Bio-degradation of shipborne fuel waste utilising a bioreactor using modified organisms

An exploratory study

Research report

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Academic year/semester: 2021-2022/semester 2

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Place of publication: Vlissingen

Date: 13/06/2022

Version: 2.2

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Abstract

This study was born out of concern that current methods for sludge disposal negatively impact the ecosystem. One method to alleviate this is to break down sludge through biological processes. This study aimed to gauge if there is sufficient impetus to develop a sludge disposal system using modified organisms. To accomplish this, two avenues of research were employed; desk research and a survey. The desk research focused on the theoretical side of the petrochemistry and biology involved and currently employed methods. This established that developing such a system is possible, but other effective systems exist. The survey measured interest in such as system was measured among shipowners. Unfortunately, very little response was received. However, it was concluded from the survey that there is no interest among shipowners in developing such a system. Thus, the final verdict is an insufficient impetus to develop a ship bourne bioreactor capable of digesting fuel sludge using modified organisms.

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List of abbreviations

As some abbreviations used may not be known to the entirety of the audience, a list of abbreviations is included to aid with understanding the text. The abbreviations listed include the page number where the abbreviation is first mentioned or defined.

Abbreviation	Meaning	page
C	Carbon	2
DNA	Desoxyribonucleic acid	4
GHG	Greenhouse gas	5
GMO	Genetically modified organism	1
H	Hydrogen	2
HFO	Heavy fuel oil	3
ISO	International Organization for Standardization	2
LNG	Liquid natural gas	23
MARPOL	The International Convention for the Prevention of Pollution from Ships	5
MDO	Marine diesel oil	15
ODME	Oil discharge monitoring equipment	6
ORI	Origin of replication	7
PAH	Poly-aromatic hydrocarbon	3
RNA	Ribonucleic acid	4
SARA	Saturates, Aromatics, Resins, asphaltenes	2

Glossary

Bactericidal: a substance capable of killing bacteria

Bacteriostatic: a substance that inhibits bacterial growth

Bioreactor: a vat where microorganisms can grow in a controlled fashion to accomplish a technical goal. This goal can be either the generation or the degradation of specific chemicals.

Carcinogenic: A substance or chemical that induces cancerous growths

Cometabolism: the simultaneous degradation of two or more substances by a single enzyme. In this, the first substance drives the metabolises of the second substance.

Cutter stock: Higher grade fuels added to residual fuels to reduce viscosity to usable levels.

Dioxygenase: An aerobic enzyme that oxidises the substrate using two oxygen atoms. These two atoms are usually sourced from the surrounding air. See: *monooxygenase*

Gene expression: The noticeable effect produced by the gene. This is usually through the production of a specific protein.

H/C-ratio: Hydrogen to carbon ratio. This is used to identify chemical bonds when conducting structural analysis.

Heteroatom: This is defined as any atom other than hydrogen or carbon within biochemistry.

High yield thermal coke: A hard, carbon-rich material formed in chemical reactions under high temperatures. This material can deposit on engine parts, reducing their performance.

Lipophilic: a substance which has an affinity for fatty tissues

Metabolites: substances that result from the biological action on a substrate.

Mitosis: The process of cell division and replication.

Monooxygenase: An enzyme that oxidises its substrate using a single oxygen atom. As this single oxygen is usually derived from diatomic oxygen, the other atom is usually used to produce water

Mutagenic: *Mutagenic*: Substances that induce genetic mutations. Note that in the context of molecular biology, these mutations can be harmful as well as positive.

π-bond: The covalent chemical bond formed in double-bonded substances. These secondary bonds are weaker than their single counterparts.

Plasmid: Circular DNA strands separate from chromosomes and can replicate independently.

Polarity (chemistry): The symmetry or asymmetry of electrostatic potential within a molecule. This property influences the interaction with other chemicals.

Sludge: The residue from purifying fuels before using them in a marine engine.

Teratogenic: Substances which induce congenital defects, particularly in foetuses.

Transcription (genetics): The process where genetic information in DNA or RNA is translated into usable protein by the cell.

1. Introduction

As it stands, sludge is a waste product that the ship cannot process on board and needs to transfer to shore. Discharging sludge to shore, however, adds to the vessel's operating costs. Another drawback is that the techniques employed by shore-based processing plants, such as stabilisation or incineration, are not environmentally friendly (Li, Lee, Mi, & Su, 1995). The one exception to this is biodegradation. However, biodegradation using current methods takes months to complete(Aguelmous et al., 2018). This timeframe is untenable given the current sludge production compared to biodegradation's capacity (Khan, Husain, & Hejazi, 2004). Various environmental effects of oil disasters demonstrate the need to safely dispose of fuel oil sludge. Add unto this, the goals set forth by the Paris Agreement (UNFCCC, 2015) and the need for an alternative means of sludge disposal is evident. This study proposes using genetically modified organisms (GMOs) to break down sludge into harmless substances as a solution. However, research into biodegradation using GMOs is still in its infancy, and a lot is still unknown about sludge properties.

This study was conducted at the behest of Anthony Veder Rederijzaken, a gas shipping company that owns and operates several gas tankers. As part of their operation, their vessels produce fuel sludge that must be disposed of. This incurs costs and logistical and operational challenges. This study aims to reduce the amount of sludge the vessel needs to dispose of ashore by processing it on board without putting additional stress on the ecosystem. Furthermore, it aims to pave the way for further research into this subject. In the long term, the hope is that this will make the industry more sustainable.

Central question:
How much impetus is there to develop a bioreactor capable of breaking down sludge?

Sub questions:

- What are the qualities of currently developed sludge disposal systems?
- How would a plasmid have to function to enable it to break down sludge?
- How willing are shipowners to implement this technology if developed?

The next chapter presents a literature review consisting of multiple parts. These parts each elucidate each sub-questions underlying theory and present the desk research findings. Furthermore, the literature review briefly introduces the nature of fuel sludge. The 3rd chapter outlines the methods used in this paper. The results then follow this in chapter four. Following this is the 5th chapter, where conclusions from the result are noted. These conclusions and their implications are then discussed in chapter 6. Lastly, references and any additional materials are referenced in this paper.

2. Literature review

Within this chapter, the basic principles underpinning this study are elucidated. Since this study encompasses multiple fields, this chapter is broken down into four sections. These four sections each expound on a different discipline bar the last section, which covers the conceptual model. These four sections cover, in order: the petrochemical nature of fuel sludge, existing methods for handling fuel sludge and genetic engineering relating to petrochemistry.

The second purpose of this chapter is to act as a consolidated repository of the results gleaned through desk research. Each section proffers its results, unbiased by the disciplines of the other sections. This then forms the foundation from which a final result can be synthesised.

2.1. Fuel sludge

Ere methods for disposing of sludge can be discussed, a synopsis of what fuel sludge is must be given. Fuel sludge is a colloquialism given to the remains of the fuel cleaning process on board. The vast range of bunker fuels used on board ships encompasses a broad range of substances. The standards for bunker fuels give some insight into this.

The ISO 8217 standard gives the general characteristics of fuel. However, the exact composition of fuels can differ as long as they comply with this standard (British Standard, 2012). For illustration purposes, consider a residual fuel of grade RMK with the worst possible degree of contamination the standards allow. For 100 metric tons of fuel, there could be up to 6.25 tons of particulate matter in ash, catalytic fines, and sediments. For this reason, fuels are cleaned on board with sludge as a result.

Since sludge is the result of a subtractive process of cleaning fuel, it can be posited that the composition of sludge is that of the bunker fuel minus that of the fuel going to the engine. In reality, a small quantity of fuel goes with the sludge, and thus all the compounds found in fuel can be found in said sludge. Whilst minimised, such losses have proven challenging to avoid (International Council on Combustion Engines, 2006). Due to this nature, attempting to identify all compounds present is impractical, if not impossible (Rahimi & Gentzis, 2007). This challenge requires generalisation since the fuel composition and, thus, the resulting sludge can differ from batch to batch.

2.1.1. SARA

Whilst the exact petrochemical nature of sludge is beyond the scope of this paper, a brief overview of the chemistry involved is in order. As stated earlier, a generalisation is required due to the varied nature of sludge. One way to accomplish this would be to categorise certain chemicals which behave similarly. For this purpose, as with any petroleum product, sludge can be described as having saturates, aromatics, resins and asphaltenes (SARA) (Fan, Wang, & Buckley, 2002). This SARA method differentiates these compounds based on polarity. This method's primary purpose is to discern the asphaltene fraction related to fuel instability (Speight, 2004).

Saturates

These are generally non-polar chemicals consisting of long and short alkanes and single-bonded cyclic structures (Baert, 1993). The chemical formula for these substances is C_nH_{2n+2} for alkanes and C_nH_{2n} for the cyclic structures (Figure 1). Of note is that none of these substances contains double or triple bonds, meaning that all carbon atoms bond with the maximum amount of hydrogen atoms possible. This property is called saturation and is where this fraction lends its name.

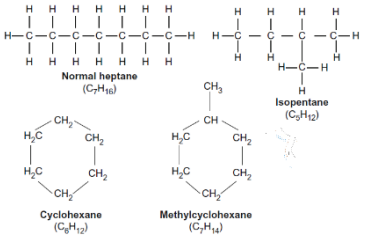


Figure 1 Example structures of Saturates

The short-chain alkanes (shorter than C12) are more volatile due to their low boiling point. Short-chain alkanes' presence seems to imply the addition of cutter stock (Garaniya, McWilliam, Goldsworthy, & Ghiji, 2018) to the fuel, as these short alkanes would have boiled off during the distillation of HFO.

Besides the short-chain alkanes, longer-chain alkanes are present as well. These present as waxy solids, commonly known as paraffin. These tend to precipitate out when chilled and determine the pour point of the fuel. As a result, some of these paraffins may be present in sludge.

Aromatics

As the name suggests, this fraction primarily contains aromatic substances characterised by low polarity. Aromatic substances are unsaturated cyclic structures with delocalised electrons because of pi-bonds. Generally, these substances consist of one or more benzene rings or structures like benzene. The molecule contains multiple ring structures and can be a polycyclic aromatic hydrocarbon (PAH). These PAHs are detrimental to the ecosystem's and humans' health. Chapter 2.1.2 explains this further.

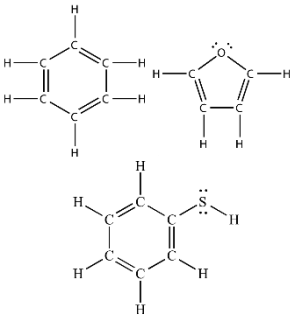


Figure 2 examples of aromatics: benzene (left), furan (right) thiophenol (bottom)

Within this fraction, several heteroatoms, such as sulphur or oxygen, start present in structures such as thiophenol and furan rings(Figure 2)(Garaniya et al., 2018).

Resins

The resin fraction contains polar cyclic structures miscible in heptane or pentane, whereas asphaltenes precipitate. These complex cyclic structures are made of multiple fused rings and contain heteroatoms such as sulphur and nitrogen. Attached to these polar cyclic structures are long non-polar alkane tails (Figure 3). Due to the low H/C ratio caused by the fused rings (Garaniya et al., 2018) combined with a high molecular mass, the resin has the potential to form high-yield thermal coke (Andersen & Speight, 2001) and can thus contribute to the fouling of engine components and the formation of carbon build-up (Kuiken, 2012). However, resins having a polar and non-polar part(Andersen & Speight, 2001) can form micelles (Luo, Liu, Zhang, & Jin, 2009). These micelles play a vital role in maintaining the asphaltene fraction's miscibility within the non-polar fractions(Andersen & Speight, 2001).

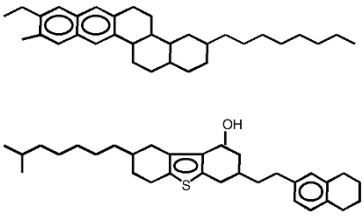


Figure 3 Example structures of resins

Asphaltenes

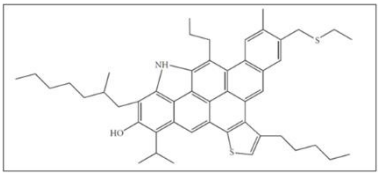


Figure 4 example of asphaltene structure (Hasanvand, Montazer, Salehzadeh, Amiri, & Fathinasab, 2018)

Like resins, asphaltenes are highly polar cyclic substances with complex structures (Figure 4). The critical difference is that they do not dissolve in pentane or heptane. They are, however, soluble in substances such as benzene (Mitchell & Speight, 1973). Garayana (2018) stated that this fraction contains the most bound sulphur of all the fractions. It is, therefore, this fraction that contributes most to sulphur-rich exhaust. Due to their complex nature and lacking solubility, asphaltenes and resins are viewed as recalcitrant fractions. This property means that very few organisms can readily metabolise these substances(Yanto & Tachibana, 2013).

2.1.2. PAH toxicity

Polycyclic aromatic hydrocarbons (PAHs) are, as the name suggests, substances that contain multiple aromatic hydrocarbon ring structures yet do not contain heteroatoms or substituents other than alkyl groups (Purcaro, Moret, & Conte, 2015). As established earlier, all but the saturate fraction contain aromatic compounds and can contain PAHs. PAHs can be due to the crude oil feedstock, chemical processes utilised during or after refining, or biodegradation.

Therefore, most substances making up fuel sludge can be PAHs or precursors. These compounds are resistant to degradation and can be mutagenic, carcinogenic and teratogenic (Bollinger et al., 2015). Add unto this that PAHs are lipophilic and, as such, bind easily to fats. They absorb readily into the food chain, forming a potential danger for the rest of the food chain and human consumption(Purcaro et al., 2015). Several PAHs are known to cause fatty tumours, adverse effects on reproduction and development, liver and kidney failure and other health effects (Nasr, Arief, & Malhat, 2010). Though the PAHs can be acutely toxic, the actual dangers lie in their reactive metabolites(products formed in cellular processes), disrupting cellular biochemical processes such as mitosis(cell division and replication) and protein formation(Bollinger et al., 2015; Costa, Cole, & Furlong, 2015). Combinations of PAHs and diol-exposed (a molecule containing two hydroxyls or "-OH" groups) metabolites can interact and damage DNA and RNA causing lasting genotoxicity and increased carcinogenic potential (Prince, 2017).

2.2. Existing sludge handling methods

The currently employed systems can be divided into three categories: incineration, store/discharge, and separation. Each of these systems has its own merits and drawbacks.

2.2.1. Storage onboard

The most widespread and straightforward system is the store/discharge system. The sludge is retained on board in separate holding tanks until filled. The sludge is discharged ashore or to a barge at the appropriate level. The main merit of this method is its simplicity; all that is required is a tank, a transfer pump and piping(Agarwal, 2021). In day-to-day operations, the operational load is limited to regular soundings and routine pumping operations with periods of increased load when transferring sludge ashore. Depending on the vessel's available systems, the levels of the tanks may need to be manually checked daily.

Furthermore, a watch stationed at the manifold is required when pumping ashore as per MARPOL regulations. Thus increased strain is placed on the crew. Moreover, since this system is entirely manual, it is subject to human error, potentially resulting in significant pollution incidents, injuries and loss of life (Hassler, 2016).

The second drawback of this system is that the entire volume, and thus mass, is retained on board. Depending on the vessel, a significant amount of sludge can be retained. Thus the cargo carrying capacity is directly and negatively impacted.

2.2.2. Incineration

The second method of sludge handling is onboard incineration. In this method, the sludge is burned at high temperatures and a modicum of pilot fuel to facilitate combustion. The primary advantages are the reduced retention capacity and the low operational load (Wankhede, 2021). However, incineration creates vast air pollution(Johnson & Affam, 2019; Murungi & Sulaimon, 2022). Whilst light or simple molecules can easily be reduced to CO_2, heavier and more complex molecules produce more complex combustion products. These complex combustion products can be highly toxic and carcinogenic (Bollinger et al., 2015; Thorsen, Cope, & Shea, 2004).

Furthermore, while some chemicals, such as vanadyl-porphyrins, may catalyse the combustion of other molecules(Jones, Agnew, Kennedy, & Watts, 1997), it is unlikely that conditions for adequate combustion of the porphyrins will occur. Regardless of whether complete combustion occurs, the introduction of MARPOL Annex VI imposes strict regulations on the incineration of waste (Amendments to the Annex of the Protocol of 1997 to amend the International Convention for the Prevention of Pollution from Ships, 1973, as modified by the Protocol of 1978 relating there to, 2021). A progressive carbon intensity reduction is implemented as outlined in the MARPOL Annex VI regulations 26 and 28. Therefore this method of sludge disposal cannot be considered for long-term application unless significant advances in flue gas cleaning are made ("Addressing climate change - a decade of action to cut GHG emissions from shipping," 2021).

2.2.3. Separation

The last method separates the fuel sludge into different components, which may be disposed of separately. One such system is the PureDry system by Alfa Laval (Alfa Laval, n.d.). This system uses a proprietary centrifugal separator to separate water, oils and solids. Large quantities of water may be present in the sludge tanks depending on the fuel treatment method(Murungi & Sulaimon, 2022). The separation and subsequent discharge via an ODME allow for a smaller retention volume (Alfa Laval, 2016). Meanwhile, the separated oils are retained, similar to the

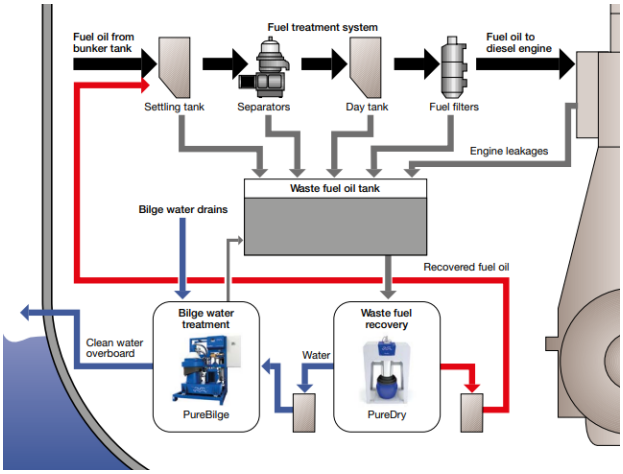


Figure 5: Alfa Laval PureDry System (Alfa Laval, 2016)

store/discharge method, or reused as fuel. Lastly, the solids are either retained or incinerated, requiring at least one of the other systems (Alfa Laval, n.d.).

2.3. Biology

Before establishing the required components of the plasmid, it is necessary to define what parts are indispensable and how they interact with the target organism.

A lab-made plasmid consists of minimally three parts: an origin of replication (ORI), a cloning site and a selection marker. Such a minimal plasmid is called an empty backbone and serves no other purpose besides providing a ready-made framework for further modification (Dale & Park, 2004). To utilise such a backbone, at least two other parts (depending on the assembly method, it may require more). These parts are a promoter and the target insert. What all these parts are and how they interact is described in the following paragraphs.

2.3.1. Origin of replication

The first and most critical part of the plasmid is the origin of replication (ORI). The ORI is a section of DNA where DNA replication starts (Lodish H, Berk A, Zipursky SL, 2000). Both the host organism and the plasmid need to have this to reproduce. However, the ORI of the plasmid is usually different from the host to avoid transcription errors. Nonetheless, both ORIs utilise the same mechanisms within the cell to replicate; thus, the ORI of the plasmid needs to be compatible with the host (Solomon, Berg, & Martin, 2011). Because the ORI can differ, the plasmid ORI can be somewhat tailored to the application. For instance, the following ORIs, pMB1, ColE1, P15A and pSC101, are utilised for E. Coli but have different characteristics. pMB1, for instance, has a copy number (how many copies are present in the cell) of 500-700, whilst pSC101 has a copy number of 5 (Addgene, 2011). Another factor is how the ORI is controlled or regulated. An ORI is regulated either by RNA or by protein. A protein-regulated ORI is called stringent, whilst an RNA regulated is called a relaxed ORI. This regulation directly affects the actual copy number present in the cell. Of note is that the copy number is not solely dependent on the ORI but is also influenced by other factors such as the growing conditions, the size of the insert, and the specific host strain (Addgene, 2011).

2.3.2. Selection marker

The selection site is needed for multiple reasons. First, a plasmid places additional strain on the host, no matter how slight. The modified host is at an evolutionary disadvantage due to the increased energy expenditure to express the plasmid alongside the natural proteins (Dale & Park, 2004). Thus an evolutionary advantage needs to be included in the plasmid. Usually, this takes the form of a specific antibiotic resistance paired with a growth medium inoculated with that antibiotic. This means that the unmodified strain cannot survive. The second reason lies in differentiating the modified strain from unmodified ones. Ensuring only the modified cells survive makes this differentiation easy (Dale & Park, 2004).

The selection of which antibiotic to use depends on many factors such as interaction with the host, the intended environment and the size of the resistance gene. However, more pragmatic factors such as the availability or cost of an antibiotic may also play a role (Addgene, 2011; Solomon et al., 2011).

Given the intended operating environment of the bacteria, it would be preferable to utilise a bacteriostatic selection antibiotic instead of a bacteriocidal one. A bacteriostatic agent allows any bacteriocidal agent to be a failsafe (Addgene, 2011). The need for such a failsafe is contingent on the nature of the final plasmid and the applicable laws regarding the unintentional release of modified organisms.

2.3.3. Cloning site and promoter

The cloning site is where the gene of interest is expressed. However, two parts are needed to express the gene correctly: the promotor and the terminator. The promotor is a region upstream of the gene of interest that indicates what to express and how. Like the ORI, the promotor is species-specific but has a greater variation (Watson et al., 2015). This variation is due to how the promotor can express a gene. This gene expression controls when the gene is expressed (either constitutive or active in the presence of certain compounds) and what happens after the gene is expressed (either it stays within the cell or is transported outside the cell) (Addgene, 2011).

Downstream of the gene of interest is the terminator region. This region indicates when the mechanisms need to stop transcribing. Without this terminator, the produced protein would not be released correctly, if at all. If the protein is not released correctly, it can be destroyed by internal mechanisms of the cell, while if it does not release, it can kill the cell (Watson et al., 2015).

2.3.4. Gene of interest

All plasmid parts thus far discussed are to produce a viable empty plasmid. However, the goal is to express a specific protein. This is accomplished by inserting the gene of interest within the cloning site. This process is called cell transformation or cloning. However, the exact process of transforming a plasmid is beyond the scope of this study. In the case of this paper, the genes of interest are those that help metabolise hydrocarbons found in fuel sludge. However, only seldom can a single protein metabolise a compound. Thus arises the need for metabolic pathways. Metabolic pathways are a series of enzymatic or biochemical operations. All pathways mentioned below are designated using Biocyc ID as employed by Biocyc (Karp et al., 2019).

Saturates

Many natural processes synthesise and degrade alkanes and cycloalkanes due to their prevalence within nature and biochemistry. Therefore, multiple pathways exist to break down these aliphatic hydrocarbons. However, most of these pathways rely on oxidising the chemical through a monooxygenase or dioxygenase (Abbasian, Lockington, Mallavarapu, & Naidu, 2015). Several bacteria have been demonstrated to degrade aliphatic compounds typically found in diesel oil. Pseudomonas aeruginosa DQ8 degraded diesel oil with solely this oil as its substrate (Zhang et al., 2011).

Aromatics

Within the aromatic fraction of keynote are the polycyclic aromatic hydrocarbons. Several PAHs have been identified(Uhler, Stout, Douglas, Healey, & Emsbo-Mattingly, 2016). Furthermore, some of these PAHs can be metabolised by known pathways.

The first of these PAHs is naphthalene. This substance can be readily metabolised via multiple pathways, namely the PWY-5427 aerobic pathway (Kulakov, Allen, Lipscomb, & Larkin, 2000) and the PWY-7620 anaerobic pathway(Estelmann, Blank, Feldmann, & Boll, 2015).

A second substance that is both identified and degradable is biphenyl. This substance can be degraded via the PWY5F9-12 pathway (Iwasaki, Miyauchi, Masai, & Fukuda, 2006). Of note is that the genes responsible for this pathway can also degrade chlorinated biphenyls through cometabolism (Gonçalves et al., 2006).

Delving into the slightly more complex molecules, fluorene is the next candidate for degradation, along with analogous structures such as dibenzothiophene and dibenzofuran. Given the structural similarities between these molecules, it is reasonable that the same mechanism can degrade these compounds. What is remarkable is that this mechanism utilises the naphthalene 1,2-dioxygenase complex, also found in the pathway for naphthalene degradation (Resnick & Gibson, 1996).

However, alternate pathways exist for dibenzofuran and dibenzothiophene. The pathway PWY-681 for dibenzothiophene first transforms dibenzothiophene into dibenzothiophene 5,5-dioxide using multiple monooxygenase enzymes. This is then transformed or reused in previous steps as FMN or $FMNH_2$, or it undergoes desulfurisation, which leads to the PWY5F9-12 biphenyl pathway (Oldfield, Pogrebinsky, Simmonds, Olson, & Kulpa, 1997).

Dibenzofuran can similarly be metabolised following the P622-PWY metabolises pathway. This pathway utilises dioxygenase enzymes and a hydrolase enzyme to convert it to a common intermediate metabolite, also found in the degradation pathways for naphthalene and toluene (Schmid, Rothe, Altenbuchner, Ludwig, & Engesser, 1997).

Resins

Petroleum resins have long been considered recalcitrant to biodegradation (Chandra, Sharma, Singh, & Sharma, 2013; Leahy & Colwell, 1990). However, some bacterial strains have been identified that can metabolise resins (Varjani, 2017). Unfortunately, it is still unclear by which mechanism these resins are degraded; thus, no information regarding the relevant metabolic pathway can be provided.

Asphaltenes

Similar to resins, asphaltenes seem recalcitrant to biodegradation. Nonetheless, certain fungal and bacterial strains have been proven capable of metabolising asphaltenes. Furthermore, these strains were capable of growing on naught but asphaltene as substrate (Hernández-López, Perezgasga, Huerta-Saquero, Mouriño-Pérez, & Vazquez-Duhalt, 2016; Yanto & Tachibana, 2013). Alas, similar problems arise as with resins. Due to the complexity of the molecules involved, few asphaltenes have been structurally identified. As such, not much is known regarding the degradation mechanism. However, cytochrome P450 (CYP450) enzymes appear essential (Hernández-López et al., 2016; Wang et al., 2011).

2.4. Conceptual model

The goal is to gauge if there is sufficient impetus to develop a biological method to break down sludge. This impetus is influenced by many factors but can be broadly classed into three classes: economic benefit, practical benefit, and ecological benefit. As such, these were the primary variables selected. Each of these variables is driven by several factors. The conceptual model below visualises the interaction of all these variables (Figure 6).

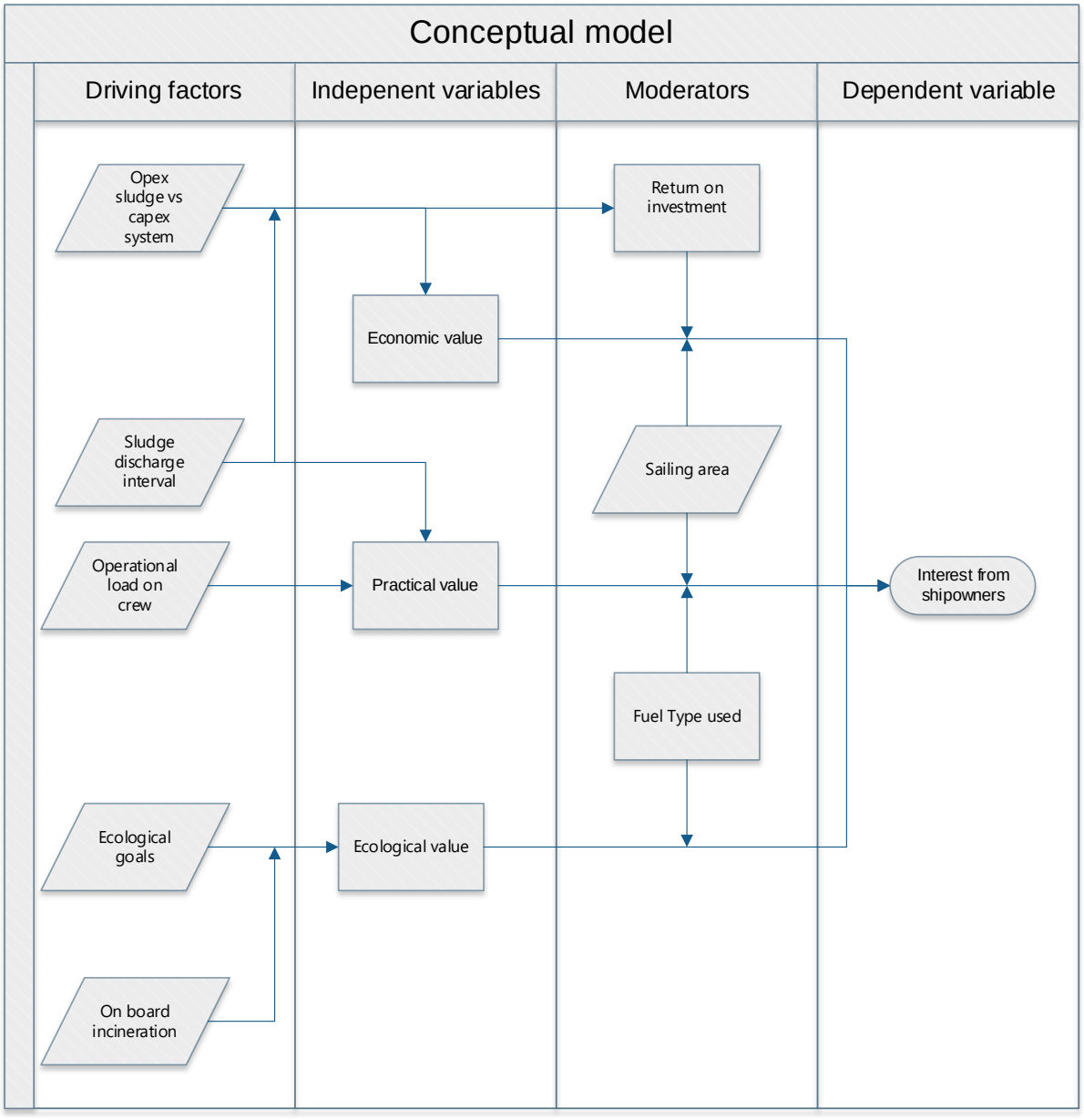


Figure 6 Conceptual model

3. Method

A mixed-method approach consisting of a qualitative survey and desk research was executed for this study. A survey was conducted among shipowners and interested parties in the qualitative segment.

In the second segment, desk research was carried out on existing sludge processing methods and sludge biodegradation. The first of these topics, existing sludge processing methods, was limited in scope to broadly applied existing systems. One caveat to this is the inclusion of the Alfa Laval PureDry™ system, as this system is still in its roll-out phase at the time of writing. All findings regarding this subject are displayed in the literature review and collated into a concrete comparison in the results section.

The second topic dealt with the groundwork for developing organisms capable of biodegrading fuel sludge. The scope of this will be limited to only breaking down the sludge found in the samples. Furthermore, the plasmid's actual synthesis is beyond the study's scope and means.

3.1. Survey

In order to gauge the receptiveness of shipowners to implementing a biological sludge breakdown system, a targeted survey was utilised. This survey consisted of open questions, scale indications, and multiple-choice questions. The complete questionnaire is available in Annexe 1: Model questionaire

3.1.1. Population vs sample

Since sludge is a factor for all shipowners utilising petroleum fuels, a large population presents itself. Thus it was not considered feasible to survey all shipowners. However, to ensure statistical rigour, a broad sample size was considered. This sample population consisted of 211 companies which were directly contacted, as well as an estimated 192 companies contacted via the independent partner “Blauwe Cluster”(“De Blauwe Cluster | Vlaanderen zet in op de innovatieve blauwe economie,” n.d.). Furthermore, additional surveys were sent to HZ University of Applied Sciences students who were completing their apprenticeships at sea. This population group consists of an estimated 12 persons. Thus the total amount of surveys sent is 415.

However, a mere two responses were received despite the efforts to ensure statistical rigour. Of these two responses, one was only a partial response. This means that the total response rate was $\frac{2}{415} \cdot 100\% = 0.4819\%$. This means that the gathered results are not statistically significant.

3.1.2. Data collection method

All data collection was accomplished through a questionnaire. This questionnaire was predominantly (211 of 415) distributed via an email linking to the survey platform "SurveyMonkey". A further 192 surveys were disseminated among members of the "Blauwe Cluster" through an internal email linking to the same online survey platform. Lastly, the remaining 12 questionnaire forms were dispersed among students as printable documents.

This document was then to be filled in by a qualified person on board and sent back. Upon receipt, the responses would be entered manually into the survey platform.

The survey consisted of 16 questions divided into four segments. Each segment gauged a different motivator, bar the first section, which provided metadata. These motivators are outlined in the conceptual model.

3.1.3. Ethics

Data was anonymised using an online survey platform to ensure this study complies with ethical controls and standards. After input into the survey platform, all physical copies of filled surveys were destroyed. The same measure was implemented for any copy received digitally outside the survey platform.

The specialised online survey platform ensured that no personal or corporate data was gathered from participants. Furthermore, the questionnaire was constructed to identify no individuals through any questionnaire responses. The list of directly contacted companies will not be made available and will be destroyed three months after publishing to protect the privacy of all involved.

3.2. Desk research

3.2.1. Existing methods for sludge treatment

Within this section, existing methods for sludge handling were identified and discussed.

In order to adequately judge whether or not there is sufficient impetus to develop a biological sludge handling system, the currently utilised systems needed to be profiled. This was accomplished using desk research. The primary sources for this were technical journals, manufacturer-specific literature and various but corroborated sources.

First, currently employed systems were identified and categorised. Next, the individual merits and demerits of each system group were examined. Lastly, each system was scored according to a relative scale in key defining categories: space effectiveness, cost, ease of use, and environmental impact.

3.2.2. Plasmid design criteria

For drafting the requirements of a viable plasmid, the method of choice was desk research. First, potential target organisms were identified from the literature based on their utilisation within contemporary industrial processes. Next, the requirements for a minimum viable plasmid were outlined using established practices as delineated by textbooks and other literature. Then, a brief assay of the constituent chemicals of fuel sludge was made. Subsequently, metabolic pathways were identified and matched with their respective chemical groups. All relevant genes were identified and verified within the literature within these metabolic pathways. Furthermore, any overlapping pathways were marked and catalogued for further investigation. Finally, all prior findings were collated into a single cohesive table and a prototype plasmid design.

4. Results

This section collates the results found in the literature and the survey results. Below are the results of each of the subquestions in order.

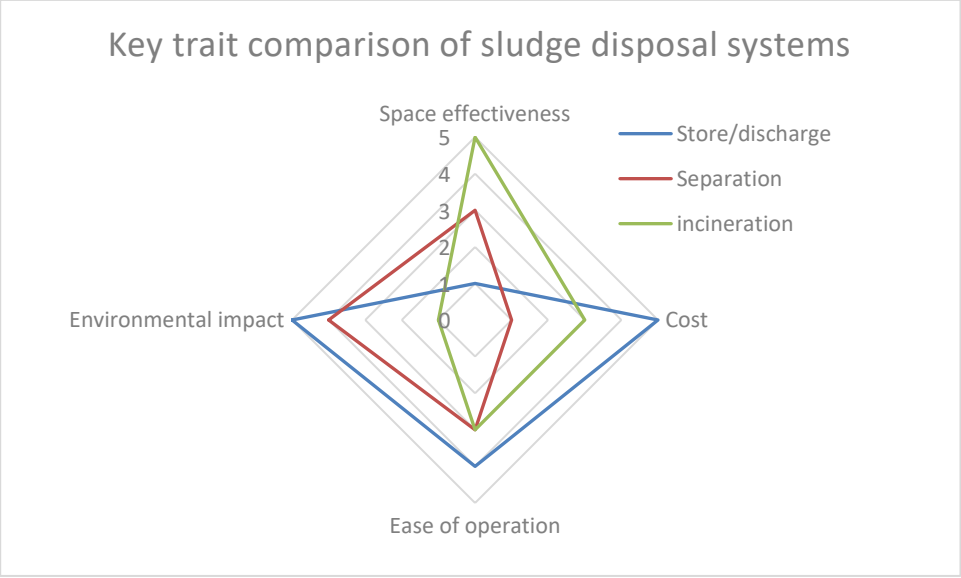
4.1. Existing systems

From the literature, three categories of systems are currently utilised. These are stored onboard, separation and incineration. Each of these systems offers its advantages and disadvantages. These advantages and disadvantages are tabulated below (Table 1).

Table 1 trait comparison for existing systems

Metric	Store/discharge	Separation	incineration
Space effectiveness	Lowest	Moderate	Highest
Cost	Lowest	Highest	Moderate
Ease of operation	High[1]	Moderate	Moderate
Environmental impact	Lowest[2]	Low[2]	highest

To better visualise the relationship between these systems, each attribute was graded on a scale of one to five. This allows a total score to be attributed to each system, encompassing each attribute. This total score is the area covered in a radar diagram (Graph 1: Radar diagram); the higher the area, the higher the score. Thus space effectiveness and ease of operation were graded where one is least beneficial (i.e. least space-efficient and most challenging to operate, respectively). Conversely, cost and environmental are rated on an inverse scale where one is the highest cost/impact, and five is the most negligible cost/impact.



Graph 1: Radar diagram

4.2. The groundwork for genetic engineering

As stated in the literature, modifying an organism to express proteins of interest is possible and has already been applied in other fields(Nielsen, 2013). Both eukaryotic and prokaryotic organisms have been used for the manufacture of pharmacology. However, the literature concludes that the cost of mammalian cell lines is prohibitive for large-scale production of recombinant protein. Other organisms used are yeast strains and E.Coli strains. Both of these organisms are used in the large-scale production of recombinant protein. However, they each have their drawbacks.

Nevertheless, E. Coli is viewed as the industry standard for modification. Furthermore, E. Coli is typically used to produce sufficient copies of a synthesised insert for safe transportation. Therefore, E. Coli is the most suited organism for initial testing and development.

As E.coli is chosen as the target organism, the basic plasmid needs are established. These are a prokaryotic compatible ORI, an appropriate antibiotic resistance gene and multiple cloning sites. Lastly, the genes of interest are needed to break down the fuel sludge.

Given the project's needs, a relaxed ORI with a moderate copy number, such as pGEX, would be advantageous for further development.

The required antibiotic resistance is initially driven out of sheer practicality. This means any antibiotic resistance or other selection method is viable for initial development. However, for application in the field, bacteriostatic resistance would be preferred.

The choice of cloning sites and promoters depends entirely on the gene expressed at that site. Because of this, the empty backbone is recommended to contain multiple cloning sites and multiple promotors so that a single organism can correctly express multiple genes. When selecting the promotors, it is imperative that they suit the target enzyme and that there is no interference between the different promotors.

Due to the complex nature of fuel sludge, many proteins will be needed to break down sludge fully. As petroleum products consist mainly of alkanes and PAHs, it is prudent to first focus on degrading these substances.

Given the ubiquity of alkanes, no specific pathway is proposed, but pathways incorporating CYP450 enzymes are recommended.

Certain substances have been identified within the aromatic fraction that can be broken down. These substances are tabulated below, along with their respective pathway and the genes needed for that pathway.

Table 2: list of chemical and their degradation pathways

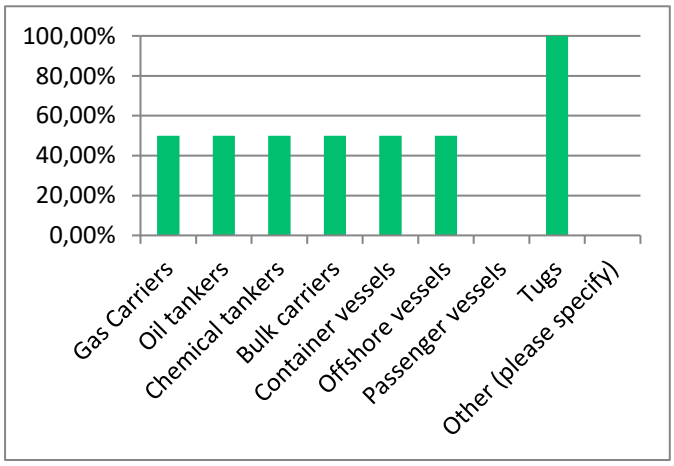
Chemical	Pathway	Genes
Naphthalene	PWY-5427 (Aerobic)	nahAd, nahAc, nahAb, nahAa, ndoA, ndoC, ndoB, ndoR, nahB, nahC, nahD, nahE, nahF
Naphthalene	PWY-7620 (Anaerobic)	N47_K27500, N47_K275400,N47_B20660, N47_G38220, N47_G38210
Biphenyl	PWY5F9-12	bphAc, bphAd, bphAb, bphAa, etbAa1, etbAb1, etbAc, etbAb2, etbAa2, bphB1
Fluorene	PWY-1381	nahAd, nahAc, nahAb, nahAa
Dibenzofuran	P662-PWY	dxnA1, dxnA2, dbfB
Dibenzothiophene	PWY-681	dszC, dszA, dszD, dszB

For resins and asphaltenes, no clear pathways have been identified yet. However, the literature has identified multiple possible enzymes which may be involved in the degradation of these substances. The fungus neosartorya fisherii and the bacterium pseudomonas aeruginosa DX8 have shown the capability to degrade these recalcitrant chemicals.

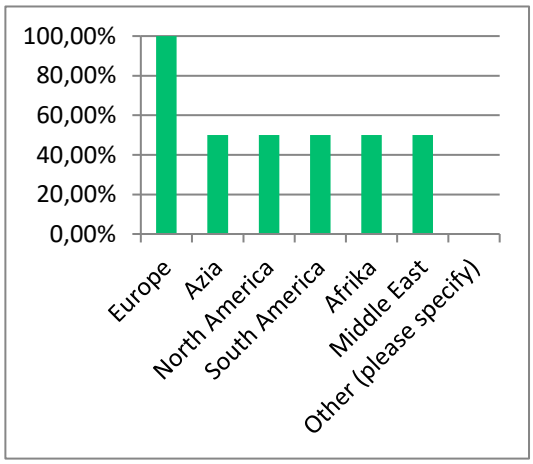
4.3. Survey

Unfortunately, the response rate (<0.5%) was such that no statistically substantiated data could be obtained. Only two responses were recorded. Of these two responses, one is a partial response with no recorded answers after the eighth question. For questions 9 to 16, only a single response is noted. Nonetheless, the accumulated data is presented below.

The first three questions were aimed at collecting metadata for this study. The first question was to determine which vessel type respondents operate. All respondents indicated that they operate towage vessels. In addition, 50% of respondents operate all other vessel types bar passenger vessels.



Graph 2: Vessel types operated

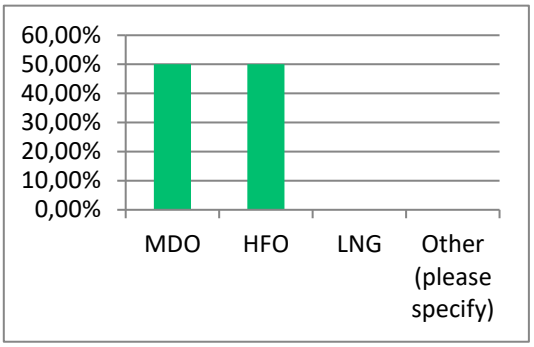


Graph 3: sailing area

The second question establishes the sailing area. All respondents indicate sailing in Europe, and 50% indicate sailing worldwide.

The third question gauges which fuel type is predominantly used. Here, 50% of respondents utilise HFO, and the other 50% utilise MDO.

Question four of the survey was implemented to enable respondents who do not use petroleum-based fuels to continue the survey if they wished. However, since all respondents indicated they sail with petroleum fuels, no responses to this question were recorded.

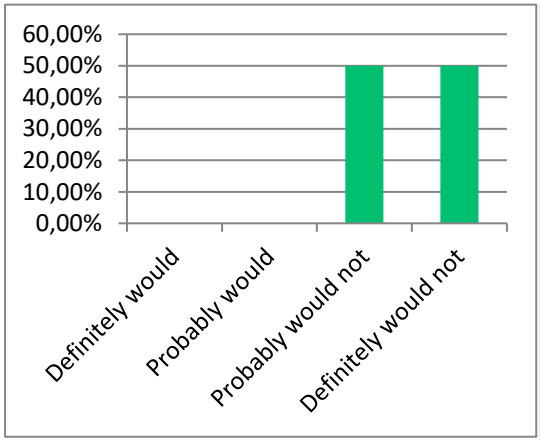


Graph 4: fuel type used

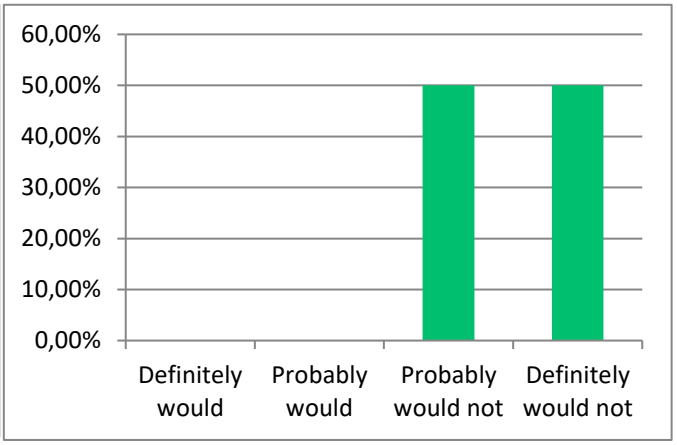
The following four questions formed the economic section. This section aimed to gauge whether or not such a system would provide an economic benefit to companies. It was possible to opt out of answering some questions if the respondent deemed the question too sensitive.

The fifth question was intended to gauge some of the direct operating costs of a sludge handling system. Given that this may be sensitive information, an option to skip this question was given. Unfortunately, no respondents were willing to share this information, and thus no responses were recorded.

Question six aimed to gauge whether participants would be willing to invest in developing a biobased system. 50% of respondents indicated that they probably would not invest, and 50% indicated that they definitely would not invest in such a system.



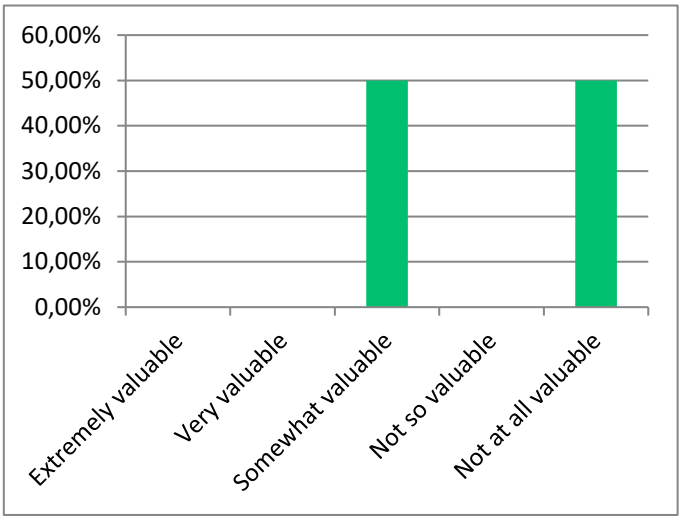
Graph 5: Willingness to invest



Graph 6:Willingness to participate in testing

The seventh question gauged whether respondents were willing to participate in testing a biobased system. Here, the responses reflect those of question 6; 50% would probably not be willing, and 50% would definitely not be willing to participate in testing.

The last question of the economic established if a biobased system would be of economic benefit for the respondent. Here, 50% indicated it would be somewhat valuable, while the other 50% indicated it would not be of any added value.



Graph 7: Expected economic benefit

The following four questions form the section dealing with practicality and operational load. This section was implemented to gauge if there are operational issues with the currently employed systems. Additionally, this section aimed to assess any additional operational load placed on the crew by implementing a biobased sludge disposal system. Unfortunately, from this section onward, only a singular respondent was recorded.

Question nine assessed whether current issues are associated with sludge handling using existing means. The only response indicates that some issues do exist. The tenth question hoped to assess if a new system would overburden the crew. The respondent noted that they do not know if such a system would increase operational load unacceptably. The next question asked if a new biobased system would alleviate current issues with sludge handling and thus improve operations. The answer given was that it would probably not improve operations. Question 12 aimed to quantify how often their vessels need to discharge sludge. This gives some perspective to previous questions regarding operations as the respondent noted that sludge is only discharged less than once per month.

The last segment dealt with environmental concerns regarding the current handling of sludge and projected environmental benefits.

The first question of this segment appraised any concerns regarding the handling of sludge once discharged ashore. The person responded that they have no concerns at all. The second question of this section asked if a biological system would benefit the environment. The respondent indicated that such a system would not be beneficial. The next question in this segment examined if a biological system would assist in reaching the company's ecological goals. The response given was that there would not be any benefit.

The last question of the survey asked which sludge disposal would be preferred. The answer given was that discharge shore was preferred above all else.

5. Discussion

Because only a few people responded to the survey, it is clear that the gathered results cannot be considered representative of the population. However, considering the number of responses relative to the intended sample size, two hypotheses form to explain this. The first is a general disinterest among shipowners in developing a biobased system. This would affirm the found results.

A second hypothesis is that the means used to spread the survey were ineffective. This may be caused by emails not reaching the appropriate persons or the accompanying email being unfit for purpose. The survey results cannot be considered valid if this is the case.

Either way, the use of a survey poses certain limitations and risks. The primary limitations are the limited target audience and the lack of organic answers. The first issue can potentially be solved by expanding the target audience, i.e., sending more surveys. However, given the resources available, this may not be possible. The second issue is that a survey utilises a very rigid structure that does not allow for delving deeper into answers the way an interview can. This can then obscure answers under ambiguity, or questions or answers are misinterpreted.

If this study were to be repeated, it is suggested that a broader target audience is used. Secondly, it is recommended that the survey data be augmented with interviews to provide more depth to the answers.

Regardless, due to ever more stringent measures against pollution and carbon footprint, the need for a sludge treatment system may be rendered obsolete simply due to vessels shifting away from petroleum-based fuels. Depending on advances with alternate fuel technologies, other systems may need development to satisfy the needs of these other fuels.

Alternatively, while a sludge biodegradation system may be obsolete shortly, the underlying genetic engineering technology may find broader applications within the maritime industry. Nonetheless, a petroleum waste degradation method could be employed when combating oil spills. Ashore may find purpose as a remediation method for petroleum-contaminated soils.

6. Conclusion

Ere an overarching conclusion can be drawn, each formative sub-questions must be answered. To reiterate, these are:

- What are the qualities of currently developed sludge disposal systems?
- How would a plasmid have to function to enable it to break down sludge?
- How willing are shipowners to implement this technology if developed?

Based on the desk research regarding sludge treatment methods, it can be surmised that the systems employed are adequate, as evidenced by a general lack of alternative disposal methods. The store/discharge systems appear to be the predominant system of the evaluated systems due to their universally applicable nature and simplicity. Situationally this system can be augmented using the Alfa Laval PureDry system. Considering more stringent legislation regarding environmental impact, onboard incineration is deemed not a long-term solution.

The first question can be answered thusly: the general trend for innovation regarding sludge handling seems to be directed at improving the capabilities of the store/discharge system. This is evidenced in the Alfa Laval PureDry system, where the volume requirements are reduced by partially reclaiming fuel oil.

Judging from the advances in biochemistry and genetic engineering, it is demonstrably possible to create organisms capable of degrading fuel sludge and other petroleum wastes. Various enzymatic processes have been identified which can degrade known petrochemical substances. This appears to be predominantly based on mono- and dioxygenase enzymes of the CYP450 family. Thence the answer to the second question can be formulated as follows; A plasmid designed for sludge breakdown would need to rely on a multitude of aerobic enzymatic processes utilising oxygenase enzymes to break down the constituents of fuel sludge. These initial enzymatic actions must then be followed up with oxidoreductase enzymes and hydroxylase enzymes specific to the metabolite.

Nonetheless, further research is required to fully break down fuel sludge as some of its constituents are recalcitrant to degradation using naturally derived enzymes. This additional research can further investigate natural degradation systems or synthetic proteins designed. However, this field still needs much work to realise the envisioned goals.

Furthermore, given the apparent dearth of innovation within fuel sludge disposal systems, a system employing such organisms may be superfluous. The results of the survey further corroborate this lack of motivation. The few responses to the survey indicate that there is currently no need nor interest for a biological disposal system from the shipowners' perspective, answering the third question. This lack of interest may also explain the low response rate.

Thus the crowning conclusion arises: there is insufficient impetus to develop a system using modified organisms to break down sludge for ships. However, the application of this technology may be considered in other fields, such as bioremediation ashore. It is recommended that further research into this technology be conducted. This may open up new avenues of approach for solutions and provide opportunities for developing new technology.

Bibliography

Abbasian, F., Lockington, R., Mallavarapu, M., & Naidu, R. (2015). A Comprehensive Review of Aliphatic Hydrocarbon Biodegradation by Bacteria. *Applied Biochemistry and Biotechnology 2015 176:3*, *176*(3), 670–699. https://doi.org/10.1007/S12010-015-1603-5

Addgene. (2011). Addgene's Plasmids 101, *2017*(3rd Edition), 193. Retrieved from https://www.addgene.org/educational-resources/ebooks/

Addressing climate change - a decade of action to cut GHG emissions from shipping. (2021, July 14). International Maritime Organization. Retrieved from https://wwwcdn.imo.org/localresources/en/MediaCentre/Documents/Addressing climate change - a decade of action to cut GHG emissions from shipping_FINAL_%2814-07-21%29_Large.pdf

Agarwal, M. (2021). An Overview Of Sludge And Bilge Management Onboard Ships. *Marine Technology*. Retrieved from https://www.marineinsight.com/tech/sludge-and-bilge-management-onboard-ships/

Aguelmous, A., El Fels, L., Souabi, S., Zamama, M., Yasri, A., Lebrihi, A., & Hafidi, M. (2018). Petroleum sludge bioremediation and its toxicity removal by landfill in gunder semi-arid conditions. *Ecotoxicology and Environmental Safety*, *166*, 482–487. https://doi.org/10.1016/j.ecoenv.2018.09.106

Alfa Laval. (n.d.). Alfa Laval PureDry Waste fuel oil recovery and sludge minimisation.

Alfa Laval. (2016). *Technical reference booklet-2016 Edition The Alfa Laval Adaptive Fuel Line BlueBook*.

Amendments to the Annex of the Protocol of 1997 to amend the International Convention for the Prevention of Pollution from Ships, 1973, as modified by the Protocol of 1978 relating there to (2021). International Maritime Organization. Retrieved from https://wwwcdn.imo.org/localresources/en/OurWork/Environment/Documents/Air pollution/MEPC.328(76).pdf

Andersen, S. I., & Speight, J. G. (2001). PETROLEUM RESINS: SEPARATION, CHARACTER, AND ROLE IN PETROLEUM. *Petroleum Science and Technology*, *19*(1–2), 1–34. https://doi.org/10.1081/LFT-100001223

Baert, R. S. G. (1993). A Mathematical Model for Heavy Fuel Droplet Vaporization and Pyrolysis in a High Temperature Inert Gas. *Combustion Science and Technology*, *90*(1–4), 125–147. https://doi.org/10.1080/00102209308907607

Bollinger, C. E., McCallister, M., Clark, R., Rhoades, R., Maguire, M., Savage, R. E., … Hood, D. B. (2015). Polycyclic Aromatic Hydrocarbons: Implications for Developmental, Molecular, and Behavioral Neurotoxicity. In *Handbook of Toxicology of Chemical Warfare Agents: Second Edition* (pp. 249–265). Elsevier Inc. https://doi.org/10.1016/B978-0-12-800159-2.00020-8

British Standard. (2012). ISO / FDIS 8217 Petroleum products - Fuels (class F) - Specifications of marine fuels. *English*, 42.

Chandra, S., Sharma, R., Singh, K., & Sharma, A. (2013). Application of bioremediation

technology in the environment contaminated with petroleum hydrocarbon. *Annals of Microbiology*, *63*(2), 417–431. https://doi.org/10.1007/S13213-012-0543-3/TABLES/5

Costa, L. G., Cole, T. B., & Furlong, C. E. (2015). Paraoxonase (PON1) and Detoxication of Nerve Agents. In *Handbook of Toxicology of Chemical Warfare Agents: Second Edition* (pp. 1089–1098). Elsevier Inc. https://doi.org/10.1016/B978-0-12-800159-2.00073-7

Dale, J. W., & Park, S. F. (2004). *Molecular Genetics of Bacteria 4th edition* (4th ed.). Chichester: John Wiley & Sons Ltd.

De Blauwe Cluster | Vlaanderen zet in op de innovatieve blauwe economie. (n.d.). Retrieved May 14, 2022, from https://www.blauwecluster.be/

Estelmann, S., Blank, I., Feldmann, A., & Boll, M. (2015). Two distinct old yellow enzymes are involved in naphthyl ring reduction during anaerobic naphthalene degradation. *Molecular Microbiology*, *95*(2), 162–172. https://doi.org/10.1111/MMI.12875

Fan, T., Wang, J., & Buckley, J. S. (2002). Evaluating Crude Oils by SARA Analysis. In *SPE/DOE Improved Oil Recovery Symposium* (pp. 883–889). Society of Petroleum Engineers. https://doi.org/10.2118/75228-MS

Garaniya, V., McWilliam, D., Goldsworthy, L., & Ghiji, M. (2018). Extensive chemical characterisation of a heavy fuel oil. *Fuel*, *227*, 67–78. https://doi.org/10.1016/j.fuel.2018.04.094

Gonçalves, E. R., Hara, H., Miyazawa, D., Davies, J. E., Eltis, L. D., & Mohn, W. W. (2006). Transcriptomic assessment of isozymes in the biphenyl pathway of Rhodococcus sp. strain RHA1. *Applied and Environmental Microbiology*, *72*(9), 6183–6193. https://doi.org/10.1128/AEM.00947-06

Hasanvand, M. Z., Montazer, M., Salehzadeh, M., Amiri, M., & Fathinasab, M. (2018). A literature review of asphaltene entity, precipitation, and deposition, introducing recently models of deposition in the well column. *Journal of Oil, Gas and Petrochemical Sciences*, *1*(2), 83–89. https://doi.org/10.30881/jogps.00016

Hassler, B. (2016). Oil Spills from Shipping: A Case Study of the Governance of Accidental Hazards and Intentional Pollution in the Baltic Sea, 125–146. https://doi.org/10.1007/978-3-319-27006-7_6

Hernández-López, E. L., Perezgasga, L., Huerta-Saquero, A., Mouriño-Pérez, R., & Vazquez-Duhalt, R. (2016). Biotransformation of petroleum asphaltenes and high molecular weight polycyclic aromatic hydrocarbons by Neosartorya fischeri. *Environmental Science and Pollution Research International*, *23*(11), 10773–10784. https://doi.org/10.1007/s11356-016-6277-1

International Council on Combustion Engines. (2006). Recommendations concerning the design of heavy fuel treatment plants for diesel engines, (25), 1–52. Retrieved from http://www.cimac.com

Iwasaki, T., Miyauchi, K., Masai, E., & Fukuda, M. (2006). Multiple-subunit genes of the aromatic-ring-hydroxylating dioxygenase play an active role in biphenyl and polychlorinated biphenyl degradation in Rhodococcus sp. strain RHA1. *Applied and Environmental Microbiology*, *72*(8), 5396–5402. https://doi.org/10.1128/AEM.00298-06

Johnson, O. A., & Affam, A. C. (2019). Petroleum sludge treatment and disposal: A review. *Environmental Engineering Research*, *24*(2), 191–201. https://doi.org/10.4491/EER.2018.134

Jones, J. M., Agnew, J., Kennedy, J., & Watts, B. (1997). Porphyrin- and metalloporphyrin-derived carbons as models for coal char combustion and pyrolysis. *Fuel*, *76*(13), 1235–1240. https://doi.org/10.1016/S0016-2361(97)00018-5

Karp, P. D., Billington, R., Caspi, R., Fulcher, C. A., Latendresse, M., Kothari, A., … Subhraveti, P. (2019). The BioCyc collection of microbial genomes and metabolic pathways. *Briefings in Bioinformatics*, *20*(4), 1085–1093. https://doi.org/10.1093/BIB/BBX085

Khan, F. I., Husain, T., & Hejazi, R. (2004). An overview and analysis of site remediation technologies. *Journal of Environmental Management*, *71*(2), 95–122. https://doi.org/10.1016/j.jenvman.2004.02.003

Kuiken, K. (2012). *Diesel Engines for ship propulsion and power plants* (2nd ed.).

Kulakov, L. A., Allen, C. C. ., Lipscomb, D. A., & Larkin, M. J. (2000). Cloning and characterisation of a novel cis-naphthalene dihydrodiol dehydrogenase gene (narB) from Rhodococcus sp. NCIMB12038. *FEMS Microbiology Letters*, *182*(2), 327–331. https://doi.org/10.1111/J.1574-6968.2000.TB08916.X

Leahy, J. G., & Colwell, R. R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiological Reviews*, *54*(3), 305–315. https://doi.org/10.1128/MR.54.3.305-315.1990

Li, C.-T., Lee, W.-J., Mi, H.-H., & Su, C.-C. (1995). PAH emission from the incineration of waste oily sludge and PE plastic mixtures. *Science of The Total Environment*, *170*(3), 171–183. https://doi.org/10.1016/0048-9697(95)04705-X

Lodish H, Berk A, Zipursky SL, et al. (2000). *Molecular Cell Biology 5th Ed*. *W.H. Freeman*.

Luo, H., Liu, G., Zhang, R., & Jin, S. (2009). Phenol degradation in microbial fuel cells. *Chemical Engineering Journal*, *147*(2–3), 259–264. https://doi.org/10.1016/j.cej.2008.07.011

Mitchell, D. L., & Speight, J. G. (1973). The solubility of asphaltenes in hydrocarbon solvents. *Fuel*, *52*(2), 149–152. https://doi.org/10.1016/0016-2361(73)90040-9

Murungi, P. I., & Sulaimon, A. A. (2022). Petroleum sludge treatment and disposal techniques: a review. *Environmental Science and Pollution Research*, *1*, 1–15. https://doi.org/10.1007/S11356-022-19614-Z/FIGURES/7

Nasr, I. N., Arief, M. H., & Malhat, F. M. (2010). Polycyclic Aromatic Hydrocarbons (PAHs) in Aquatic Environment at El Menofiya. *Journal of Applied Sciences Research*, *6*(1), 13–21.

Nielsen, J. (2013). Production of biopharmaceutical proteins by yeast: Advances through metabolic engineering. *Bioengineered*, *4*(4), 207–211. https://doi.org/10.4161/BIOE.22856

Oldfield, C., Pogrebinsky, O., Simmonds, J., Olson, E. S., & Kulpa, C. F. (1997). Elucidation of the metabolic pathway for dibenzothiophene desulphurisation by Rhodococcus sp. strain IGTS8 (ATCC 53968). *Microbiology (Reading, England)*, *143 (Pt 9)*(9), 2961–2973. https://doi.org/10.1099/00221287-143-9-2961

Prince, U. (2017). Environmental Effects of Polycyclic Aromatic Hydrocarbons Environmental Effects of Polycyclic Aromatic Hydrocarbons, (January 2015).

Purcaro, G., Moret, S., & Conte, L. S. (2015). Polycyclic Aromatic Hydrocarbons. In

Encyclopedia of Food and Health (pp. 406–418). Elsevier Inc. https://doi.org/10.1016/B978-0-12-384947-2.00550-X

Rahimi, P. M., & Gentzis, T. (2007). The Chemistry of Bitumen and Heavy Oil Processing. In *Practical Advances in Petroleum Processing* (pp. 597–634). Springer New York. https://doi.org/10.1007/978-0-387-25789-1_19

Resnick, S. M., & Gibson, D. T. (1996). Regio- and stereospecific oxidation of fluorene, dibenzofuran, and dibenzothiophene by naphthalene dioxygenase from Pseudomonas sp. strain NCIB 9816-4. *Applied and Environmental Microbiology*, *62*(11), 4073–4080. https://doi.org/10.1128/AEM.62.11.4073-4080.1996

Schmid, A., Rothe, B., Altenbuchner, J., Ludwig, W., & Engesser, K. H. (1997). Characterisation of three distinct extradiol dioxygenases involved in mineralisation of dibenzofuran by Terrabacter sp. strain DPO360. *Journal of Bacteriology*, *179*(1), 53–62. https://doi.org/10.1128/JB.179.1.53-62.1997

Solomon, E. P., Berg, L. R., & Martin, D. W. (2011). *Biology (9th Edition)*. *Cengage Learning* (Vol. 2011).

Speight, J. G. (2004). Petroleum Asphaltenes - Part 1: Asphaltenes, Resins and the Structure of Petroleum. *Oil & Gas Science and Technology*, *59*(5), 467–477. https://doi.org/10.2516/ogst:2004032

Thorsen, W. A., Cope, W. G., & Shea, D. (2004). Bioavailability of PAHs: Effects of Soot Carbon and PAH Source. *Environmental Science and Technology*, *38*(7), 2029–2037. https://doi.org/10.1021/ES0306056

Uhler, A. D., Stout, S. A., Douglas, G. S., Healey, E. M., & Emsbo-Mattingly, S. D. (2016). Chemical character of marine heavy fuel oils and lubricants. *Standard Handbook Oil Spill Environmental Forensics*, 641–683. https://doi.org/10.1016/B978-0-12-803832-1.00013-1

UNFCCC. (2015). *Report of the Conference of the Parties on its twenty-first session, held in Paris from 30 November to 13 December 2015*. *Addendum-Part two: action taken by the Conference of the Parties* (Vol. 01194). Paris. Retrieved from https://unfccc.int/resource/docs/2015/cop21/eng/10a01.pdf

Varjani, S. J. (2017). Microbial degradation of petroleum hydrocarbons. *Bioresource Technology*, *223*, 277–286. https://doi.org/10.1016/J.BIORTECH.2016.10.037

Wang, X. B., Chi, C. Q., Nie, Y., Tang, Y. Q., Tan, Y., Wu, G., & Wu, X. L. (2011). Degradation of petroleum hydrocarbons (C6-C40) and crude oil by a novel Dietzia strain. *Bioresource Technology*, *102*(17), 7755–7761. https://doi.org/10.1016/j.biortech.2011.06.009

Wankhede, A. (2021). Construction and Working of Waste Oil Incinerator. Retrieved May 4, 2022, from https://www.marineinsight.com/guidelines/construction-and-working-of-waste-oil-incinerator/

Watson, J. D., Baker, T. A., Bell, S. P., Grann, A., Levine, M., & Losick, R. (2015). Molecular Biology of the Gene (7th edition). *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York*.

Yanto, D. H. Y., & Tachibana, S. (2013). Biodegradation of petroleum hydrocarbons by a newly isolated Pestalotiopsis sp. NG007. *International Biodeterioration and Biodegradation*, *85*, 438–450. https://doi.org/10.1016/j.ibiod.2013.09.008

Zhang, Z., Hou, Z., Yang, C., Ma, C., Tao, F., & Xu, P. (2011). Degradation of n-alkanes and polycyclic aromatic hydrocarbons in petroleum by a newly isolated Pseudomonas aeruginosa DQ8. *Bioresource Technology*, *102*(5), 4111–4116. https://doi.org/10.1016/J.BIORTECH.2010.12.064

Annexe 1: Model questionaire

1. Questionnaire regarding sludge

This questionnaire will assess the demand for a new sludge disposal system. This system is similar to a sewage treatment plant in operation but utilises genetically modified bacteria to degrade sludge to a point where it is harmless to the environment. However, the development of this system is still in its concept phase. The responses to this questionnaire will directly affect the development of this system.

2. General information

In this survey section, some general information is asked regarding your vessels. This information helps to process further questions correctly.

1. What kind of vessels does your company operate?
    - Gas Carriers
    - Oil tankers
    - Chemical tankers
    - Bulk carriers
    - Container vessels
    - Offshore vessels
    - Passenger vessels
    - Tugs
    - Other (please specify)

2. What are the predominant sailing areas of your vessels?
    - Europe
    - Azia
    - North America
    - South America
    - Afrika
    - Middle East
    - Other (please specify)

* 3. What type of fuel do your ships predominantly operate on?
    - MDO
    - HFO
    - LNG
    - Other (please specify)

* 4. Even though your ships do not sail on petroleum fuels, do your ships encounter the issue of fuel sludge?
    - Yes
    - No

4. Economic benefits

This section explores if there are economic benefits attached to this system.

5. What are the current average costs of sludge disposal on your vessels?

* 6. How willing would your company be to invest in this system?
    - Definitely would
    - Probably would
    - Probably would not
    - Definitely would not

* 7. If this system enters development, how willing would your company be to participate in testing?

- Definitely would
- Probably would
- Probably would not
- Definitely would not

* 8. Do you feel that this system can provide economic benefit for your company?
  - Extremely valuable
  - Very valuable
  - Somewhat valuable
  - Not so valuable
  - Not at all valuable

5. Practical benefits

This section aims to gauge is this system has practical value.

* 9. Are there problems associated with sludge handling or disposal on your ships?
  - A great deal
  - A lot
  - A moderate amount
  - A little
  - None at all

* 10. Do you think a new sludge disposal system would increase the load on the ship's crew beyond acceptable levels?
  - Yes
  - No
  - Don't know

* 11. How much do you think a new sludge processing system would improve operations?
  - Definitely would
  - Probably would
  - Probably would not
  - Definitely would not

* 12. How often do your vessels need to discharge sludge?
  - Every day
  - A few times a week
  - About once a week
  - A few times a month
  - Once a month
  - Less than once a month
  - Other (please specify)

6. Ecological benefits

This section appraises whether there is a demand from an ecological standpoint.

* 13. How concerned are you regarding the handling of sludge ashore?
  - A great deal
  - A lot
  - A moderate amount
  - A little
  - None at all

* 14. How beneficial for the environment do you think a sludge processing system would be?
  - Extremely helpful
  - Very helpful
  - Somewhat helpful
  - Not so helpful
  - Not at all helpful

* 15. How well would such a system help accomplish the company's ecological goals?

- A great deal
- A lot
- A moderate amount
- A little
- None at all

16. If given the option, which system would be preferred on your ships?
    - Incinerator
    - on board processing
    - discharge ashore
    - dumping
    - Other (please specify)

7. Thank you for your participation

Thank you for completing this survey.