of CT has been reported to be relatively low compared with MFT, with common phyla found including Gamma- and Beta-proteobacteria, *Bacillota*, *Actinomycetota*, and microbes capable of sulfur metabolism (Warren et al 2016). Thickened tailings (TT) which have been augmented with organic polymers to flocculate the solids to recover more water may also occur (Foght et al 2017).

Tailings ponds can harbor a diverse range of microbiota. Facultative and/or aerobic microorganisms are reported to occur in high numbers (10^6-10^8 cells mL^{-1}) (Foght et al 2017). Within the bacteria, members of the Beta-, Delta- and Epsilon- Proteobacteria have been found (An et al 2013; Yergeau et al 2012). For example, *Thauera, Acidovorax, Thiobacillus, Brachymonas, Rhodoferax, Thiobacillus, Smithella, Schumannella, Hydrogenophaga, Azonexus, Salinimicrobium, Achromobacter, Gillisia*, *Alcaligenes* sp. and *Acinetobacter* sp. (Yergeau et al 2012; Del Rio et al 2006; Holowenko et al 2000; Ramos-Padron et al 2011). In addition, enrichment cultures established from OSPW and oil sands have recovered *Pseudomonas* sp., *Alcaligenes*, *Acinetobacter*, and *Kurthia* species among others (Herman et al 1994; Wyndham and Costerton, 1981a,b). However in some ponds, increased NA concentrations and/or limited carbon sources may select for a less diverse bacterial community compared to natural NA-contaminated ecosystems such as Athabasca River sediments (Yergeau et al 2012). Within the Archaea, Euryarchaeota (primarily *Methanomicrobia*) have been found, with the Crenarchaeota almost absent (Yergeau et al 2012). Phototrophic and heterotrophic Eukarya including Fungi, Metazoa, Chlorophyta, Alveolates, Stramenopiles and Excavata occur (Aguilar et al 2016). Specifically, 18S rRNA gene sequencing has revealed that the majority of sequences were from heterotrophs related to Rhizaria, Ciliata and Fungi which is consistent with other low-light and low-oxygen environments (Aguilar et al 2016). In addition, photosynthetic taxa such as *Euglena* and Chlorophyta have also been reported (Aguilar et al 2016).

The diverse microbial communities found in tailings ponds are capable of various metabolic functions including hydrocarbon biodegradation, element cycling (S and Fe) and gas production (CH_4, CO_2, H_2S) (Wilson et al 2016; Warren et al 2016). Anaerobic metabolism dominates OSTPs, particularly methanogenesis, due to the anoxic conditions during biodegradation of hydrocarbons such as residual bitumen (Siddique et al 2008; 2015; Fedorak et al 2003; Holowenko et al 2000). Indeed, many of the microbiota found in tailings are involved in anaerobic hydrocarbon degradation, including fermentative and syntrophic bacteria, nitrate reducers, iron reducers, sulfate-reducing bacteria (SRB), hydrogenotrophic and acetoclastic methanogens (An et al 2013; Golby et al 2012; Ramos-Padron et al 2011). However, the presence of sulfate from the addition of gypsum by some operators may promote sulfidogenic activity and sulphate-reducing bacteria (e.g. *Desulfocapsa*, *Desulfurivibrio*, *Desulfobacterium*, and *Desulfuromonas*), may outcompete methanogens for available substrates inhibiting methanogenesis when sulfate is abundant (Holowenko et al

2000; Fedorak et al 2003; Ramos-Padron et al 2011). Yet, in the deep anaerobic zones (where sulfide and sulfate concentrations increases), methanogens (e.g. *Methanosaeta* (acetoclastic) and *Methanolinea* and *Methanoregula* (hydrogenotrophic) have been found (Ramos-Padrón et al 2011).

Shifts in the microbial communities may also occur depending on the NAs source, the hydrocarbon substrates present and the *in situ* conditions (Aubu Laban et al 2015 c;d; Tan et al 2013). For example, when aromatics are present, *Desulfosporosinus* (under methanogenic conditions) or *Desulfobulbaceae* (under sulfidogenic conditions) were found to dominate, whereas when alkanes were present, other taxa such as *Peptococcaceae* dominated (Aubu Laban et al 2015 c;d; Tan et al 2013). Furthermore, under nitrate-, and sulfate- reducing conditions, the relative abundance of *Rhizobium* increased when enriched with AEOs, whilst *Steroidobacter* and *Methanosarcina* increased under nitrate-reducing conditions in the presence of a commercial NA mixture (Lv et al 2020). Shifts in the microbiome may also occur as a result of the various bioremediation treatments for tailings ponds, which can include UV irradiation, filtration, carbon adsorption, coagulation and ozonation (Quinlan and Tam 2015). For example, following ozonation treatment, a decrease in the abundance of planktonic microbes and *Pseudomonadota* was reported (Islam et al 2015). Specifically, *Pseudomonadales* which are generally dominant, were sensitive to ozonation and their abundance decreased, whilst others such as *Burkholderiales* increased in abundance, as ozonation reduced the toxicity of the OSPW and amount of labile hydrocarbons (Islam et al 2015). In another study, treatment of OSPW by ozonation, also decreased the abundance of *Pseudomonadota* compared to the untreated community (Zhang et al 2020). Cyanobacteria and *Chloroflexota* have also been found to increase following ozonation of OSPW biofiltrates (Zhang et al 2020a). Furthermore, *Bacteroidota* increased only in ozonated biofiltrated OSPW, whereas *Actinomycetota* increased only after biofiltrated raw OPSW (Zhang et al 2020a). Thus, the functionally diverse microbiomes may alter through the various bioremediation treatment strategies and this is an important consideration for successful pond reclamation.

3.2 Oil sands process affected water (OSPW)

OSPW is composed of a mixture of various source waters including river water, formation water, recycled water and surface run-off (Mahaffet and Dubé 2016), and is stored in ponds of various sizes and ages (Fennell and Arciszewski 2019). It has been reported previously that the NA concentrations found in OSPW may be as high as 120 mg L^{-1} (Clemente et al 2003b; Holowenko et al 2002;). The constant recycling of OSPW may select for an enriched and adapted microbiome. Indeed, the surface waters of OSPW may harbor more diverse taxa (*Methyloversatilis*, *Azospirillum* and *Gemmata*) than the deeper layers, where strict anaerobes such as methanogens, sulfate reducers, nitrate reducers and iron reducers occur (An et al 2013; Ramos-Padrón et al 2011; Foght et al 2017; Stasik *et al* 2014). However, it has been reported that both methanogens and sulfate reducers are only minor members of OSPW communities (An et al 2013). Additionally, thiosulfate oxidizers (Stasik *et al* 2014), sulfur oxidizers (e.g. *Chromatiales* (*Thiocapsa*) and *Desulfuromonadales* (*Geobacter*)) and facultative species of *Rhodocyclales* (*Thauera*), *Burkholderiales* (*Acidovorax*, *Hydrogenophaga*, Alcaligenaceae) and *Flavobacteriales* (*Flavobacterium*) have been found in OSPW (An et al 2013). In another study, enrichments of anoxic OSPW in the presence of model NAs were dominated by *Terrabacter*, *Derxia, Limnobacter, Lutibacter* and *Pseudomonadota* under nitrate-reducing conditions, while *Anaerobacter, Clostridium, Syntrophus, Desulfobulbus, Desulfobaceterium* and *Desulfomicrobium* dominated under sulfate-reducing conditions (Clothier and Gieg, 2016). Also found in OSPW enriched with diamondoid NAs were *Pseudomonadota, Bacteroidota, Actinomycetota* and *Bacillota* (Folwell et al 2020); and algae, such as *Chlorellales* and *Acutodesmus* (*Scenedesmus*) (Paulssen and Gieg, 2019). Methane oxidation to CO_2 by aerobic

methanotrophic bacteria (predominantly *Methylocaldum*) has also been reported in OSPW, with ~17% of biogenic CH_4 at the pond surface being microbially oxidized, and potentially mitigating against some of the greenhouse gas emissions from OSTPs (Saidi-Mehrabad et al 2013).

The microbiota found within OSPW also has the potential to aerobically biodegrade hydrocarbons (An et al 2013). In the uppermost OSPW layer where it is aerated, aerobic hydrocarbon degradation has been reported, but is slower in the winter when the ponds are covered with ice (Foght et al 2017). Data from metagenome analysis has found several putative mono-, di- oxygenases and O_2-dependent ring cleavage enzymes in the genomes of pond surface waters, suggesting that the microbial communities within OSPW have the potential for aerobic biodegradation of hydrocarbons (An et al 2013). The yeast *Rhodotorula mucilaginosa* and fungus *Trichoderma harzianum* have also been enriched from OSPW amended with commercial NAs, demonstrating the capacity for mycoremediation of NAs including the diamondoid, 1-adamantane carboxylic acid (Miles et al 2020). It is well known that microbial biodegradation decreases OSPW toxicity over time (Frank et al 2009; Johnson et al 2011) and therefore bioremediation using microorganisms is a potential strategy for both the detoxification and removal of OSPW.

3.3 Mature Fine Tailings (MFT)

Within the bacterial community, the MFT microbiome is dominated by *Pseudomonadota* (Penner and Foght 2010). Notably, *Betaproteobacteria*, particularly *Albidoferax* (formerly *Rhodoferax*), *Acidovorax* sp., *Thauera*, *Desulfatibacillum, Burkholderiales* and *Hydrogenophilales* (Foght et al 2017; Penner and Foght 2010). In some MFT samples, a dominance of bacteria rather than archaea was found, with an increase in abundance of fermenters within the *Chloroflexota* (*Anaerolineales*) and *Actinomycetota* (*Coriobacteriales*) (Siddique et al 2015; Collins et al 2016; Mohamad et al 2016). However, it has also been shown that in MFT the relative abundances of archaea and bacteria change with increasing depth, and the proportions of archaeal sequences having a broad range of hydrocarbons and exhibiting active methanogenesis, decreased with depth where labile hydrocarbons were depleted, whilst the bacteria (notably, Alpha-, Beta- and Gamma-proteobacteria) increased, at the expense of *Bacillota* and Deltaproteobacteria (Foght et al 2017, Stasik et al 2014). This supports the hypothesis that ‘dormant’ MFT tend to be dominated by bacteria, whilst in ‘active’ MFT archaea are dominant (Foght et al 2017). It has also been reported that the deepest zones of the tailings pond harbor bacteria not found elsewhere (e.g. *Brachymonas, Thiobacillus*, and *Cellulomonas*), and that these microorganisms may help in some way with tailings densification or consolidation (Ramos-Padron et al 2011). Eukaryotes have also been found in MFT including Amoebozoa and Rhizaria (Aguilar et al 2016).

Using mesocosms and laboratory incubations of MFT, it has been shown that bacterial and archaeal community succession patterns occur, whereby the original MFT is dominated by bacteria but shifts towards sulfidogenesis and a dominance of archaea following bioreactor manipulation (Chi Fru et al 2013). In terms of the functional microbiome of MFT, an abundance of methanogens and sulphate reducers have been found with both methanogenesis and sulfidogenesis occurring in tailings ponds with their overall dominance depending on the operational practice (Ramos-Padrón et al 2011; Foght et al 2017). For example, where gypsum has been added, sulphate reduction may dominate (Foght et al 2017). However, the methanogenic community is generally considered the most active in MFT (Lv et al 2020; Fedorak et al 2002; 2003). Indeed MFT enrichments have the potential for methanogenic degradation of hydrocarbons such as naphtha and short chain *n*-alkanes among other compounds (Foght et al 2017). Within the methanogens, *Methanomicrobiales, Methanosaeta, Methanoregula* and *Methanosarcinales* were found to dominate (Penner and Foght 2010; Foght et al 2017). In the deeper anaerobic zones where sulfide and sulfate

concentrations increase, the microbial community was dominated by syntrophs (*Pelotomaculum*, *Syntrophus*, and *Smithella* sp.) and sulfate- and sulfur-reducing bacteria (*Desulfocapsa, Desulfurivibrio* sp.*, Desulfomicrobium, Desilfomonile*, *Desulfobulbus* and *Acetobacterium*) (An et al 2013; Lv et al 2020; Fedorak et al 2002; 2003). A range of other anaerobic microbiota have also been found including: Comamonadaceae, Hydrogenophilaceae, Anaerolineaceae, iron reducers, fermenters, and nitrate reducers (e.g. Rhizobium, Steroidobacter and Ignavibacterium) (Lv et al 2020; Fedorak et al 2002; 2003). Although nitrate reduction occurs, it is generally less dominant as nitrate and nitrite concentrations are typically low (Fedorak *et al* 2002; Penner and Foght 2010; Stasik and Wendt-Potthoff 2014). Despite this, facultative nitrate reducers are abundant and widely distributed throughout tailings depth profiles (Fedorak et al 2002). Other anaerobic hydrocarbon-degrading taxa have also been recovered from MFT enrichments, including members of the *Clostridia* (*Peptococcaceae*, *Desulfosporosinus*), *Deltaproteobacteria* (*Desulfobulbaceae*), *Rhodocyclales* (An et al 2013; Tan et al 2014a,b, 2015a, b; Abu Laban et al 2015a,b). Metagenome analysis has revealed genes for aerobic aromatic hydrocarbon biodegradation and aerobic methane oxidation (An et al 2013). Strictly aerobic genera in this environment included the methanotrophic *Methylococcales*/ Methylocaldum (An et al 2013). Indeed, it is estimated that 20% of the methane formed in the deeper layers of tailings ponds is oxidized by methanotrophs in surface waters, which is promising for mitigation against the greenhouse gas emissions from ponds (Saidi-Mehrabad et al 2013).

3.4 End pit lakes (EPLs) and reclaimed ponds

One of the major environmental challenges arising from the oil sands mining operations is the vast amount of tailings and OSPW that require reclamation (Foght et al 2017). Under government regulations, it is mandatory for tailings ponds be reclaimed within 10 years of the end of a mining operation, either as terrestrial landscapes, wetlands or end-pit lakes (EPLs) (Paulssen and Gieg 2019). Reclamation of tailings ponds however, remains a challenge due to slow rates of fine tailings densification and the presence of toxic, recalcitrant components associated with OSPW (Foght et al 2017). Moreover biogenic greenhouse gas emissions remain a challenge with OSTPs accounting for 45 % of total CH_4 emissions measured from the major surface mining facilities in the AOSR, while emissions from operations in the open pit mines accounted for ~ 50 % (Baray et al 2018). Adopting nature-based solutions for OSPW and tailings bioremediation is attracting significant interest, but requires a thorough understanding of the *in situ* communities and their processes.

The microbiomes of tailings ponds are critical for pond reclamation and a diverse community is crucial for effective tailings bioremediation (Headley and McMartin 2004; Yergeau et al 2012; Ajaero et al 2020). The flexibility and ability of the microbial community to adapt is also an important consideration for pond reclamation, especially as certain substrates are being selectively removed through the various microbial activities. Furthermore, pond reclamation and remediation must also consider issues such as H_2S production from constructed wetlands containing sulfate-rich tailings (Reid and Warren 2016), acid mine drainage from surface deposition of tailings and the wider implications of EPLs (Foght et al 2017). Under the Albertan Government's zero effluent discharge policy, operators undertake both 'wet' and 'dry' reclamation approaches (Foght et al 2017). One strategy is to place de-watered tailings in exhausted mine pits and cover them with sand, soil and vegetation to regenerate the landscape. For dry landscaping, the MFT sediments may require chemical and/or physical manipulation to remove excess water and increase solids content, whereas MFT intended for wet reclamation (freshwater ecosystems) does not require additional dewatering (Foght et al 2017). However, for the success of both wet and dry reclamation approaches, suitable microbial communities are required.

Wet capping, is one reclamation technique used with EPLs, whereby soft tailings or fluid fine tailings (FFT) are placed within decommissioned open pits and capped with freshwater (ca. 3-10 m depth) (Dompiere and Barbour 2016). The layers of freshwater are continually replenished until the tailings are settled into a thick layer and the freshwater has diluted all potential contaminants to ecologically acceptable levels and oxygen levels are comparable to that found in natural lakes (Dompiere and Barbour 2016; White et al 2018). The design and configuration of EPLs and the type of tailings stored varies with operators, but their use allows oil sands operators to decrease the volumes of FFT stored in tailings impoundments. One of the major challenges associated with reclaiming oil sands tailings in EPLs is the slow consolidation rates due to the fine-grained nature of FFT (Cossey et al 2021). To assess the efficacy of pond reclamation, heterotrophic protists have been suggested as bioindicators as they are highly resilient and represent a trophic level intermediate between bacteria and macrofauna (Richardson and Dacks 2019). Another major issue surrounding EPLs is that high concentrations of dissolved constituents, NAs, unrecovered bitumen and petroleum hydrocarbons in the FFT pore water may be transported into the lake water (Dompiere and Barbour 2016). It is proposed that this movement occurs via two key processes: (1) advective-diffusive mass transport with upward pore water flow caused by dewatering of the soft tailings; and (2) mixing by wind events or unstable density profiles through the lake water and upper portion of the FFT (Dompiere and Barbour 2016). Heat transfer between the water cap and the FFT may also occur (Dompiere and Barbour 2016). The fluid movements in the lake may cause mixing within the tailings influencing the *in situ* biogeochemistry, which in turn shape the indigenous microbiomes (Dompiere and Barbour 2016). However, establishing a sustainable biological community in EPLs is yet to be fully assessed and more research is required in this area (Dompiere and Barbour 2016).

One of the largest reclaimed sites for FFT in Canada is called Base Mine Lake (BML), which was established in 2012, and is approximately 780 hectares in size, comprising ca. 40m depth of FFT overlain with 10 m of a freshwater cap (Risacher et al 2018; Albakistani et al 2022). Between 2014 and 2019, methane emissions averaged 610 kg ha^{-1} y^{-1} with the highest levels during spring thaw, but have since gradually declined over time (Albakistani et al 2022). Methane/O_2 counter gradients occur in the water column. Specifically, dissolved methane concentration in the sediment is very high, and increases rapidly with depth (i.e. < 0.1 mM at the sediment-water interface increasing to > 2 mM between 1.0 -1.5 m depth in ~4 mM at 1.5-3.5 m depth in the FFT layer (Albakistani et al 2022). During summer stratification, a methane gradient occurs in the water column (i.e. 20-40 µM in the hypolimnion; to 0.38-1.2 µM in the epilimnion (Albakistani et al 2022). However, dissolved O_2 concentrations decrease with increased depth in the water column, (i.e. 70-85% saturation at the metalimnion to 1-5% saturation at the sediment interface (Albakistani et al 2022).

This dimictic pit lake exhibits methane cycling in the metalimnion, hypolimnion, and sediment-water interface and methanotroph population dynamics are similar to that found in natural boreal lakes (Albakistani et al 2022). Specifically, aerobic methanotrophic bacteria comprise up to 58% of the total bacterial communities (based on 16S rRNA gene sequencing reads) (Albakistani et al 2022). Moreover, seasonal changes in methanotrophic activity and population abundances were also found in the lake water, with autumn and winter highs, which declined during summer stratification, especially in the epilimnion (Albakistani et al 2022). Of the methanotroph genera found were: *Methylobacter*, *Methylovulum*, and *Methyloparacoccus* (Albakistani et al 2022). Specifically, *Methylobacter* and *Methylovulum* populations peaked in the winter/spring, when methane oxidation activity was psychrophilic, and *Methyloparacoccus* populations increased through summer and autumn, when methane oxidation was mesophilic, which suggested that there was a

temporal niche differentiation based on temperature, oxygen and methane concentration (Albakistani et al 2022).

Seasonal changes in turbidity have also been reported in BML, which are thought to be due to the settling of fine particles suspended in the surface water and the resuspension of fine particles from the FFT-water interface (Lawrence et al 2016). However, even small changes in turbidity may negatively impact on the microbial metabolic processes occurring (Shen et al 2019). Biofilms which are formed on the FFT-water interface have a biostabilization effect, reducing the resuspension of sediments, and thus reduce turbidity in EPLs (in some cases by up to 99% depending on the biofilm age and mixing speed) (Cossey et al 2019). The microbiomes of biofilms formed on the FFT-water interface contain diverse communities including photoautotrophs, such as cyanobacteria and Chlorophyta (green algae), and several heterotrophs including members of the Gammaproteobacteria, Desulfobulbia, and Anaerolineae (Cossey et al 2019). In another study, it has been shown that when water from BML was used in a co-culture with *Chlorella kessleri* biodegradation of model NAs was enhanced and the presence of *C. kessleri* concomitantly increased the diversity of the microbial community found in the BML water, with many known hydrocarbon and NA-degraders (Yu et al 2019).

Diverse microbial communities that contribute to nutrient cycling have also been found in other pond reclamation sites (Risacher et al 2018). For example, methanotrophy and nitrification are important oxygen consuming process in these ecosystems (Risacher et al 2018). However, during the early stages of pit lake establishment at low oxygen concentrations, nitrifiers appear to be outcompeted by methanotrophs (Risacher et al 2018). Yet within four years of commissioning, active nitrification has been reported, affecting oxygen concentrations in the water column of pit lakes (Risacher et al 2018). This is in contrast to OSTPs, where nitrification is often low, even when ammonium levels may be high (Risacher et al 2018).

A promising low-energy method for OSPW treatment are constructed wetland treatment systems (CWTSs) which are designed to simulate natural wetlands (Foote 2012; McQueen et al 2017; Hendrickse et al 2018; Ajaero et al 2020; Simair et al 2021). One pilot-scale CWTS was found to decrease the concentration of NAs, hydrocarbons, metals, and toxicity (McQueen et al 2017). Indeed, several mature wetlands in the AOSR have been reported to promote NA degradation and detoxification (Vander Meulen et al 2022). However, one laboratory scale constructed wetland, showed that the more complex NAs (i.e. those with increased carbon numbers such as 17–20) were more recalcitrant to biodegradation (Toor et al 2013a,b). In addition, wetland reclamation involving CT deposits may increase sulfur levels, resulting in microbial sulfur cycling and H_2S generation (Reid and Warren 2016). Indeed, high concentrations of H_2S (>500 uM or 18 mg/L) have been found in the sand cap of a constructed wetland due to active dewatering causing an upward migration of sulfur rich water, and downwelling of labile organic carbon from the developing wetland which stimulated microbial H_2S production (Reid and Warren 2016). In contrast, to H_2S levels, another study showed that methane emissions were lower at a constructed fen north of Fort McMurray compared to a nearby reference fen (Murray et al 2017). Wetland reclamation after mining may also generate marshes with elevated salinity and residual hydrocarbons (Mollard et al 2015). Bacterial diversity and abundance has been found to be relatively low in constructed wetlands with phyla including Gamma- and Beta-proteobacteria, *Bacillota*, *Actinomycetota*, and *Chloroflexota*) and some taxa distinct from OSTPs (Warren et al 2016). Despite potential issues surrounding the long-term stability of constructed wetlands (Reid and Warren 2016), they are currently being tested by the oil sands industry to treat OSPW, as operators face the challenge of reclamation under the Albertan Government's water-use restrictions.

3.5 Biofilms, Bioreactors and biofilters

Often mixed cultures perform better at NA biodegradation than single cultures. For example, *Pseudomonas putida* and *Pseudomonas fluorescens* were better at NA degradation than as individual cultures (Del Rio et al 2006). Moreover, mixed microbial consortia grown as biofilms may be effective in the removal of NAs, particularly the more recalcitrant NA compounds found in OSPW and tailings ponds (McKenzie et al 2014; Golby et al 2012; Demeter et al 2015; Folwell et al 2016). Indeed, differences in the NA-degrading microbiota has been reported between planktonic and biofilm microbiomes, with increased NA degradation rates associated with biofilm communities (Demeter et al 2015; Folwell et al 2016). Furthermore, overexpression of the proteins involved in biofilm formation may facilitate NA removal in OSPW and tailings (McKew et al 2021). Indeed, a poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase protein, which is known to be involved in biofilm formation (Wang et al 2004) was found to be significantly upregulated during NA biodegradation (McKew et al 2021).

One approach to enhance biofilm formation is the use of bioreactors. Indeed it has been suggested that biofiltration of OSPW in fixed-bed biofilm reactors would allow the development of NA-degrading microbial communities within the biofilter allowing for more successful removal (Zhang et al 2020a). Bioreactors are engineered systems that can be used to enhance biodegradation rates of many recalcitrant organics including NAs (Islam et al 2014a,b). These technologies may use materials such as clays to facilitate biofilm formation of NA-degrading microorganisms as well as to concentrate the NAs (Choi et al 2014; Golby et al 2012; Islam et al 2014a; Paslawski et al 2009). The use of petroleum coke (PC) adsorption to biofilters has also been shown to be effective in reducing the more structurally complex NAs (i.e. $12 \geq n \geq 18$ and $z = -10, -12$) and their toxicity in OSPWs, depending upon the PC content, pH and temperature (Kannel and Gan 2012). Many different types of bioreactors have been reported with differing degrees of success. For example, a modified Ludzack-Ettinger membrane bioreactor (MLE-MBR) removed ~25% of NAs after 361 days of operation (Xue et al 2016). Whilst sequencing batch reactors (SBR) inoculated with MFT achieved ~16% removal of NAs in OSPW (Choi et al 2014). Granular activated carbon (GAC), which has a high adsorptive capacity for organics, removed >86% and 99.5% of NAs from raw and ozonated OSPW, respectively (Islam et al 2014b).

Characterization of the GAC biofilm community found *Pseudomonadota* dominated the biofilm community compared to planktonic samples (Islam et al 2014b). Specifically, Alphaproteobacteria (e.g. *Rhizobiales, Rhodospirillales*, and *Rhodobacterales*) and Gammaproteobacteria (e.g., *Pseudomonadales, Alteromonadales, Chromatiales, Xanthomonadales, Oceanospirillales, Legionellales*, and *Methylococcales*) were abundant (Islam et al 2014b). In addition, *Burkholderiales*, *Pseudomonadales*, *Sphingomonadales*, *Acidobacteriota*, *Verrucomicrobia, Bacteroidota*, and *Chloroflexota* were also recovered (Islam et al 2014b). In another study which treated both raw and ozonated OSPW using biofilters, a shift in the indigenous microbial community was reported, with an increase in alpha diversity which corresponded with enhanced NA degradation (Zhang et al 2020a). These findings suggested that continuous operation of OSPW in the bioreactors was in favor of shaping the overall microbiome towards better NA degradation (Zhang et al 2020a). Therefore, to improve the design and efficiency of bioreactors, a better understanding of the functional microbiomes is required.

NA toxicity is reported to be one of the many factors that shape the functional microbiomes of biofilms used to treat NAs. Indeed, reduced photosynthetic activity has been observed in biofilms exposed to NA-contaminated sediments (Yergeau et al 2013). In the GAC biofilm community, an accumulation of toxic NAs on the GAC surface resulted in only those members of the community with high tolerances to NAs surviving (Islam et al 2014b). Interestingly, Deltaproteobacteria were not able to grow on the GAC biofilm, which may be due to an increased sensitivity to higher concentrations of NAs found on the GAC surface

(Islam et al 2014b). In aerobic biofilms, a positive correlation between members of the *Pseudomonadota* and NA concentration has also been reported (Yergeau et al 2013). Nitrospirae, which are potentially involved in denitrification, sulfur oxidation, and sulfate reduction, have been reported and may have been metabolizing NA compounds containing nitrogen and sulfur (Islam et al 2014a). Immobilized soil/sediment bioreactors (ISBRs) were also dominated by ammonium- and nitrite- oxidizing bacteria, suggesting that nitrification was occurring during the treatment of NAs in OSPW (McKenzie et al 2014). The dominant genera included *Nitrosomonas, Candidatus Nitrotoga, Arenimonas, Mesorhizobium, Bradyrhizobium, Nitrosospira, Mycobacterium, Limnobacter*, and *Commamonas*, with *Truepera, Flexibacter*, and *Saprospiraceae* considered to be involved in the biodegradation of the more recalcitrant NAs (McKenzie et al 2014). Another approach using the Calgary Biofilm Device (CBD) (a technology to facilitate the development of biofilms), demonstrated a dominance of *Pseudomonadota* (particularly Alphaproteobacteria) in the biofilms (Demeter et al 2015b; Golby et al 2012). *Pseudomonadota* particularly members of the Beta- and Gamma- Proteobacteria were also dominant in a bioreactor inoculated with MFT (Choi et al 2014).

Different aerobic and anerobic communities may occur, which affect NA removal rates. For example, anoxic biofilm reactors were reported to have at least twofold higher rates of NA removal (coupled with the reduction of nitrate) compared to aerobic biofilm reactors where bioreactor performance deteriorated (Gunawan et al 2014). Within aerobic biofilms, *Rhodoferax, Acidovorax, Acinetobacter, Pseudomonadota*, and *Thioalkalispira* have been found, whilst *Hydrogenophaga, Rhodoferax, Methyloversatilis, Magnetospirillum*, and *Acidovorax* were present in anaerobic biofilms, but Archaea were absent (Golby et al 2012). One study which used fixed-bed biofilters to biodegrade NAs in OSPW, found a high abundance of aerobic bacteria including (*Porphyrobacter, Legionella, Pseudomonadota*, and *Planctomycetota*) (Arslan and El-Din 2021). However, redox conditions within the biofilters were anoxic and selected anaerobic bacteria such as *Ruminicoccus, Eubacterium, Faecalibacterium*, *Dorea* and hydrogenotrophic methanogens (e.g. *Methanobrevibacter*, *Methanomassiliicoccus*) suggesting that methane production was likely occurring by syntrophic processes during OSPW remediation (Arslan and El-Din 2021).

Bioaugmentation of fixed bed biofilters with NA-degrading bacterial strains showed the presence of potential hydrocarbons degraders (e.g. *Pseudomonadota, Pseudoxanthomonas*) and a high abundance of methylotrophs, notably *Methylobacillus* sp. (Arslan and El-Din 2022). Bioaugmentation of the biofilters not only rapidly improved OSPW remediation but aeration likely contributed to methane consumption in the top layer, minimizing its release into the environment (Arslan and El-Din 2022). In another study, a modified Ludzack-Ettinger membrane bioreactor (MLE-MBR) was dominated with hydrocarbon-degrading Betaproteobacteria; notably *Rhodocyclales* and *Sphingobacteriales* (Xue et al 2016). In addition to increasing NA biodegradation rates, bioreactors may also provide a route for obtaining functional inoculants of NA degraders for application to further enhance NA bioremediation (Lemire et al 2015). For example, two biofilm-associated biochar samples were shown to achieve 87% removal of NAs (Frankel et al 2016). Thus, the use of bioreactors for enhancing mixed microbial communities grown as biofilms, and/or their use in obtaining functional isolates, may prove useful in future NA bioremediation strategies.

4. Biodegradation of NAs

Both model and commercial NAs have been widely used in NA biodegradation studies to better understand the microorganisms involved in NA biodegradation (Whitby, 2010). Model NAs are chemically synthesized in the laboratory, whilst commercial NA mixtures are commercially-available mixtures of NA fractions that are obtained during petroleum processing and vary in composition and purity, with brands such as Kodak, Fluka and

Merichem available (Whitby 2010). Our previous understanding of the microorganisms driving NA biodegradation was largely through enriching consortia or obtaining isolates grown on either model or commercial NAs (Whitby 2010). Such studies have been important, for example in providing a better understanding of how NA structure relates to rate of biodegradation, levels of NA persistence and the microorganisms involved in NA biodegradation (Whitby, 2010; Johnson et al 2011; 2012). Yet model and commercial NAs, are not representative of those NAs found in the environment (Rowland et al 2011a). In general, model and commercial NAs are degraded at a faster rate than those acid-extractable NAs that are recovered from environmental samples (Del Rio et al 2006; Johnson et al 2011; Scott et al 2005). Since environmental NAs are often more recalcitrant than model or commercial NAs to biodegradation, more research is needed in this area (Bartlett et al 2017; Lv et al 2020; Johnson et al 2011; 12;13; Folwell et al 2015; Scott et al 2005; Bataineh et al 2006; Headley et al 2010). The real challenge however, lies in identifying the individual components of environmental NA mixtures, and the *in situ* microorganisms and processes involved in their respective metabolism.

4.1 Factors affecting NA biodegradation rates

Several biotic and abiotic factors have been shown to affect NA biodegradation rates. For example, chemical structure, where linear and lower molecular weight compounds (<22 carbons) are degraded preferentially, resulting in an increase in the relative amounts of recalcitrant hydrocarbons such as PAHs, NAs, branched and cyclic carboxylic acids, resins, and asphaltenes (Skeels and Whitby 2018; Folwell et al 2016; Clemente and Fedorak 2005; Shuqing et al 2008). Generally, lower molecular weight acids are more readily degraded leaving higher molecular weight compounds to persist in the environment (Biryukova et al 2007; Whitby, 2010). For example, Biryukova et al (2007) demonstrated that lower molecular mass NAs were preferentially degraded whilst the proportion of high molecular mass acids increased.

Generally, the most persistent NAs found in the environment contain multiple branched alkyl chains and methyl substitution of the cycloalkane rings (Han et al 2008; Smith et al 2008; Johnson et al 2012) as well as the highly branched and multi-ringed diamondoid NAs found in tailings ponds (Demeter et al 2015; Ahad et al 2018; Paulssen and Gieg, 2019; Folwell et al 2020; Rowland et al 2011a). For example, the microbial biotransformation of branched and diamondoid NAs was found to be more difficult to achieve (Demeter et al 2015; Johnson et al 2011; Scott et al 2005) than of those NAs that have single rings (Skeels and Whitby, 2018; Whitby, 2010). Recently, degradation of diamondoid NAs has been demonstrated by bacteria (e.g. *Pseudomonas* sp.) (Folwell et al 2020) and algae (e.g. members of the genus *Acutodesmus* (*Scenedesmus*) and order *Chlorealles*) (Paulssen and Gieg, 2019).

In addition to the number of cycloalkane rings present, *cis*-isomerism in alicyclic acids also influences biodegradation rates and different degradation rates have been reported for different geometric isomers (Whitby, 2010). Specifically, heterotrophic microorganisms from Athabasca river samples were reported to degrade trans-isomers more rapidly than the cis-isomers (Whitby, 2010). The surfactant properties of NAs and their associated toxicity may also impede microbial biodegradation (Zou et al 1997). Abiotic factors such as nutrient availability (e.g. phosphorus, nitrogen), temperature, oxygen concentration, pH, salinity, redox potential, and light may also reduce NA degradation rates (Herman et al 1994; Lai et al 1996). Furthermore, addition of other carbon sources, such as glucose, may be important for maintaining the populations of NA degraders (Demeter et al 2015). Indeed, the addition of organic compounds as co-metabolites may enhance NA degradation by some microorganisms (Dutta and Harayama 2001). However, the added compounds or their metabolites may be preferentially degraded by the indigenous microbiota and/or may be toxic to other NA-degraders (Dutta and Harayama 2001). The biodegradation of NAs by

microorganisms is therefore crucial for the removal of recalcitrant NAs in the environment (Lai et al 1996; Whitby, 2010). Yet, indigenous NA-degrading microbial populations must be able to thrive in the complex and toxic environments associated with NAs (Brient et al 1995).

4.2 Aerobic versus anaerobic biodegradation of NAs

NA biodegradation may occur both aerobically and anaerobically (Whitby 2010). Generally, aerobic NA biodegradation occurs very rapidly (i.e. within days) (Smith et al 2008; Biryukova et al 2007), with fused ring structures notably susceptible to aerobic microbial degradation (Herman et al 1993; Lai et al 1996). One study using a commercial NA mixture found significant degradation (~85%) under aerobic conditions, but only a small fraction of the NA mixture was completely mineralized to CO_2 (Misiti et al 2013). The use of phototrophic organisms therefore has great bioremediation potential (Kobayashi and Rittman, 1982). For example, phototrophic algae, not only may biotransform or bioaccumulate organic compounds but they have the added advantage of providing a constant supply of oxygen for the synergistic NA-degrading microbial communities (Kobayashi and Rittman, 1982). For example, one study showed that the addition of *Chlorella kessleri* increased the *in situ* microbial diversity, resulting in faster and more complete degradation of model NAs (Yu et al 2019). Another study demonstrated that both *cis*- and *trans*-isomers of a model NA (4-methylcyclohexaneacetic acid) were completely degraded at high concentrations (5.5 mg L^{-1}) by the diatom *Naviculla* sp. within two weeks, although the strain was unable to degrade NAs found in oil sands mixtures (Headley et al 2008). Degradation of NAs in oil sands mixtures however, was demonstrated by the green alga, *Selenastrum* sp. (Headley et al 2008). Furthermore, Quesnel et al (2011) showed a removal of tailings associated NAs (i.e. 11–17 carbon compounds of the *Z* family -2) with the unicellular alga *Dunaliella tertiolecta*. These observed differences in degradation may be linked to different transport mechanisms in algae and diatoms, or the NAs inducing osmotic stress, or as a result of differences in NA concentration and structure (Headley et al 2008).

In contrast to aerobic NA biodegradation, anaerobic NA biodegradation has received less attention. Transcriptome and enzyme activity data reported that the degradation pathway for the model NA, cyclohexylacetic acid (CHAA) induced under aerobic conditions could still work in anaerobic conditions with *Pseudoalteromonas* sp. (Zan et al 2022). Another study which enriched a microbiome from an OSTP under anaerobic conditions (either sulfate-reducing or methanogenic) found no significant degradation of the NAs tested (i.e. adamantane-1-carboxylic acid and the acid-extractable NAs from OSPW) (Folwell et al 2015). Folwell et al (2016) further reported the slow anaerobic degradation of 2-methylnaphthalene (but not pyrene) by OSPW microbes and suggested that high molecular weight NAs and PAHs may persist in OSPW under anaerobic conditions (Folwell et al 2016). In general the mechanism for anaerobic NA biodegradation remains unclear (Whitby 2010). However, it is known that under anaerobic conditions, the type and availability of electron acceptors is important in shaping the microbial NA-degrading community. One study that using model NAs showed that degradation depended on the anaerobic electron-accepting conditions (Clothier and Gieg 2016). Specifically, under sulfate-reducing conditions *Desulfobulbus* and *Desulfomicrobium* were dominant, while under nitrate-reducing conditions, *Pseudomonadota, Terrabacter, Limnobacter, Lutibacter*, and *Derxia* were abundant (Clothier and Gieg 2016). Under methanogenic conditions, *Clostridium* and *Methanosaeta* were dominant, while under iron-reducing conditions, methanogenic Archaea such as *Methanosarcina* and *Methanoculleus* were found (Clothier and Gieg 2016). The study also showed that most abundant bacterium under iron-reducing conditions was *Trichococcus*, a known citrate-fermenting microorganism with a shift in the microbiome in the NA-amended Fe (III)-reducing cultures from initially biodegrading NAs to utilizing citrate (Clothier and Gieg 2016). Previously, Siddique et al (2014) postulated that methanogens

using H_2 to produce CH_4 may transfer some electrons to Fe(III), reducing it to Fe(II), thus linking iron reduction and methanogenesis. Clothier and Gieg (2016) therefore suggested that the microbial communities in the NA-amended Fe(III)-reducing cultures most likely shifted over time from initially biodegrading NAs to utilizing citrate.

4.3. NA-degrading microorganisms

If *in situ* microbial processes are to be exploited for the bioremediation of NA-contaminated environments, then a better understanding of NA-degrading microorganisms and their processes is required. Using a range of model NAs, several NA-degrading microorganisms have been identified, including *Mycobacterium* sp., *Brevibacterium* sp., *Achromobacter* sp., *Corynebacterium* sp., *Rhodococcus* sp., *Acinetobacter* sp., *Alcaligenes* sp., *Pseudomonas* sp., *Flavobacterium* sp., *Moraxella* sp., *Micrococcus* sp., and *Bacillus* sp. (Whitby 2010). One study found a dominance of *Pseudomonas* sp., *Burkholderia* sp., and *Sphingomonas* sp. during NA biodegradation (Johnson et al 2011). Whilst other NA-degraders, such as *Ochrobactrum* sp., *Brevundimonas* sp., and *Bacillus* sp. have also been shown to have higher metabolic activity on polycyclic aromatic NAs compared to other classes of NAs (Yue et al 2015). Using model NAs, several different microorganisms, including *Pseudomonas citronellolis* and *Mycobacterium austroafricanum*, have demonstrated specialized metabolic capabilities to degrade carboxylic acids with alkyl-substituted aliphatic chains (Smith et al 2008; Johnson et al 2011). Studies have also shown that diverse bacteria utilize different commercial NAs as sole sources of carbon and energy, including *Corynebactrium*, *Arthrobacter*, *Acinetobacter*, *Alcaligenes*, *Pseudomonadota*, and *Bacillota* (Scott et al 2005). One study using a commercial NA mixture showed that 80% of the 16S rRNA gene sequences belonged to the Gammaproteobacteria, including *P. putida* and *P. fluorescens* as well as known hydrocarbon-degrading bacteria found in OSPW, such as *Microbulbifer* and *Xanthomonas* (Misiti et al 2013). *Methylophilus* and *Methylobacillus*, which are known methanol-utilizing bacteria, were also present in a large proportion (63–64%) of the community, although their role in NA biodegradation is not yet fully understood but likely as a result of methanol used in the synthetic wastewater (Misiti et al 2013).

Many previous studies have demonstrated the ability of *Pseudomonadota* to degrade a range of different NAs (Whitby 2010). For example, *P. fluorescens* and *P. putida* can degrade various model NAs (Blakeley and Papish, 1982; Del Rio et al 2006; Johnson et al 2013). *Pseudomonadota* were also reported to increase in abundance during the degradation of the highly branched aromatic NAs by mixed enrichment cultures (Johnson et al 2011). Furthermore, *Pseudomonadota*, especially *P. stutzeri*, dominated enrichment cultures degrading the diamondoid adamantane-1-carboxylic acid (A1CA) (Folwell et al 2020). Whereas other organisms, such as *Rhodococcus sp.* were not degrade the diamondoid tricyclic adamantane carboxylic acid, despite being able to degrade aliphatic and alicyclic carboxylic acid compounds (Presentato et al 2018). In another study, a 17% reduction in the 10-18 carbon compounds of the *Z* family -2 to -14 was reported by *P. fluorescens* Pf-5 (McKew et al 2021). It is therefore possible that under certain conditions, *Pseudomonadota* have a competitive advantage, not only by withstanding NA toxicity, but as likely NA-degrading genera. Thus, targeting pseudomonads in OSPW may be a bioremediation strategy for oil sands operators for enhanced NA removal. Indeed the use of *Pseudomonadota* has provided a further insight into the NA biodegradation mechanisms and the cellular stress responses following exposure to NAs (see Section 4.5).

4.4. Metabolic pathways of NA biodegradation

In general, the pathways for aerobic NA biodegradation includes alpha-oxidation, beta-oxidation, and aromatization (Clemente and Fedorak 2005; Whitby, 2010). Based on the metabolic intermediates detected one study suggested that biodegradation of a model NA (CHCA) followed three possible metabolic pathways, namely: (1) via beta-oxidation, (2)

via a pathway similar to benzoate degradation, and (3) via the aromatization of the cyclohexane ring (Clothier and Gieg 2016). Previous work on aerobic NA biodegradation using model NAs showed that it proceeds via beta-oxidation of the carboxyl side chain, and depends on the degree of alkyl side chain branching (Johnson et al 2011). Several microorganisms (e.g. *Pseudomonas putida, Acinetobacter anitratum, Alcaligenes faecalis*) among others can metabolise NAs by the beta-oxidation pathway (Blakley and Papish, 1982; Smith et al 2008; Johnson et al 2013; Clothier and Gieg, 2016). Biodegradation of aromatic alkanoic NA butyl phenyl butanoic acid (BPBA) by *Pseudomonas putida* KT2440 also followed the beta-oxidation pathway (Johnson et al 2011). During the biodegradation of the model NA *n*-BPBA, a metabolite (4′-*n*-butylphenyl)ethanoic acid (*n*-BPEA) was produced, which as has also been found previously with mixed enrichment cultures (Johnson et al 2011); by pure cultures of *Mycobacterium* sp. (Johnson et al 2012); and *Pseudomonas putida* KT2440 (Johnson et al 2013). It was proposed that the biodegradation of the *n*-BPBA by *P. fluorescens* Pf-5 involved the removal of two carbons from the carboxyl side chain, which is indicative of beta-oxidation (Johnson et al 2011; 2012; 2013). In addition, a *Mycobacterium* sp. isolate was shown to degrade aromatic NAs by switching between the beta- and omega-oxidation pathways (Johnson et al 2012).

4.5. Mechanisms involved in NA biodegradation and detoxification

Despite their toxicity and ubiquitous nature, very little is known about the microbial mechanisms involved during NA biodegradation. Until recently, the genes, proteins and mechanisms involved in NA biodegradation were largely unknown. Yet, such information is crucial to facilitate the rapid removal of these toxic, persistent pollutants from contaminated environments. Given that NAs are highly toxic to several organisms, including microorganisms (Frank et al 2009; Whitby, 2010), the microbiota must first be able to protect itself from the toxic effects of NAs during NA metabolism. Recently, new insights have been reported into the general cellular responses to oxidative stress and cell detoxification mechanisms during NA degradation.

One study which used LC-MS/MS shotgun proteomics with the model organism *Pseudomonas fluorescens* Pf-5 reported multiple putative membrane porins and membrane transporters that were upregulated during the biodegradation of a model NA and a commercial NA mixture (McKew et al 2021). Specifically, ATP (energy-dependent efflux pumps)-binding cassette (ABC) transporters were upregulated (McKew et al 2021). Such findings are not surprising given that ABC transporters actively transport chemicals and their metabolites out of cells, protecting the cell from its toxicity effects (Bard, 2000; Klaassen and Lauren, 2010; Hessel et al 2013; Alharbi et al 2016). However, the ABC superfamily of transporter proteins may be inhibited by OSPW (and any NAs therein) (Alharbi et al 2016). Other cellular responses to NA toxicity include the upregulation of putative outer membrane proteins (McKew et al 2021), such as OmpA that allows slow membrane penetration by small compounds (Van der Heijden et al 2016) and OprG (part of the OmpW family) involved in Fe transport and facilitates the repair of redox stress-induced damage (Andrews et al 2003; McPhee et al 2009). Several methyl-accepting chemotaxis proteins which are involved in cell motility also play a protective role following NA exposure, as a chemotactic response to NA toxicity (McKew et al 2021). Indeed, a positive correlation between chemotaxis and aerobic biodegradation genes has also been found in the marine environment with *Pseudoalteromonas* sp., whereby chemotaxis enhanced bacterium movement and NA biodegradation (Zan et al 2022). Molybdenum and molybdoenzymes which are also involved in chemotaxis (Schwartz and Mendel, 2006; Baraquet et al 2009; Leimkuhler and Lobbi-Nivol, 2016), and pollutant detoxification (Islam et al 2004; Chovanec et al 2012; Slyemi and Bonnefoy, 2012; Kruger et al 2013) were also found as an environmental stress response (McKew et al 2021). Moreover, molybdoenzymes require high affinity ABC transporters (Hagen, 2011), which supports the upregulation of ABC

transporters and overexpression of a molybdenum (Moco) cofactor biosynthesis protein reported by McKew et al (2021). Molybdenum metabolism is tightly connected to Fe-S cluster synthesis (Mendel, 2013) and bacterioferritin (Bfr2) which provides resistance to hydrogen peroxidase (Ma et al 1999; Rivera, 2017), and was also found to be significantly expressed during NA biodegradation (McKew et al 2021). In *P. aeruginosa*, it was suggested that *Bfr2* (along with molybdoenzyme) upregulation was an oxidative stress response to NA toxicity (McKew et al 2021).

Several other proteins relating to inorganic ion metabolism were also overexpressed as a cellular response to redox stress including a copper-containing nitrite reductase (CuNiR) and a Cu-containing cupredoxin protein capable of electron transfer reactions including being an electron donor to CuNiR in the denitrification pathway (Zumft, 1997; Vijgenboom et al 1997). However, the role of CuNiR and MoCo biosynthesis proteins in NA degradation remains unknown (McKew et al 2021). In another study, genome sequence analysis of *Cupriavidus gilardii* strain CR3, isolated from a natural asphalt deposit, and shown to utilize NAs, identified genes associated with xenobiotic biodegradation and metal resistance (Wang et al 2015). Indeed, *C. gilardii* strain CR3 demonstrated heavy metal tolerance, achieved by self-detoxification through ion efflux, metal-complexation and metal-reduction and DNA self-repair mechanisms, suggesting that *C. gilardii* strain CR3 is well adapted to survive the harsh natural asphalt environments that contain high concentrations of NAs and heavy metals (Wang et al 2015).

Recently, advances have been made into understanding the genetic and metabolic mechanisms involved in NA degradation. McKew et al (2021) identified several proteins associated with fatty acid, lipid and amino acid metabolism during NA biodegradation. Notably, the formation of oleic, linoleic, palmitic, and steric acids increases during NA biodegradation and these fatty acids are common constituents of prokaryotic and some eukaryotic membranes and predominate in NA-degrading microorganisms (Biryukova et al 2007; Clemente et al 2004). Hopanoic and steroidal acids also increase during degradation, and are thought to be derived from the microorganisms responsible for oil biodegradation (Meredith et al 2000). Specifically, acyl-CoA dehydrogenase, acyl-CoA thioesterase II, and an enoyl-CoA hydratase which metabolise various fatty acids by alpha- and beta-oxidation (Hunt et al 2012) were identified during NA biodegradation (McKew et al 2021). It is not surprising that proteins associated with alpha- and beta-oxidation of lipids were overexpressed, given that the aerobic NA biodegradation occurs via alpha-/ beta-oxidation pathways (Blakeley and Papish, 1982; Johnson et al 2011) (See Section 4.4). Several putative dehydrogenases, oxidoreductases, carboxylases and transferases were also proposed to be involved in NA biodegradation, especially multiple proteins involved in sequential reactions in fatty acid degradation (McKew et al 2021). It is not surprising that proteins involved in lipid metabolism were overexpressed during NA biodegradation, given the structural similarity between alkyl-carboxylic acid side chains in some model NAs and fatty acids (McKew et al 2021). Moreover, it was suggested that *P. fluorescens Pf-5* was using its existing fatty acid catabolic pathways for NA biodegradation (McKew et al 2021). Interestingly, since these fatty acid pathways are conserved, a wide variety of species are likely to have the enzymatic potential to biodegrade NAs (Whitby, 2010; Skeels and Whitby, 2018).

Recently, a marine *Pseudoalteromonas* strain was reported to degrade the model NA (cyclohexylacetic acid (CHAA) directly under aerobic conditions, but also anaerobically when the bacterium was induced with CHAA in aerobic conditions (Zan et al 2022). Degradation was activated by acetyl-CoA transferase to form the corresponding cyclohexene, alcohol, and ketone and finally cleaved by hydroxymethylglutarate-CoA lyase (Zan et al 2022). The biodegradation pathway involved the activation of the hydroxyl group by *atoB* and the anaerobic degradation pathway was consistent with the aerobic pathway after CHAA was activated by *atoB* (Zan et al 2022). Finally, the aliphatic pimeloyl-CoA was

converted into short-chain fatty acid by multistep beta-oxidation, and the product eventually entered into the TCA cycle (Zan et al 2022). Furthermore, genome sequence analysis of *Cupriavidus gilardii* CR3 revealed that degradation of cyclohexane carboxylic acid (CHCA) undergoes an initial ring-cleavage, and that the products of which are further oxidised by beta-oxidation via several pathways including a mechanism similar to that used for fatty acid oxidation (Wang et al 2015). The peripheral ring-cleavage process in *C. gilardii* CR3 observed by Wang et al (2015), was similar to the proposed process for aerobic oxidation of CHCA by aerobic oxidation pathways in *Pseudomonas putida* (Blakeley and Papish, 1982). In another study, the gene cluster *chcpca* was transcriptionally induced during the biodegradation of the model NAs CHCA and cyclopentanecarboxylic acid (CPCA) by *Rhodococcus aetherivorans* BCP1 and has been proposed to be involved in the beta-oxidation pathway (Presentato et al 2018). In *C. gilardii*, homologs to *bad /ali* genes were suggested to be involved in the peripheral ring-cleavage pathway for NA degradation (Wang et al 2015). The *badH* gene, whose predicted product is a member of the short-chain dehydrogenase/ reductase family of enzymes, was reported to be involved in anaerobic benzoate degradation in *Rhodopseudomonas palustris* (Pelletier and Harwood 2000). Other genes which play a role in CHCA metabolism in the *Arthrobacter* strain ATCC 51369, (previously *Corynebacterium cyclohexanicum*) include *pobA*, a regulator (*pobR*) and transporter (*pobK*) (Iwaki et al 2005). Using RT (reverse Transcription)-qPCR, a gene cluster (*chpca*) was identified in *Rhodococcus aetherivorans* BCP1 that was transcriptionally induced during the growth on two model NAs, (cyclohexane carboxylic acid (CHCA) and cyclopentane carboxylic acid (CPCA)) (Presentato et al 2018). The predicted products of the *chcpca* gene cluster are proposed to be involved in aerobic NA degradation in *R. aetherivorans* BCP1. Whole-genome analysis also revealed the presence of *pobA* and *chcpca* gene clusters putatively involved in NA degradation in *Rhodococcus opacus* R7 (Zampolli et al 2020). Gene expression analysis demonstrated the specific induction of R7 *aliA1* gene, encoding for a long-chain-fatty-acid-CoA ligase, in the presence of CHCA and hexanoic acid and that *aliA1* could be targeted as a biomarker for NA degradation (Zampolli et al 2020). Another study which used metatranscriptomics to investigate the treatment of both raw and ozonated OSPW using biofilters, identified genes involved in the degradation of organic acids and petroleum-related compounds, specifically, those involved in the metabolism of aromatic compounds and benzoate transport and degradation pathway (Zhang et al 2020a). All of these studies have enhanced our knowledge of the genes, proteins and mechanisms involved in NA biodegradation. Yet further work is still needed to apply this information for improved bioremediation strategies in the future.

5. Conclusions

NAs are an important group of toxic pollutants that are found in naturally-occurring bitumen-rich environments (e.g. oil sands ores, river/wetland sediments, surface and ground waters) or may accumulate as a result of anthropogenic activities from oil sand mining operations (e.g. process streams and tailings). These bitumen-rich ecosystems harbor dynamic microbiomes that not only have a profound effect on the composition and behavior of the NAs present, but are also major players in influencing their management and reclamation. It is therefore important to better understand the NA-degrading microbiomes across the different bitumen-rich ecosystems. Yet there is a lack of knowledge on their ecology, metabolic diversity and the mechanisms involved during NA degradation.

Both natural and anthropogenic bitumen-rich ecosystems have a rich taxonomic diversity (often regardless of the degree of NA contamination). Some microorganisms such as *Pseudomonas* sp. dominate across many NA-exposed ecosystems, whilst others, including certain methanogenic Archaea, occur in a few limited NA-contaminated environments. Many microbial processes occur in ecosystems associated with NAs,

including sulfate and nitrate reduction, methanogenic, methanotrophic, sulfidogenic and hydrocarbon degradation. There are many drivers that shape these bitumen-rich microbiomes and their processes. For example, the structure and composition of NAs may select for various microbial groups. Nutrient limitations, water quality, co-occurrence of trace metals, and competition for substrates from other non-NA-degrading microbes may also play a role.

Until recently, little was known about the NA biodegradation mechanisms, and the enzymes and genes involved. Yet such information is crucial for the rapid removal of these toxic, persistent pollutants from NA-contaminated environments. However, to thrive in such bitumen-rich environments, the microbiota must first protect itself from the toxic effects of the NAs. Recently, proteomics and transcriptomics approaches have provided new insights into the general cellular responses to oxidative stress and the cell's detoxification mechanisms following exposure to NAs. Specifically, several putative membrane porins and membrane transporters, alongside methyl-accepting chemotaxis proteins, molybdenum and molybdoenzymes are known to be involved as a general stress response to NA toxicity.

During NA degradation, several proteins associated with fatty acid, lipid and amino acid metabolism have also been identified. Specifically, multiple proteins involved in sequential reactions in fatty acid metabolism were found. Given the structural similarity between some NAs and fatty acids, it is not surprising that proteins involved in lipid metabolism were over expressed during NA biodegradation. It has therefore been suggested that microorganisms use their existing fatty acid catabolic pathways for NA biodegradation. More importantly, since these fatty acid pathways are conserved, a wide variety of microbial species are likely to have the enzymatic potential to biodegrade NAs which is important for future bioremediation strategies.

6. Future Perspectives

Biologically based treatments, which exploit the degradative ability of microorganisms, have both clear cost and environmental advantages to remove and detoxify NAs. Yet the real challenge is the removal of those NAs that are more persistent in the environment. In order to better understand NA degradation, it is important to elucidate the microbial ecology of NA degraders across different environments. Typically, microbial communities from environments that have a history of NA contamination, such as those found in oil sands and OSPW, may degrade NAs, especially the more recalcitrant NAs, more readily. However, prior exposure to NAs may not necessarily select for NA-degrading communities and NA-degraders have been identified from non-NA contaminated environments. Thus, more research is needed into the distribution, ecology, and mechanisms of NA-degrading microorganisms found in natural systems, especially more detailed spatial and temporal studies that focus on the interactions between natural processes and the anthropogenic stressors across the different bitumen-associated habitats.

Significant advances in analytical and molecular methods, including high-throughput sequencing and "omics" approaches, have made some headway in elucidating the NA compounds and the microbiota present across natural and anthropogenic ecosystems. Despite this, we are still unable to unequivocally differentiate between contaminants supplied from natural and anthropogenic contaminant sources, and more research into using biologically-based markers (e.g. genes, proteins, indicator species) alongside new modelling tools may enable the sector to better predict and assess ecosystem health in the AOSR. The rich taxonomic and metabolic diversity found across both natural and anthropogenic ecosystems has provided fundamental knowledge applicable to other environments, particularly regarding anaerobic hydrocarbon biodegradation, and possible biotechnological applications therein. Although, microorganisms found in bitumen-rich environments are well known to degrade NAs, microorganisms without any prior exposure to NAs may also metabolize NAs. Therefore for future remediation strategies, it may be

possible to manipulate environmental conditions to select for only those microbial communities that show higher rates of NA degradation as well as degrade the more persistent NAs. Indeed, identification of factors that affect the growth of NA-degrading microorganisms (either enhancing or inhibiting growth), such as the addition of nutrients, aeration, and ozonation, has important implications for the development of more effective NA bioremediation strategies. Although there are still significant challenges for developing effective NA bioremediation approaches, information on the distribution, ecology, and *in situ* microbial processes involved in NA-biodegradation will improve the effectiveness of new bioremediation technologies in the future. In conclusion, unlocking the metabolic potential of the microbiomes within natural and anthropogenic bitumen-rich environments will be fundamental to developing new energy technologies and managing our natural resources in the future.

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