

The Toxicity Identification Evaluation assay as a tool for Wastewater Management

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Abstract

As terrestrial and marine ecosystems increasingly experience detrimental stress from pollution and contamination, contemporary waste management should consider wastewater as more than just trash to discard, but instead as a crucial resource to be developed. The Toxicity Identification Evaluation (TIE) is one of more comprehensive ecotoxicological methods which can evaluate and address toxic wastewater mitigation without knowledge of the specific toxic components. The aim of this study was to explore the effectiveness of TIE from a wastewater management standpoint by a) Testing the TIE method on a specific toxic aqueous effluent produced from a Danish offshore oil drilling operation, and b) assess its efficacy in the broader context of environmental management through a comparison of scientific literature on wastewater management. By applying TIE in conjunction with a Microtox™ equivalent toxicity test, this study found that an initial biological treatment followed by the application of activated carbon, is effective for addressing the toxic fractions of the oil produced wastewater. An analysis of the literature reveals that the strength of TIE compared to other more chemically specific techniques is in its broad application and ability to be applied to unfamiliar toxins and situations. Overall, the TIE is a critical tool to be considered and recommended for wastewater management as well as broader environmental management.

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Introduction

As human civilization settles into the 21st century, the ramifications of decades of pollution, resource overconsumption, and waste mismanagement are just beginning to be felt. Terrestrial and marine ecosystems are increasingly experiencing detrimental stress from pollution and contamination. Much of the solid waste, sludge, and wastewater we produce is not recycled or reused, but instead placed in landfills or discharged into natural environments. In 2016, 242 million metric tons of plastic waste and an estimated 1.6 billion metric tons of CO₂ - equivalent GHG emissions from solid waste were generated worldwide (Kaza, 2018). Additionally, the 2.01 billion metric tons of municipal solid waste produced annually is expected to rise steadily as the total quantity of solid waste generated in developing countries is expected to triple by 2050 (Kaza, 2018). Literature regarding global wastewater flows is scarce or relatively incomplete, as comprehensive reviews and assessments are missing. Nevertheless, recently several global organizations including FAO/IWMI¹, UN-Habitat and Global Water Intelligence are beginning to produce more thorough reports (Mateo-Sagasta, 2015). Escalating trends in human population, climate change, and water use indicate that freshwater resources will become and remain threatened well into the future (Vörösmarty, 2010). Waste, and wastewater specifically, contain valuable resources including water itself, organic matter, energy, and nutrients, which can be recovered for diverse economic, social, political, and environmental purposes. Comprehensive waste decontamination, material recycling, and informed reuse will be critical tools in meeting the resource demands of the future.

¹ Food and Agriculture Organization / International Water Management Institute

Agricultural practices, urban and coastal development, coastal tourism, river damming, mining, fisheries, and manufacturing all contribute to waste production, which threatens the wellbeing of the hydrosphere. Land-based sources, including agricultural run-off and untreated sewage, account for 80% of global marine pollution. Plastic and other buoyant trash that enters marine environments has a chance of getting caught in the five major oceanic gyres². Heavier substances and dissolved compounds enter deeper waters, where oxygen levels are insufficient to oxidize pollutants, which instead gradually accumulate (Bohn, 1972). In 2006, the United Nations Environmental Program (UNEP) estimated that there were 46,000 pieces of litter on the surface of every square mile of ocean (Gjerde, 2006). The Great Pacific garbage patch (GPGP) that has formed in the North Pacific gyre as a result of marine waste has at least 79,000 metric tons of ocean plastic floating inside an area of 1.6 million km² (Lebreton, 2018).

Holistic and sustainable management of natural resources is needed to address issues of waste and waste disposal. Conventional availability of water as a resource has led to the expectation of unlimited, cheap, large-scale, and centralized access (Schäfer, 2006). Such strategies, without informed prudence, have led to the over-exploitation, depletion, and pollution of water resources that should be protected and maintained (Al-Jayyousi, 2003). Today, supplying fresh clean water has become a complex and often expensive management issue. Not only is quantity of concern but maintaining quality has become equally critical. A greater awareness of population and economic growth, changing weather patterns, and environmental and societal impacts on inadequate water

² A large system of circulating ocean currents

resources is promoting the benefits of restoration and conservation for remaining water resources.

Contemporary waste management should consider wastewater as more than just trash to discard, but instead as a crucial resource to be developed. Access to potable fresh water is highly threatened for nearly 80% of the world's population, heightening the need for sustainable and effective management (Vörösmarty, 2010). Wastewater has been used as a supporting resource to great success, particularly in regions where water is scarce and population and economic growth is rapid (Yang, 2007). Reclaimed water can be used for many purposes, including agricultural irrigation, groundwater recharge, landscaping, and lavatory flushing (Jayyousi, 2003; Yang, 2007). Wastewater can be broken into several sub-categories, each with different methods of use and resulting benefits. Common examples include grey water and black water. Greywater is largely composed of soapy water from washing machines, bathtubs, showers and bathroom sinks. It does not include water collected from kitchen sinks dishwashers or toilets (Al-Jayyousi, 2003). Greywater is distinct from black water, which is comprised of more highly contaminated waters including sewage flows, among other sources. The different subcategories of wastewater should be assessed with suitable standards for aesthetics, hygienic safety, as well as environmental, technical, and financial feasibility. According to the EPA, wastewater reclaimed for toilet flushing should be filtered, disinfected, and contain no detectable fecal bacteria (Al-Jayyousi, 2003). The simplest treatment of greywater, the largest potential source of water savings in domestic residences, involves direct introduction of freshly generated greywater into an active, live topsoil environment. Swift treatment stops pathogenic microorganisms from multiplying which otherwise render

such wastewater more difficult and dangerous to reuse, recycle, or dispose of safely.

Greywater typically has less suspended solids and turbidity than the average wastewater, indicating a greater proportion of dissolved contaminants (Al-Jayyousi, 2003). It can be envisaged that one day consumers will willingly pay a similar price for recycled water to that of freshwater, not only for continued water conservation but also for preserving a better future environment (Schäfer, 2006).

Judicious integration of a policy to reduce, re-use, and recycle (RRR) for resource and waste management is critical for maintaining water resources and encouraging more sustainable lifestyles. Most waste treatment in developed nations is driven by environmental concerns, as the economic gains of reuse are relatively low. As of 2003, Japan, the US and Australia maintained the best record of greywater reuse, with many European countries maintaining higher than average levels (Al-Jayyousi, 2003). In Singapore and California, indirect potable reuse is preferred through the reclamation and treatment of wastewater. For Arizona, where the arid climate restricts the availability of water, grey water can be particularly cost saving, as the average household can generate between 30,000 and 40,000 gallons of greywater a year. Yet in the rest of the US, only an estimated 9.8 gegalitres of water is reused daily, which accounts for only 7.4% of total water used (Miller, 2006). Although developing countries tend to have much lower rates of wastewater treatment, and frequently industry and domestic waste is dumped without treatment, Namibia uses direct potable reuse, the recycling of water back into the water source or back into the potable water system (Yang, 2007).

Conventional management approaches have been unable to adequately address the escalating water crisis that humanity faces, but perhaps considering concepts like the

precautionary principle (PP) might contribute to change. The PP can be defined as a concept that evaluates the proportionality of risk against the cost and feasibility of a proposed action in order to anticipate harm before it occurs. When adhering to the PP in decision-making, establishment of lack of harm for a proposed activity is the responsibility of the activity's proponent. In the context of wastewater management, it allows managers to discern the wisest course of action in the context of the total water cycle, minimizing risk (Schäfer, 2006). A simple way to understand the precautionary principle would be through informed prudence. As Albert Einstein once said, "The significant problems we face cannot be solved at the same level of thinking we had when we created them" (Schäfer, 2006). This type of progressive and adaptable problem solving is what makes it important to include the PP as a guideline for wastewater management. When addressing the discharge of marginally treated sewage, whereby sewage is treated to a quality intended for potable reuse, or non-potable reuse, application of PP can prove useful when pressured by economics, political agendas, or environmental circumstances (Schäfer, 2006). In Europe, the PP has begun evolving into a principle of international law and has been included in almost all treaties and international policy documents. In the US, the incorporation of PP has been more controversial, as corporate interests have spread confusion and distrust about the principle's implications and message (Schäfer, 2006). Nevertheless, legislature introduced to protect environmental resources and water specifically, such as the EPA's Clean water act, continue to stress utilization of the PP. Continued and improved application of the PP will allow regulators, managers, and scientists to properly address future wastewater concerns and encourage sustainable development.

To address toxic pollution and contamination in wastewater management, managers can apply the PP in risk assessment strategies that precisely direct ecotoxicological methods to mitigate the impacts of industrial and agricultural chemicals on ecological systems without ignoring hazardous side effects. Ecotoxicology is a combination of ecology and toxicology that is concerned specifically with the relationship between human products and their impact on natural ecosystems. Its studies are typically concerned with the impact of a dose or concentration of a substance on its surrounding environment, often tested through mortality testing, of bacteria or other organisms. The mortality of the test organisms is typically expressed through the median lethal dose (LD_{50}) or median lethal concentration (LC_{50}). These both give a measure of the dose/concentration which causes mortality in 50% of the sample population. Similarly, the effective concentration (EC_{50}), gives a measure of the potency of a drug, antibody or toxicant that gives the half-maximal response after a given exposure time (Calow, 2014). In ecotoxicology, hazard assessment is made up of three key variables: 1. the potential of chemicals to cause harm by estimating their toxicity (T); 2. their capacity to persist in the environment (P); and 3. their propensity to be bioaccumulated in organisms (B) (Calow, 2014). Together, these distinct approaches to measuring and defining toxicity are particularly useful for making wastewater assessments and management decisions.

Ecotoxicological methods that can evaluate and address toxic waste mitigation for risk assessment can be divided into chemically specific approaches and broader multi-treatment practices. Chemically specific mitigation techniques are essentially tailor-made to the specific properties of the pollutant itself, requiring

knowledge of which contaminants are polluting. This allows for the most effective and appropriate treatment to be selected in order to address the issue. On the other hand, broader multi-treatment mitigation strategies don't require knowledge of the exact compound or compounds that are pollutants. Instead, they apply a series of different treatments that remove or neutralize compounds with specific properties. Physical and chemical properties that particular treatments might be used to target toxicity neutralization and removal include pH (acids/bases), temperature, polarization state, organic/non-organic compounds, and metals. The toxicity of the raw and treated samples can be measured through Microtox™ equivalent toxicity tests (Johnson, 2005). In this test, bioluminescent marine bacteria, *Allivibrio fischeri*, are pipetted into a solution to be analyzed for toxicity. The light production is proportional to the metabolic activity of the bacterial population. Exposure of bacteria to toxic substances results in a decrease in luminescence and the percent change in light production is correlated to toxicity. After identifying key contributing fractions of the total toxicity, treatment can be better focused on other requirements including cost, practicality, etc. Together, the two toxic waste mitigation methods, chemically-specific mitigation and broader multi-treatment mitigation, cover most situations that require risk assessment and toxicity mitigation.

Toxicity Identification Evaluation (TIE) is a prime example of the broad evaluative methods for assessing and mitigating pollutants, as described above. TIE was developed in the 1980s by the US EPA for the assessment of toxicity in both municipal and industrial effluent samples (Ankley, 2011). The TIE procedure includes an array of different physical and chemical treatments aimed to identify key groups of compounds causing toxicity. The evaluation is composed of three different levels that systematically

isolate different compound groups. TIE level 1 is focused on an initial aeration of the sample that attempts to remove volatile compounds from the sample. In addition, this ensures that the effluent is oxygenated, which is a requirement for Microtox equivalent toxicity testing. Following level 1, TIE Level 2 includes flocculation, metal binding, absorption of organic compounds, and extraction of organic compounds. Finally, TIE level 3 is composed of polar and non-polar compound extraction and pH based non-polar extraction. After every treatment, the sample is tested for toxicity via a Microtox equivalent toxicity test as described above.

In this report, a toxic aqueous effluent from a Danish offshore oil drilling operation was tested to evaluate the effectiveness of TIE and to identify effective treatments for the wastewater. The wastewater was too toxic to be pumped to a nearby treatment facility, so the preferred solution for the company involved was an intermediary in-house treatment step, which would allow the partially purified wastewater to be transferred to the public treatment facility. To assess whether the toxicity of the wastewater source fluctuated, two different wastewater samples were obtained and received for testing. Although, the samples originated from the same source, the second sample was collected several months after the first sample. For the two wastewater samples received, a chemically specific detoxification could not be attempted, as it was uncertain which compounds were present at toxic levels. Additionally, based on the interactions between the different compounds and the toxicities of the degradation products, the effluent could not simply be evaluated by a chemical analysis. Instead, selecting TIE methodology was considered the most effective strategy for assessing the effluent toxicity. In the present study, the two samples were assessed for potentially

effective treatments and evaluated for the effectiveness of TIE in conjunction with the Microtox toxicity detection assay.

Methods

A detailed description of the TIE procedure applied to the oil wastewater samples is as follows: The treatments in TIE were split into three levels, with a basic overview of the procedure highlighted in Figure 1. For TIE level 1, 2 liters of each sample were aerated at 21°C over the course of 24 hours³. Prior to the toxicity testing, and at the beginning and end of every treatment step, the pH, conductivity, salinity, and temperature were measured. For TIE level 2 and 3, the untreated aerated sample was included as a blank. Following the first level, TIE level 2 protocol involved dividing up the aerated sample and subjecting each division to a different treatment, each attempting to reduce toxicity by taking advantage of different groupings of physical and chemical properties. First, to assess whether the toxic compounds can be precipitated, flocculation of toxic compounds was attempted through the addition of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ at concentrations of 0.01, 0.03, and 0.1 g / L, to 100 ml of the effluent sample. To pinpoint whether toxic metal ions were present and could be sequestered into non-toxic complex compounds, Ethylenediaminetetraacetic acid (EDTA) (Grčman, 2001), a chelating agent, was added in concentrations of 0.03, 0.3, and 10 g / L, to 100 ml of the sample. If a reduction in toxicity was seen at this point, it was considered likely that toxic metals contribute to the overall toxicity level. Finally, to identify whether potentially toxic organic compounds can be absorbed and extracted, 10, 30, and 100 g / L of activated carbon (AC) were each

³ Appendix 5: Figure 1

added to 100 mL of the aerated sample^{4,5}. After each treatment step in the level 2 protocol, the solution in its bottle was placed on an end-over-end shaker for 24 hours at 13 rpm⁶. Then, after 24 hours the sample was filtered through a 0.16 µm filter to separate the AC from the treated sample in preparation for toxicity testing.

The two remaining second level treatments included heat treatment and heptane-based liquid-liquid extraction. To identify, through

heat stressing, whether the effluents toxic contribution stems from weakly bonded covalent compounds, 100 ml of stripped sample from TIE level 1 was placed in an oven at 40°C for 24-hours. The lid of the bottle was left ajar so volatile compounds could evaporate, potentially resulting in reduced toxicity. Filtration was deemed unnecessary after this step, so after cooling down to room temperature, the sample was ready for toxicity testing. Finally, through heptane-based liquid-liquid extraction, the toxicity contribution of extractable organic compounds was tested. Here, 30 ml of heptane was

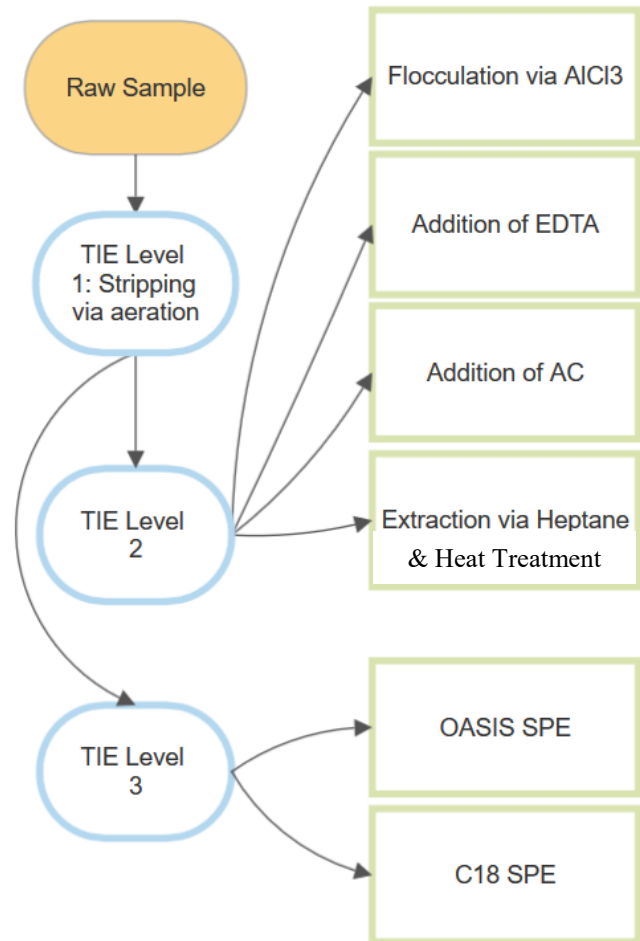


Figure 1: A procedural representation of TIE

⁴ Filtrasorb 400, Chemviron Carbon, Brussels

⁵ Concentration: 10, 30, 100 g / L

⁶ Appendix 5: Figure 2

added to 30 ml of aerated sample. The sample was then placed on an end-over-end shaker for 24 hours at 13 rpm. In preparation for toxicity testing, the sample fraction of the solution was carefully separated from the heptane fraction using a funnel.

TIE level 3 treatments similarly followed after the procedure for level 1 treatment and assessed the relative toxicity of organic acids and bases, as well as polar and non-polar compounds. The results from the third TIE level can potentially support or modify the results from the AC treatment or liquid-liquid extraction from TIE level 2. To absorb polar hydrophilic and non-polar hydrophobic compounds, solid phase extraction (SPE) through OASIS HLB⁷ columns was attempted. The OASIS HLB resin has the ability to bind both polar hydrophilic and non-polar hydrophobic molecules. The resin needs to be conditioned by several solvents before the treated sample solution is passed through. As such, 3 ml ethyl acetate, 3 ml methanol, and 6 ml Milli-Q water at pH 7 were passed consecutively through the resin via gravity. Then, 50 ml of the aerated sample from level 1 was added to the column, adjusted to pH 2, 7, and 12 by application of HCL or NaOH, before being collected and subsequently left at room temperature for toxicity testing. The second TIE level 3 treatment also involved SPE at pH 2, 7, and 12, for the aerated sample from the TIE level 1 treatment, but instead using C18⁸ hydrophobic columns that retain non-polar hydrophobic compounds and require pre-treatment with different preparatory solvents. For conditioning of the C18 columns, 3 ml heptane, 1.5 ml acetone, 3 ml of methanol, and finally 6 ml milli-Q water at pH 7 were added consecutively to each column, before the treated pH adjusted sample solutions were passed through. The pH

⁷ OASIS PRiME HLB: Waters, Massachusetts, USA

⁸ LC-18 SPE Tube: Supelclean™, Supelco, USA

manipulation in the two TIE level 3 treatments neutralizes either acidic or basic compounds, making them non-polar and increasingly retainable by the C18 columns. If more toxicity is removed by the OASIS HLB columns than by the C18 columns it provides evidence that not only non-polar organic compounds, which are retained by both types of columns, but also polar compounds, selectively retained by OASIS HLB columns, contribute to the overall toxicity of the test samples.

To assess the toxicity of the treated samples, a Microtox equivalent test, known as the Biotox luminescence inhibition test, was used to evaluate luminescent inhibition of the marine bacteria *Allivibrio fischeri*, after exposure to toxins or other contaminants in the samples (Lappalainen, 2001). The test is applicable for a multitude of samples including fresh, brackish, and marine based samples originating from chemical compounds, wastewater, aqueous extract, leachates, and pore water. When addressing toxic compounds from diverse sources with unique physical and chemical properties, the bacterial strain employed for the test must match the salinity of the sample, in addition to pH and temperature. Specific strains have been developed for salinity levels matching typical environmental or industrial conditions. The Biotox test can be performed immediately after the individual TIE treatments have been completed, and the results of the test are available within 30 mins of test initiation.

This study applies the Biotox test⁹ protocol guided by the ISO standard¹⁰ to characterize and compare toxicity treatments, with key steps and modifications outlined

⁹ Labsystems Oy, formerly Bio-Orbit Oy, Helsinki, Finland

¹⁰ ISO 11348-3 (2007)

below. Initially, the freeze dried Biotox bacteria¹¹ were acclimated to standard temperatures before Biotox testing. To do so, the bacteria were first suspended in 12.5 ml of 4% sea salt¹² and stored at 4°C for 15 mins, before being moved to room temperature for 20 minutes. Following acclimation, the post-treatment stock solution was prepared as a dilution series, with 100% being the highest concentration of the treated sample, followed by 50%, 25%, 12.5%, and 6.25% concentrations. Additionally, a 4% sea salt solution was prepared as a control, and a blank consisting of untreated toxic sample - was added for reference (for a total of 7 samples). Two replicates were obtained for each dilution, as well as the blank and control. To begin data collection, 100 µl of bacteria were added to 14 empty 500 µl luminosity test vials, including 2 replicates of the 7 different solutions. After being left to rest for 5 minutes, the first luminescence measurement is taken¹³ for each vial to establish the pre-toxicity baseline measurement ($t = 0$). After the initial bioluminescent measurement of all the vials, 100 µl of test solution is added to each test vial and left for 10 minutes at which point the luminescence is measured again ($t = 10$). Finally, the test vials are measured after another 10 minutes ($t = 20$).

In order to account for the natural time decay of light emission from the bacteria, a correction factor (KF_x) is calculated for both the second ($T=10$) and third ($T=20$) luminosity measurements from the control replicates in each dilution series. In total, each Biotox test will result in two KFs, one for the 10-minute mark and one for the 20-minute

¹¹ 1243-500 Biotox™ kit, ABOATOX, Finland

¹² Sera™ Marin Salt, Sera, Germany

¹³ Luminosity measuring device: Luminoskan TL Plus, ThermoFisher

mark, which can then be used to adjust the rest of the measurements to better account for the decay. The formula for KF_x is provided below:

$$KF_x = \frac{IC_x}{IC_0}$$

Where IC_x is the average luminescence of the two control sample replicates after x min and IC_0 is the average luminescence of the control replicates at $T = 0$.

From the correction factor KF , the relative response ($REL\%$) can be calculated for each toxicity measurement at 10 and 20 minutes. The $REL\%$ can be calculated through this formula:

$$REL\%_x = \frac{IT_x}{KF_x * IT_0} * 100$$

Where IT_x is the luminescence of each test sample after contact time of x minute, and IT_0 is the luminescence of each test sample after 0 minute. The $REL\%$ can be produced for every replicate of the dilution series from the 10 min and 20 min toxicity measurements.

Statistics

The observed inhibition data measurements were fitted to a concentration-response curve with estimated 95% confidence intervals using a LN statistical program (Christensen, 2009. Andersen, 1994). The statistical program requires data from continuous rather than discrete responses, allowing for a more detailed analysis. An example of the post-Biotox test data for 1 g of AC treatment, before being transformed through the statistical program, is shown below in Table 1:

Conc. (%)	10 g / L AC Treatment										Control	
	100		50		25		12.5		6.25			
REL10	0.58 5	0.53 8	0.85 7	0.90 1	0.84 3	0.97 1	0.96 5	0.95 6	0.95 9	1.02 8	0.900	1.120
REL20	0.52 5	0.43 6	0.63 5	0.73 8	0.83 3	0.93 7	0.98 1	1.07 5	1.03 4	1.25 3	0.931	1.082

Table 1: Bacterial survival after treatment with 10 g /L AC

In table 1, the data quantifies how the different dilutions of the treated solution inhibit the bioluminescent bacteria. Values closer to the control (control trends towards 1.0) indicate higher bioluminescence observed and thus a higher survival rate of the bacteria, consistent with a less toxic sample. However, the best use of the dilution series is to determine the EC₂₀ and EC₅₀, i.e. the effective concentration of the treatment that gives the 80% and 50% drop in luminescence, through the Concentration response curve.

The data transformation which the statistical program processes, gives not only a fitted concentration curve, but also provides further statistical data estimated from the curve. An example of the fitted curve and the resulting statistical data is shown in Figure 2.

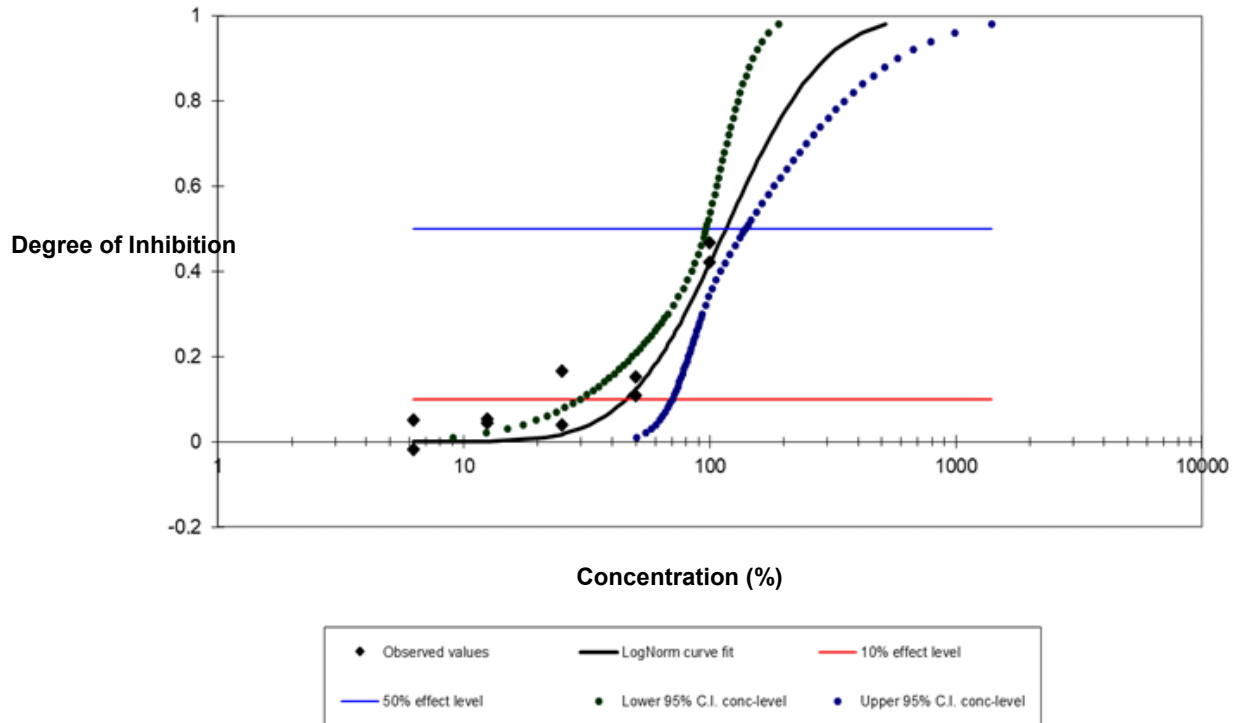


Figure 2: Concentration curve for toxicity after purification with 10 g / L of AC

In Figure 2, the y-axis gives the degree of bacterial inhibition from untreated or treated solutions, as a function of the sample's concentration or dilution level for the sample, given on the x-axis. The solid black line is the fitted curve provided by the program for the data set. The curve is approximated through nonlinear regression, by taking the smallest distance between observed data and the theoretical curve for logarithmic-normal distribution data. The program also gives the lower and upper 95% confidence level curves for each data set. For data with strong statistical backing, the 95% confidence curves will be nearly touching the fitted curve and will mirror its shape as well. Finally, the program calculates the EC_{10} , the EC_{20} , and EC_{50} based on the fitted curve and the data. The EC_{10} and EC_{50} are placed onto the graph as horizontal lines, the former being the red line and the latter the blue line. The various aspects of the graph

combine to provide the relevant data for comparing visually and quantitatively, treatments both within a data set and between data sets.

Estimated EC values for 10 g / L AC (%)		95 % Confidence Limits	
		Lower	Upper
EC ₁₀	45.66	29.67	70.27
EC ₂₀	62.91	48.41	81.75
EC ₅₀	116.0	96.40	139.7

Table 2: Statistical estimations for treatment with 10 g / L of AC

The statistical estimations are also incorporated into a table, which gives the EC₁₀, the EC₂₀, and the EC₅₀, in addition to the 95% confidence limits for each EC (Table 2). The fitted curves and the statistical estimations of the different EC's allow this study to compare the various treatments in order to recommend a potentially effective treatment for the toxic aqueous effluent from the Danish offshore oil drilling operation.

Research Results

Toxicity of the two Samples

In order to compare the different treatments of samples, it was necessary to first establish a measure of the toxicity of the two original untreated samples. These two samples were collected from the same location of offshore oil drilling, but they were obtained several months apart and differed in the severity of toxicity. Therefore, the second sample was measured to be of a significantly higher toxicity than the first sample, as reflected in results of toxicity assays¹⁴. The effective concentration of Sample 1 that gives a half maximal response (half survival or EC₅₀) of the standard test bacteria was

¹⁴ Appendix 1: Figure 1

assessed to be 43% concentration of the original sample while the EC₂₀ for Sample 1 (20% survival) was about 11% of the original sample. In contrast, for Sample 2, the EC₅₀ is only 1.848 % of the original untreated sample and the EC₂₀ is reached at a miniscule 0.076%. The difference in toxicity between the two samples is supported by the confidence intervals, which give relatively small error between lower and upper confidence estimates¹⁵. Further comparison between Sample 1 and 2 requires a broader overview of the treatment effectiveness and is therefore included towards the end of the results. All further toxicity measurements for Sample 1 and 2 were compared to an aerated but otherwise untreated toxic blank of the sample as well as a non-toxic 4% sea salt control.

Sample 1

For Sample 1, the TIE level 1 treatment, had little effect on toxicity, suggesting that aeration for 24 hours at 21°C did not remove a significant proportion of toxic compounds. However, for TIE level 2 treatments, one method provided results consistent with a significant mitigation of toxicity. The aerated samples from the TIE level 1 experiment were tested through the application of AlCl₃·6H₂O, EDTA, AC, heptane, and heat. With the exception of AC, the other treatments were all ineffective at reducing the toxicity levels of the sample, compared to the untreated toxic blank. Consequently, the contribution to toxicity by compounds that precipitate in the presence of AlCl₃·6H₂O, were metals ions, heptane extractable or volatile, is likely limited. In contrast, extraction by AC had a marked toxicity reducing effect. While, the blank sample had an EC₂₀ of

¹⁵ Appendix 1: Figure 2 and 3

approximately 11%, treatment with 10 g / L of AC increased the EC₂₀ fourfold, from 11% for the blank to nearly 41%¹⁶. The higher concentrations of AC, 30 g / L and 100 g / L, however, resulted in lower EC₂₀ values than for the blank. Overall, for TIE level 2, the 10 g / L AC treatment provided the least toxic end product.

The results for TIE level 3 analyses for Sample 1, gave incomplete results due to a technical problem encountered at high pH. For the TIE level 3 treatments, aerated samples from TIE level 1, were passed through C18 columns or OASIS columns at three different pH's. The acidic (pH 2) and neutral (pH 7) treatments for both C18 and OASIS columns, resulted in EC₅₀ values being reached at concentrations ranging from 17.9 to 25.5 % and was more toxic than the blank sample, indicating that the treatment enhanced rather than decreased the toxicity of the samples¹⁷. Unfortunately, passing the sample adjusted to pH 12 through the columns, resulted in an extensive precipitation, which blocked the columns and did not produce sufficient liquid for testing. Given the limited time period allotted for experimentation, a second attempt at the OASIS and C18 columns for the pH 12 sample could not be achieved.

Considering all of the TIE level 1, 2, and 3 treatments for Sample 1, the AC treatment provided the only significant reduction in sample toxicity. While the EC₂₀ for the 10 g / L AC treatment reached 40.57 %, the next highest EC₂₀, 8.08 % for OASIS at pH 7, was still below the value of the Sample 1 blank, at ~ 11 %¹⁸. The Sample 1 EC₅₀ values, demonstrate two shifts in the ordering of treatments effectiveness. Thus, the 100 g

¹⁶ Appendix 2: Figure 1

¹⁷ Appendix 2: Figure 2

¹⁸ Appendix 2: Figure 3

/ L AC and 30 g / L AC treatments were the second and third most effective treatments at 103.3 % and 54.97 % respectively compared to the untreated blank at 43.15 %¹⁹.

Comparing the EC₂₀ and EC₅₀ values for Sample 1, it appears that the AC treatments were more effective than any other treatment, although it remains unknown at this point whether SPE treatments at pH 12 could have been effective at reducing the toxicity of Sample 1.

Sample 2

For Sample 2, special measures were taken to ensure that the full spectrum of treatments could be analyzed and compared. Similarly, to the Sample 1 results, the TIE Level 1 treatment for Sample 2, did not remove a significant proportion of the toxicity and the TIE Level 2 treatments provided similar results as for the first sample. As such, the AlCl₃·6H₂O, EDTA, heptane, and heat treatments were ineffective at reducing the toxicity levels relative to the blank sample suggesting again that a large fraction of the toxicity contribution were not provided by compounds precipitable by the flocculant AlCl₃·6H₂O, by metals ions, by Heptane extractable compounds or by compounds volatile at 40°C. The EC₂₀ values for AC treatments, were respectively, 63% for 10 g / L, 55% for 30 g / L, and 43% for 100 g / L²⁰. These values were orders of magnitude higher than the 0.08% value for the blank sample. Overall, for Sample 2 EC₂₀ values, the three AC treatments mitigated the highest degree of toxicity, with 10 g / L AC measured as the most effective EC₂₀ of the three AC concentrations. While, for the EC₅₀, 30 g / L of AC was the most effective at reducing toxicity. Following filtration, the shift in both the

¹⁹ Appendix 2: Figure 4

²⁰ Appendix 3: Figure 1

turbidity but especially the color of the AC treated solutions could easily be distinguished visually from the toxic blank sample²¹.

For TIE Level 3, the aerated Sample 2 was passed through the C18 and OASIS columns at three different pH's, producing more complete results than for Sample 1 TIE level 3. The acidic and neutral conditions for both C18 and OASIS columns, again resulted in values for the EC₅₀ and EC₂₀ that approached or were below that of their respective blank. In contrast, the toxicity of Sample 2 was significantly reduced by SPE treatments at pH 12. This time, the precipitation in the high pH columns was prevented by adding an additional filtration step for the sample prior to passing it through the pH 12 columns. This provided a smooth, uninterrupted flow through the column, and enabled the collection of sufficient volume of treated sample for analyses of toxicity. The contrast in turbidity for Sample 2 SPE treatments pre-²² and post-²³ filtration is marked and shows the significance of a physical filtration step before adding to the columns. The resulting EC₂₀ and EC₅₀ values obtained after SPE at pH 12 using OASIS HLB chromatography, were orders of magnitude less toxic than the blank sample for the OASIS columns, with EC₂₀ of ~52% compared to the blanks 0.08%, and an EC₅₀ of ~388% vs ~1.85% for the blank²⁴. Additionally, the level of toxicity mitigation achieved at pH 12 by the C18 columns was markedly higher, albeit less so than the OASIS columns. Finally, the gave similar purification results as the AC treatments, suggesting that a large portion of the toxic contribution originates from polar organic compounds, in addition to a smaller

²¹ Appendix 5: Figure 3

²² Appendix 5: Figure 4

²³ Appendix 5: Figure 5

²⁴ Appendix 3: Figure 2

contribution by non-polar toxic compounds. Overall, the AC treatment at 10g/L or 30g/L and the SPE treatments at pH12 were the most effective purification methods for mitigating toxicity for sample 2.

Comparing Sample Results

The different test results between the two samples and the various treatments, provide significant information regarding the differences and similarities of Sample 1 and Sample 2. Firstly, as mentioned earlier, of the two samples, the second sample, with an EC_{20} of 0.08%, had a much higher starting toxicity than the first sample, with an EC_{20} of 10.97%. Secondly, the AC treatments for the Sample 2, with EC_{20} values of 62.91 % for 10 g / L AC, 54.89% for 30 g / L AC, and 42.62 % for 100 g / L AC compared to 0.08% for the untreated blank, had a much stronger purification effect than for Sample 1 AC treatments, which showed EC_{20} values of 40.57 % for 10 g / L AC, 4.21 % for 30 g / L AC, and 3.57 % for 100 g / L AC, compared to ~ 11 % for the untreated blank²⁵. Although, the EC_{20} values for Sample 1 AC treatments, do not seem as effective as for Sample 2, comparing the EC_{50} values reveal that all the AC treatments reduced the toxicity of both samples. Of the AC treatments, 30 g / L AC had the least significant effect at 54.97 % EC_{50} , compared to the 43.15 % for the Sample blank²⁶. For the SPE treatments, the low and medium pH EC_{20} values for both C18 and OASIS are lower for the second treatment than the first, maintaining the correlation between the potentially elevated toxicity of the second sample compared to the first sample. Finally, the lack of sample 1 data for the elevated pH SPE treatments, does not allow for elevated SPE

²⁵ Appendix 4: Figure 1

²⁶ Appendix 4: Figure 2

comparison between sample. However, the ostensive effectiveness of the elevated pH SPE for sample indicate that they likely would have been at least somewhat effective compared to the blank for sample 1. The marked difference in the toxicity measurements for the two samples and the difference efficacy of AC and SPE treatments, likely indicates that the composition of the toxins has significantly changed relative to the first sample or that the second sample happened to have a greater proportion of toxic elements which respond to the AC treatments.

Discussion

Evaluation of Results

The aim of this study was to a) Identify an effective remediation for a highly specific toxic oil wastewater and b) Assess the effectiveness of the Toxicity Identification Evaluation (TIE) for wide-ranging wastewater management. The research into an effective remediation technique found that two treatments were able to significantly reduce the toxicity. The data suggests that the primary fractions of toxicity in the effluent are non-volatile organic compounds with both polar and nonpolar bonds. While, the contribution of metals and salts to the toxic effluent fractions is minor at best, the results from the Solid Phase Extraction (SPE) treatments indicate that smaller water-soluble organic compounds, such as methanol, that are not picked up in the columns, may also contribute to the overall toxicity profile. This analysis is supported by the AC results from the TIE Level 2 treatments. Thus, the addition of activated carbon (AC) resulted in a marked decrease in the toxicity of the samples, consistent with the suspected organic nature. Overall, the AC treatments proved to be the most effective in both the quality and

quantity of significant results, with the elevated pH SPE following as a secondary candidate.

Based on scientific literature, the research results, as well as economic and practical feasibility of large-scale treatment, the most appropriate treatment for the toxic effluent is recommended to be purification via AC treatment. While the highest concentration of AC tested in the experiment (100 g / L) is approaching the limits of an economically viable solution, it was actually less effective than the lower concentrations of AC for sample 1. Treatment with 10 or 30 g / L AC seems to be a more reasonable alternative. Additionally, given that the TIE procedure did not identify any substances that could be problematic for an initial biological treatment in TIE steps 1 – 3, such a step could be implemented in conjunction with TIE treatment. With the large volume of toxic oil produced wastewater at the source, the recommended treatment is to apply an initial biological treatment to eliminate easily degradable organic compounds, like methanol, to lower the chemical oxygen demand (COD), before attempting toxicity mitigation via AC filtration for the remaining toxicity. Finally, persistent toxins leftover after the two previous steps can be extracted normally at a standard wastewater treatment facility. Notably, the Danish oil company does have a wastewater pipe to such a facility.

This study showed how TIE can be an effective wastewater management strategy. Being able to identify differences in contaminants involving unknown toxic chemicals, as well as to compare treatments, demonstrates the flexibility of TIE. In addition, TIE assays have proven to be effective in several studies both as a management technique and as a toxicology assay. In terms of management, one study applied TIE methodology to compare and assess Cr(VI) toxicity and its inhibitive properties, along with societal

feasibility of using activated carbon converted from coconut shells for treatment in Bangkok, Thailand. The study found that coconut shells are an abundantly available unused resource that can provide new economic value upon conversion to AC, thereby helping to reduce waste disposal costs, and provide an alternative to commercial activated carbon (Babel, 2004). Showing how locally supplied solutions, and employment of the precautionary principle (PP), can be effectively used to address management issues while being cost-effective and sustainable. If Denmark does import coconuts, the unused coconut shells could be recycled to produce CAC and potentially reduce costs when enacting the toxicity remediation recommendation suggested earlier. Alternatively, Denmark can potentially produce Charcoal from excess woody biomass and use that input to improve cost efficiency of treatment.

In addition to the standardized aqueous TIE methodologies, there are several alternative applications of TIEs. There are TIE reports that apply “focused”, carefully selected TIE regimens to particular toxicity samples based on strong historical evidence of specific toxicants (Weston, 2009). In this case, the study proposed a focused TIE approach to pyrethroid insecticides and organophosphate insecticide chlorpyrifos. Other studies have adapted the aqueous nature of the TIE methods to address whole sediment toxicity in marine sediment samples (Burgess, 1999), or analyze leachates²⁷ from municipal solid waste landfills in order to identify toxicants (Isidori, 2003). Non-aqueous TIE is incredibly important strategy to develop for the propensity of Solid waste we produce. Finally, alternatives to TIE or other biochemical assays have been discussed, such as the advantages and potential of various electrochemical wastewater treatment

²⁷ Liquid which has, in the course of passing through, has extracted suspend-able/soluble solids

technologies (Chen, 2004). Electrochemical wastewater techniques include electrodeposition to recover heavy metals, Electroflotation to separate oil, grease, and organic pollutants, and electrooxidation to degrade refractory pollutants.

Limitations

A major limitation in the study was the excess turbidity of the oily and salty wastewater effluent, which could have been accounted for with careful examination of the scientific literature. A physical filtration process is recommended for treatment of oily wastewaters originating from offshore sea platforms, due to the high salt content and inherent turbid characteristics (Fakhrul-Razi, 2009). While the source of the oily wastewater in this study was not from harsh Arctic Offshore platforms, it did have similar characteristics in terms of salinity and abundance of oily organic compounds. This would have been particularly useful to have considered for the pre- Sample 1 SPE treatments, as the excess turbidity clogged the elevated pH columns, invalidating the resulting product. For the Sample 2 elevated pH SPE, a physical filtration process was implemented before being added to the columns, which did alleviate the problem in much same way as the Fakhrul-Razi paper (2015) might suggest.

A secondary limitation potentially originates from the results for Sample 1's EC₂₀ AC treatments. Unlike, for Sample 2 AC results and Sample 1 EC₅₀ results, the 30 and 100 g / L AC treatments experienced lower bacterial survival values relative to the blank and AC's effectiveness on the two samples as a whole. These lower bacterial survival rates could indicate that the extraction of the treated liquid for those two steps failed due to human error in treatment. Another possible reason for this would be that the higher

concentrations of AC might have been clogging the 16-micron filters. This possibility could be tested by repeating the experiment and inserting a crude filtering step to remove the larger magnitude of AC before the 16-micron filtering step.

Broader Implications

Wastewater management is becoming increasingly central in today's drastically changing world. The results of this study are pertinent to the goal of guiding research questions and in particular demonstrated the potential effectiveness of TIE for wastewater toxicity management, to address the growing quantity of waste produced. Produced wastewater is the largest waste stream generated in oil and gas industries and is of increasing concern due to the increasing volume of waste worldwide and the impact of its environmental discharge (Fakhru'l-Razi, 2009). This concern is especially an issue for offshore platforms, where space is limited, and compact solutions are cost-ineffective. Offshore oil and gas development in harsh environments near the Arctic require higher efficacy of water management, as these environments are incredibly sensitive to changes in water quality, even more so than in more temperate regions (Zheng, 2015). The application of high potential emerging technologies is significantly limited by the offshore setting and harsh climate experienced in the Arctic and Antarctic regions.

As no single technology can produce suitable effluent characteristics for all oil and gas produced waters, a combination of two or more in series is the recommended strategy (Fakhru'l-Razi, 2009). TIE can address a variety of toxic wastes and complications which may stump other water quality management solutions, as TIE methodology is particularly useful at identifying several potentially effective toxicity or

water quality control remediation treatments. For water and wastewater management, beyond preventative management through the PP or other methodologies, TIE is well suited for addressing specific issues, but also for looking at the bigger picture. As Albert Einstein once said, “In the same way in which the world evolves around us, people must adapt and evolve to best account for the new and unfamiliar.” This quote exemplifies the mindset with which this paper recommends enacting solutions for broad environmental management and focused wastewater management.

Bibliography

Al-Jayyousi, Odeh R. "Greywater Reuse: Towards Sustainable Water Management."

Desalination 156, no. 1-3 (2003): 181-92. doi:10.1016/s0011-9164(03)00340-0.

Andersen, H. Statistical methods for evaluation of toxicity of waste water. M.Sc. Thesis

7/94. ISSN 0105-8568. *Institute for Mathematical Statistic and Operation*

Analysis. Technical University of Denmark, 1994. (Danish, Statistical Program ref).

Ankley, Gerald T., et al. “Early Evolution of the Toxicity Identification Evaluation

Process: Contributions from the United States Environmental Protection Agency

Effluent Testing Program.” *The Handbook of Environmental Chemistry Effect-*

Directed Analysis of Complex Environmental Contamination, 2011, pp. 1–18.,

doi:10.1007/978-3-642-18384-3_1.

Babel, Sandhya, and Tonni Agustiono Kurniawan. "Cr(VI) Removal from Synthetic

Wastewater Using Coconut Shell Charcoal and Commercial Activated Carbon

Modified with Oxidizing Agents And/or Chitosan." *Chemosphere* 54, no. 7
(2004): 951-67. doi:10.1016/j.chemosphere.2003.10.001.

Bohn, Hinrich L., and Robert C. Cauthorn. "Pollution: The Problem of Misplaced Waste: Inefficiency of Resource Use and Misplacement of Spent Resources Are the Physical Causes of the Pollution Phenomenon." *American Scientist* 60, no. 5 (1972): 561-65. <http://www.jstor.org/stable/27843364>.

Burgess, Robert M., Mark G. Cantwell, Marguerite C. Pelletier, Kay T. Ho, Jonathan R. Serbst, Howard F. Cook, and Anne Kuhn. "Development of a Toxicity Identification Evaluation Procedure for Characterizing Metal Toxicity in Marine Sediments." *Environmental Toxicology and Chemistry* 19, no. 4 (2000): 982-91. doi:10.1002/etc.5620190427.

Calow, Peter, and Valery E Forbes. "Ecotoxicology." *ELS*, (2014)
doi:10.1002/9780470015902.a0003245.pub2.

Chen, Guohua. "Electrochemical Technologies in Wastewater Treatment." *Separation and Purification Technology* 38 (2004): 11-41. doi:10.1016/j.seppur.2003.10.006.

Christensen, E.R., Kusk, K.O. and Nyholm, N. Dose-response regressions for algal growth and similar continuous endpoints: Calculation of EC10 based on EC₅₀ and slope at 50% inhibition. *Environ. Toxicol. Chem.* 28 (4): 826-835. (2009)

Fakhru'L-Razi, Ahmadun, et al. "Review of Technologies for Oil and Gas Produced Water Treatment." *Journal of Hazardous Materials*, vol. 170, no. 2-3, 2009, pp. 530–551., doi:10.1016/j.jhazmat.2009.05.044.

- Gjerde, Kristina M. *Ecosystems and Biodiversity in Deep Waters and High Seas*. UNEP Regional Seas Reports and Studies No. 178 (2006).
- Grčman, H., Š. Velikonja-Bolta, D. Vodnik, B. Kos, and D. Leštan. "EDTA Enhanced Heavy Metal Phytoextraction: Metal Accumulation, Leaching and Toxicity." In *Plant and Soil*, 105-14. Vol. 235. (2001) doi:10.1023/A:1011857303823
- Hunt, John, Brian Anderson, Bryn Phillips, Ron Tjeerdema, Bryan Largay, Melanie Beretti, and Amanda Bern. "Use of Toxicity Identification Evaluations to Determine the Pesticide Mitigation Effectiveness of On-farm Vegetated Treatment Systems." *Environmental Pollution* 156, no. 2 (2008): 348-58. doi:10.1016/j.envpol.2008.02.004.
- Isidori, Marina, Margherita Lavorgna, Angela Nardelli, and Alfredo Parrella. "Toxicity Identification Evaluation of Leachates from Municipal Solid Waste Landfills: A Multispecies Approach." *Chemosphere* 52, no. 1 (2003): 85-94. doi:10.1016/s0045-6535(03)00298-4.
- Johnson, B. Thomas. "Microtox® Acute Toxicity Test." *Small-scale Freshwater Toxicity Investigations*, (2005): 69-105. doi:10.1007/1-4020-3120-3_2.
- Kaza, Silpa, Lisa Yao, Perinaz Bhada-Tata, and Frank Van Woerden. "What a Waste 2.0: A Global Snapshot of Solid Waste Management to 2050" *The World Bank: Urban Development Series* (2018) doi:10.1596/978-1-4648-1329-0.
- Lappalainen, Juha, Risto Juvonen, Johanna Nurmi, and Matti Karp. "Automated Color Correction Method for *Vibrio Fischeri* Toxicity Test. Comparison of Standard and

Kinetic Assays." *Chemosphere* 45, no. 4-5 (2001): 635-41. doi:10.1016/s0045-6535(00)00579-8.

Lebreton, L., et al. "Evidence That the Great Pacific Garbage Patch Is Rapidly Accumulating Plastic." *Scientific Reports*, vol. 8, no. 1 (2018): doi:10.1038/s41598-018-22939-w.

Mateo-Sagasta, Javier, Liqa Raschid-Sally, and Anne Thebo. "Global Wastewater and Sludge Production, Treatment and Use." *Wastewater*, 2015, 15-38. doi:10.1007/978-94-017-9545-6_2.

Miller, G. Wade. "Integrated Concepts in Water Reuse: Managing Global Water Needs." *Desalination* 187, no. 1-3 (2006): 65-75. doi:10.1016/j.desal.2005.04.068.

Schäfer, A.i., and S. Beder. "Relevance of the Precautionary Principle in Water Recycling." *Desalination* 187, no. 1-3 (2006): 241-52. doi:10.1016/j.desal.2005.04.083.

Vörösmarty, C. J., and P. B. McIntyre. "Global Threats to Human Water Security and River Biodiversity." *Nature* 467 (2010): 555-61. doi:https://doi.org/10.1038/nature09440.

Weston, Donald P., and Michael J. Lydy. "Focused Toxicity Identification Evaluations to Rapidly Identify the Cause of Toxicity in Environmental Samples." *Chemosphere* 78, no. 4 (2010): 368-74. doi:10.1016/j.chemosphere.2009.11.017.

Yang, Hong, and Karim C. Abbaspour. "Analysis of Wastewater Reuse Potential in Beijing." *Desalination* 212, no. 1-3 (2007): 238-50.

doi:10.1016/j.desal.2006.10.012.

Zheng, Jisi, et al. "Offshore Produced Water Management: A Review of Current Practice and Challenges in Harsh/Arctic Environments." *Marine Pollution Bulletin*, vol.

104, no. 1-2, 2016, pp. 7–19., doi:10.1016/j.marpolbul.2016.01.004.

Appendices

Appendix 1: Untreated Samples

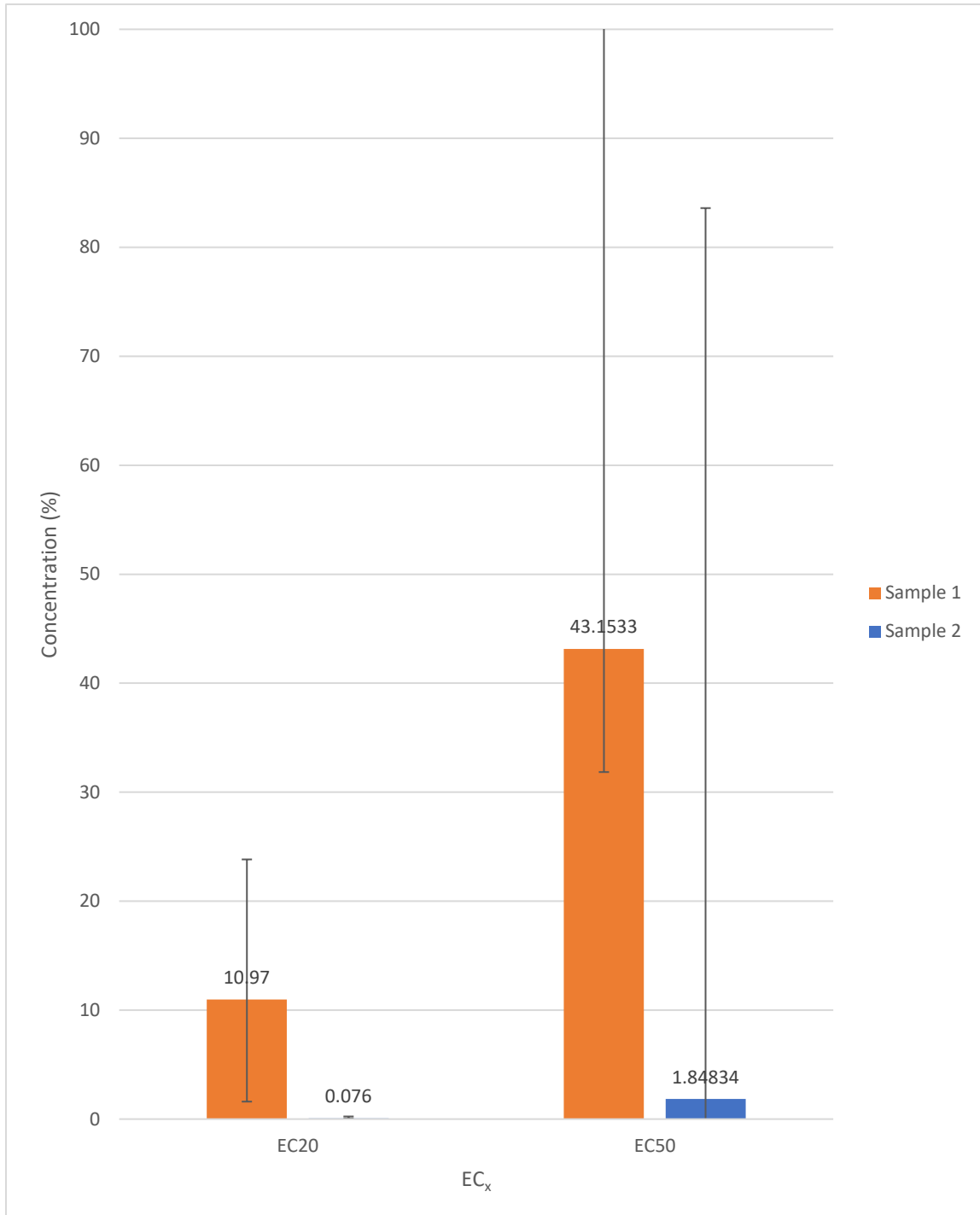


Figure 1: Comparing the EC₂₀ and EC₅₀ of the two untreated samples

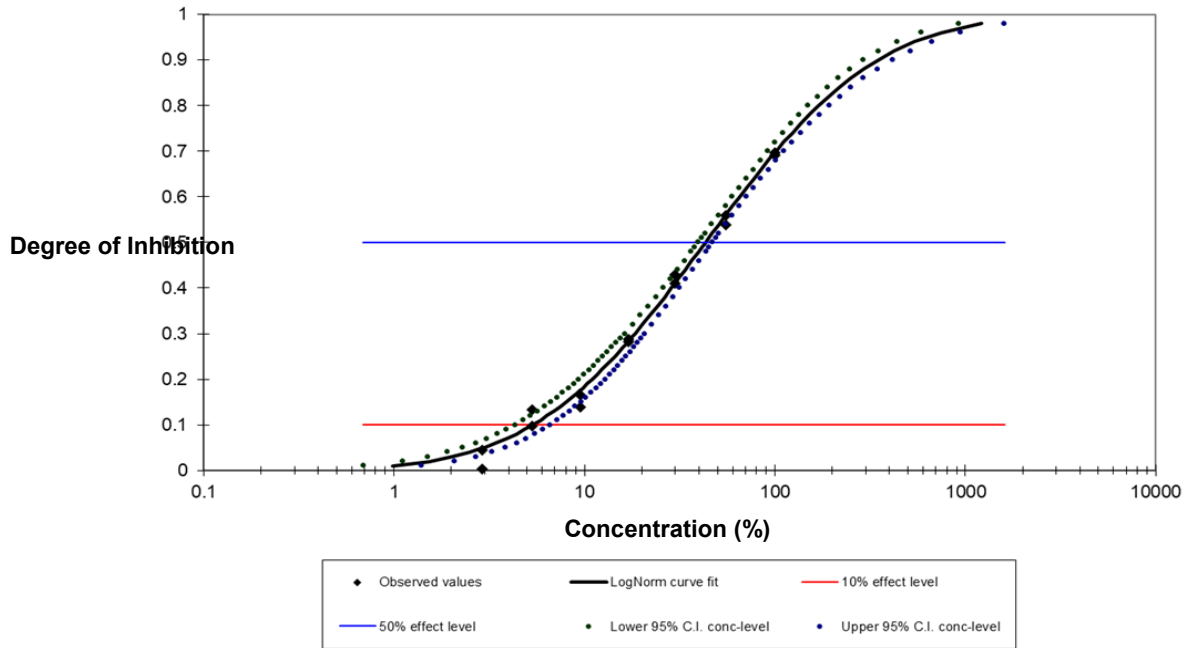


Figure 2: Concentration curve for Sample 1 prior to treatment

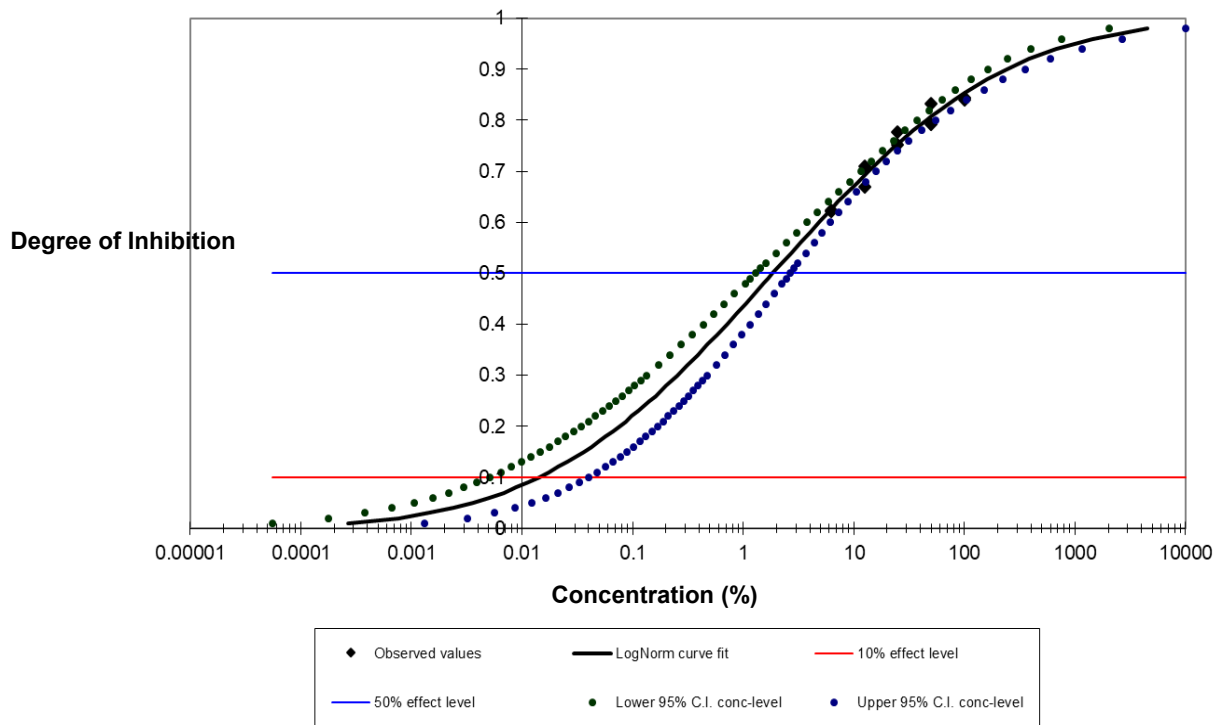


Figure 3: Concentration curve for Sample 2 prior to treatment

Appendix 2: Sample 1 TIE Level 1 - 3

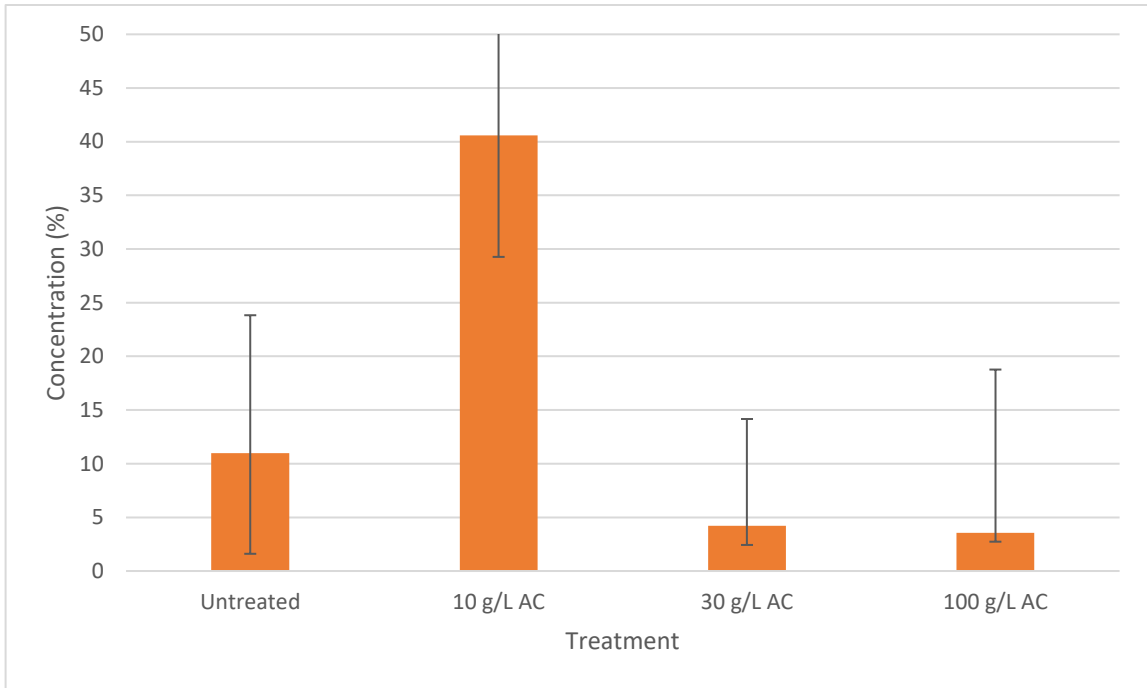


Figure 1: Sample 1 AC treatments EC₂₀ values

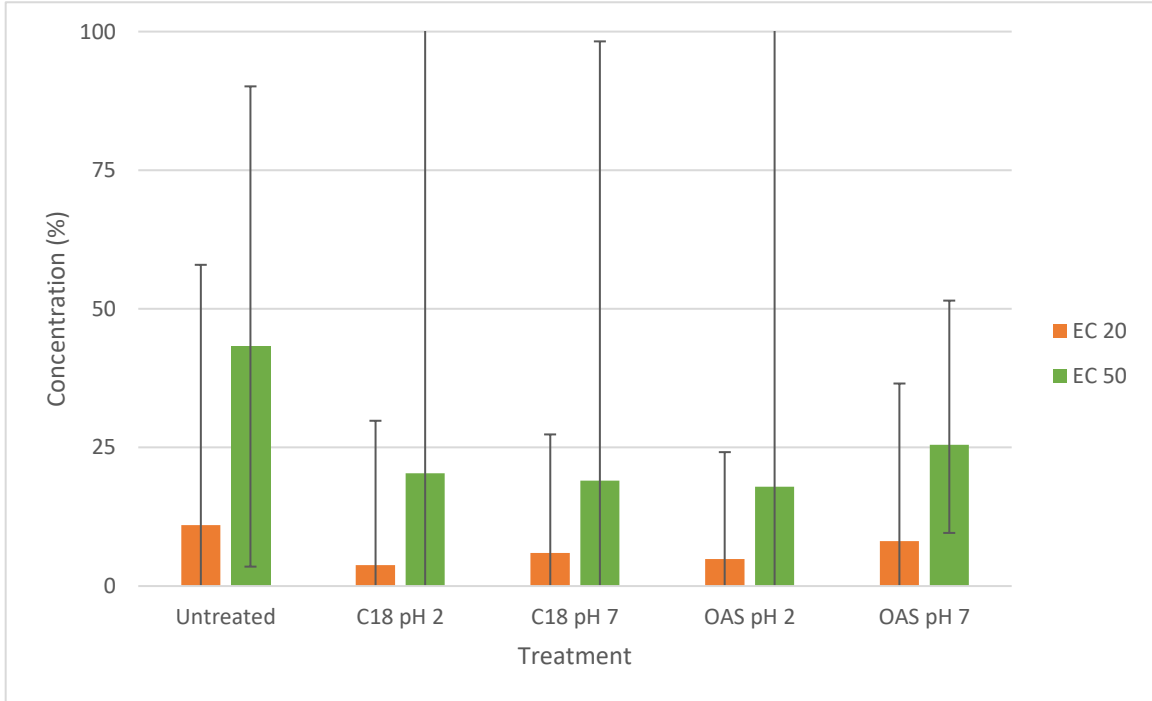


Figure 2: Sample 1, TIE Level 3 EC₅₀ and EC₂₀

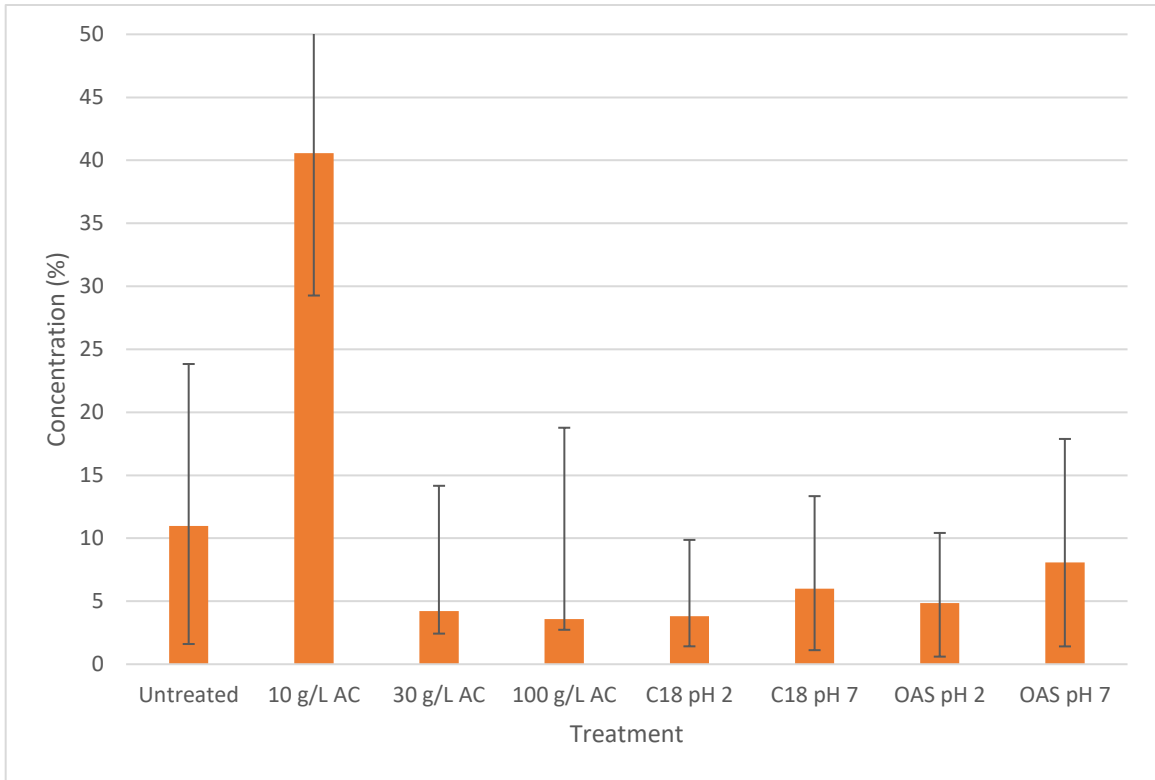


Figure 3: Sample 1, TIE Level 1 – 3, comparing EC₂₀ values among treatments

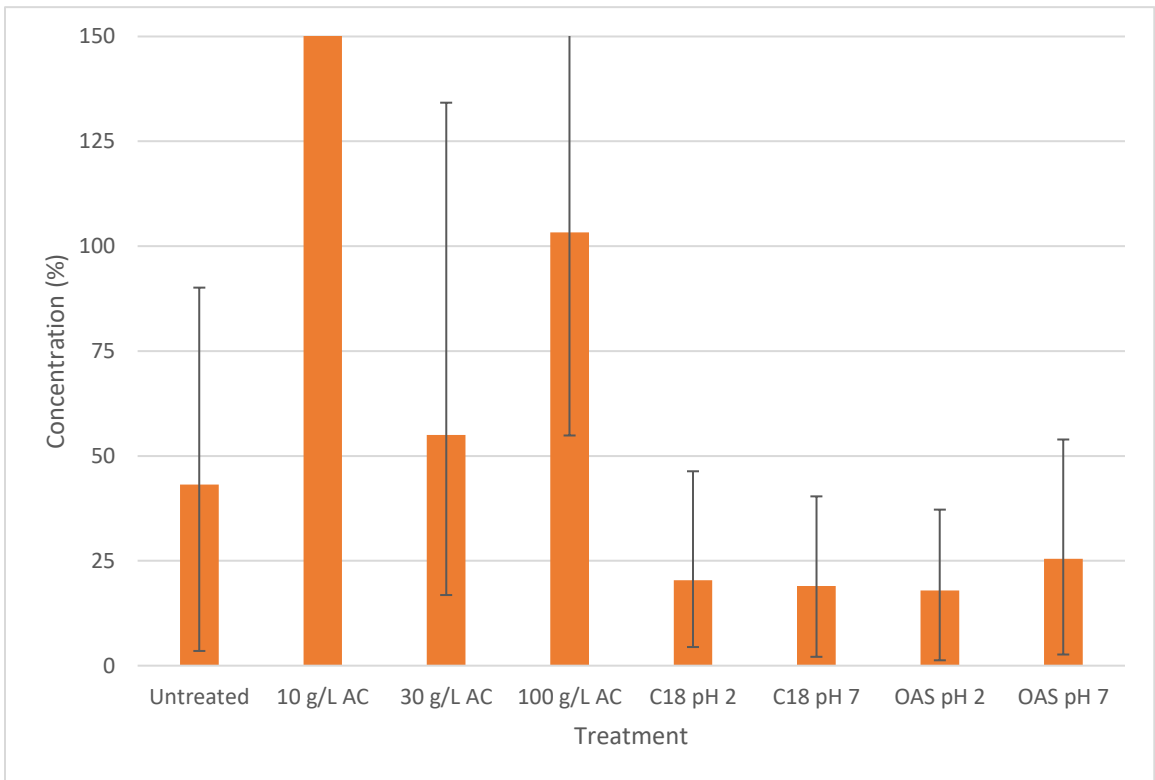


Figure 4: Sample 1, TIE Level 1 – 3, comparing EC₅₀ values among treatments

Appendix 3: Sample 2 TIE Level 1 - 3

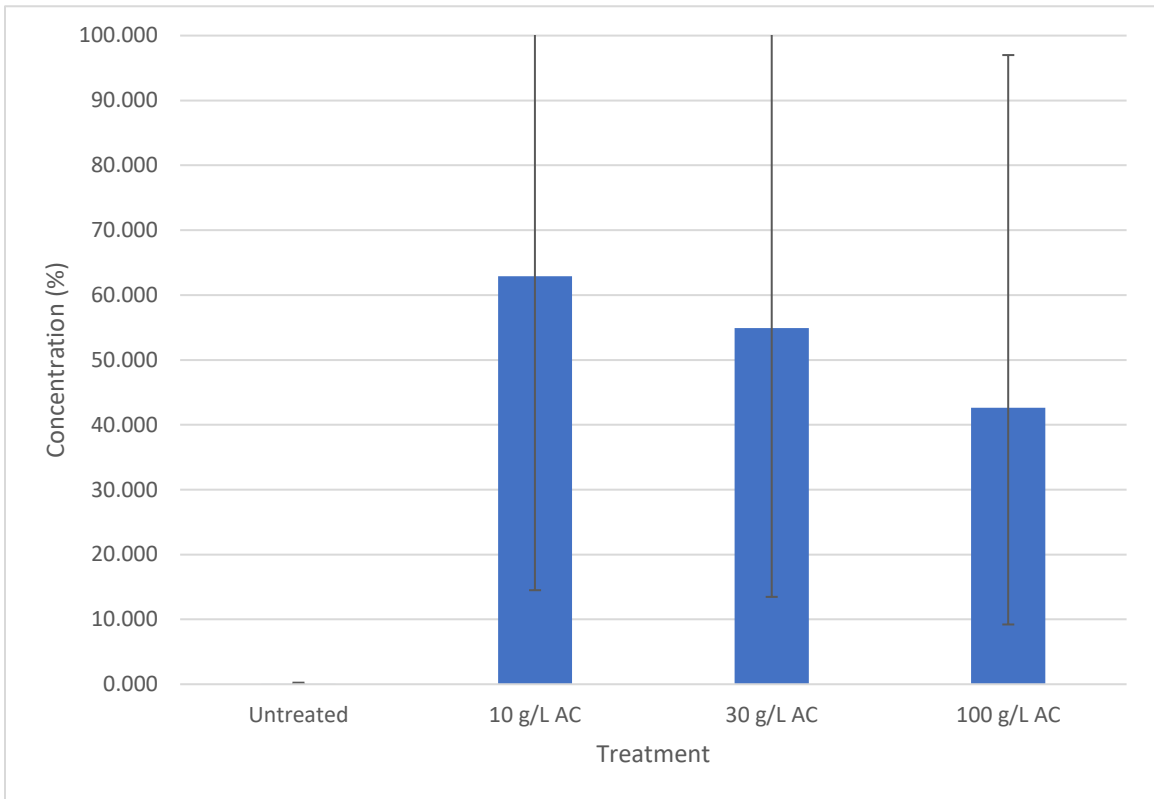


Figure 1: Sample 2, Comparing EC₂₀ values between AC treatments

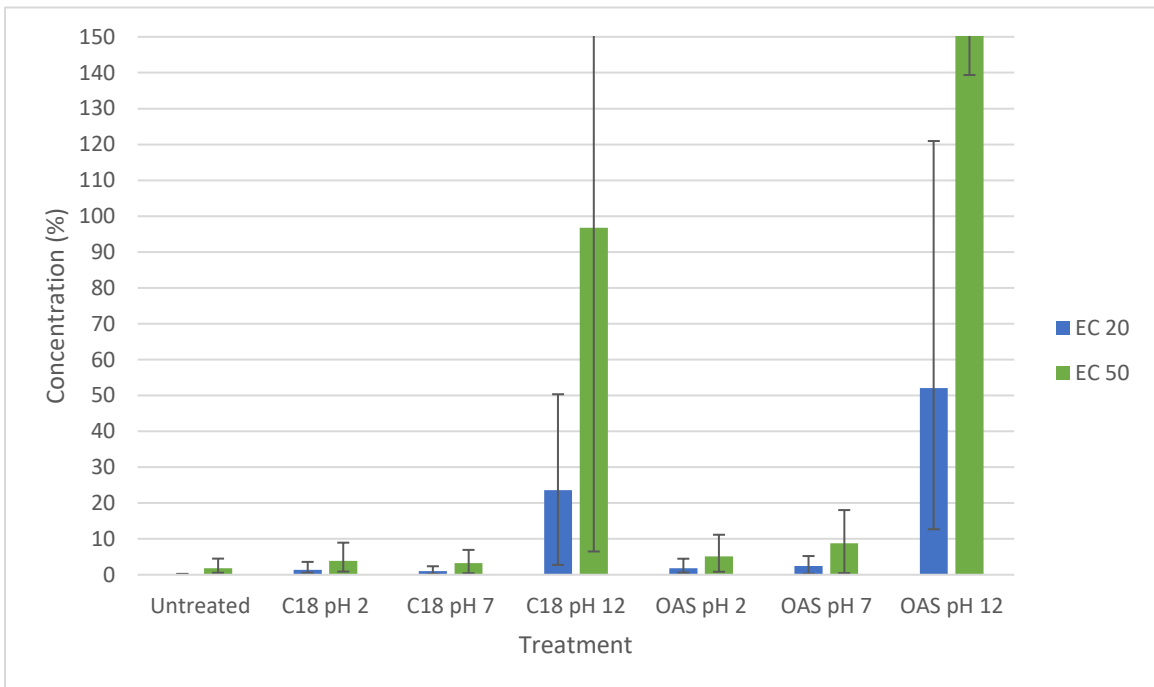


Figure 2: Sample 2, Comparing OASIS and C18 EC_{xS}

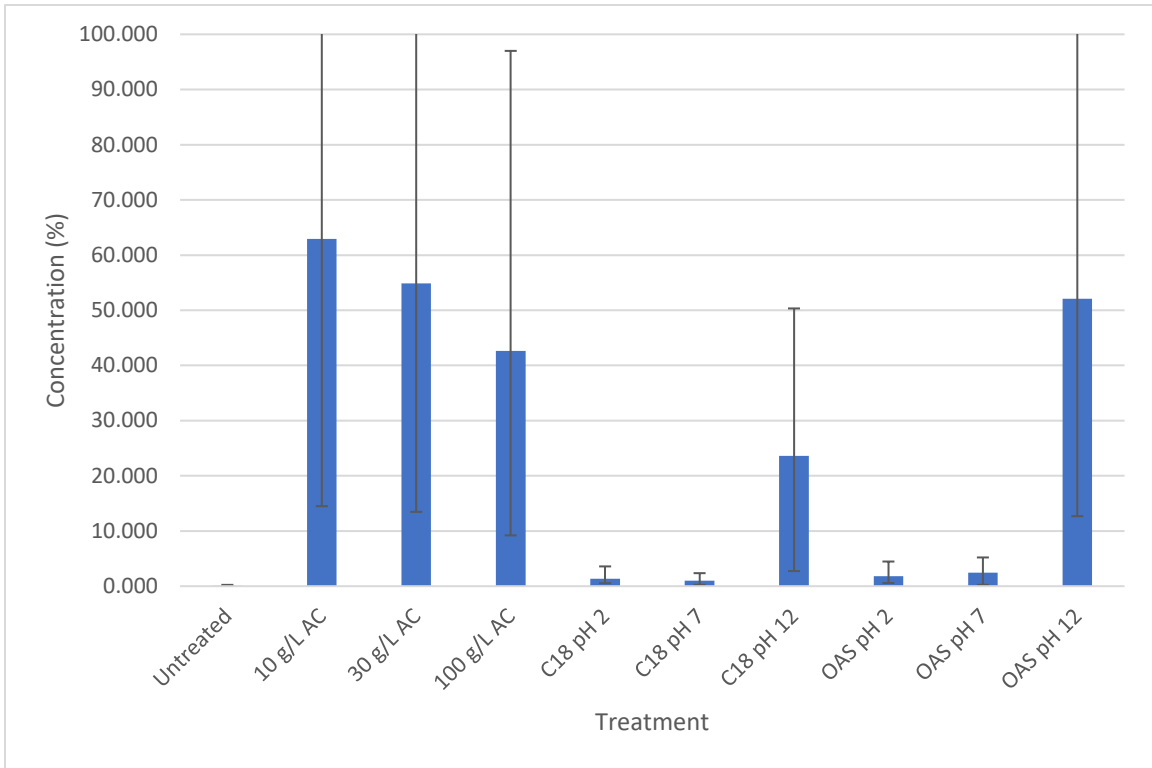


Figure 3: Sample 2, TIE Level 1 – 3, comparing EC₂₀ values among treatments

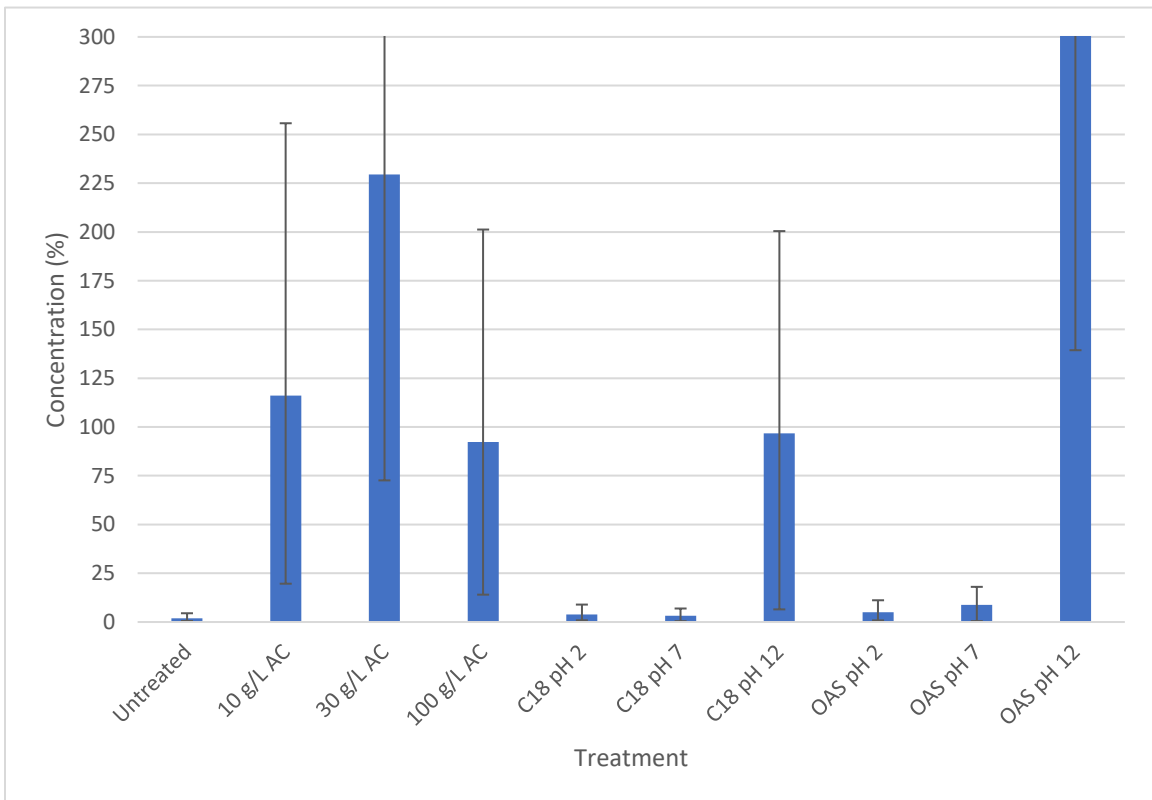


Figure 4: Sample 2, TIE Level 1 – 3, comparing EC₅₀ values among treatments

Appendix 4: Comparing Samples

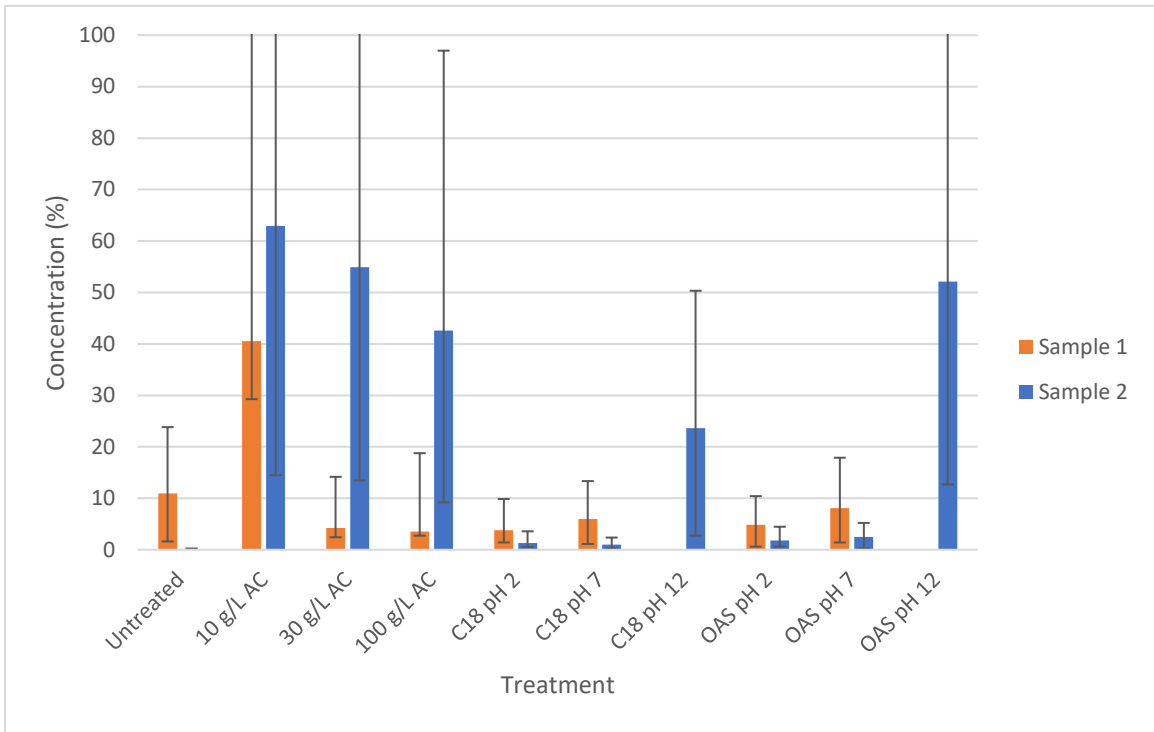


Figure 1: Comparing EC₂₀ values between treatments and samples

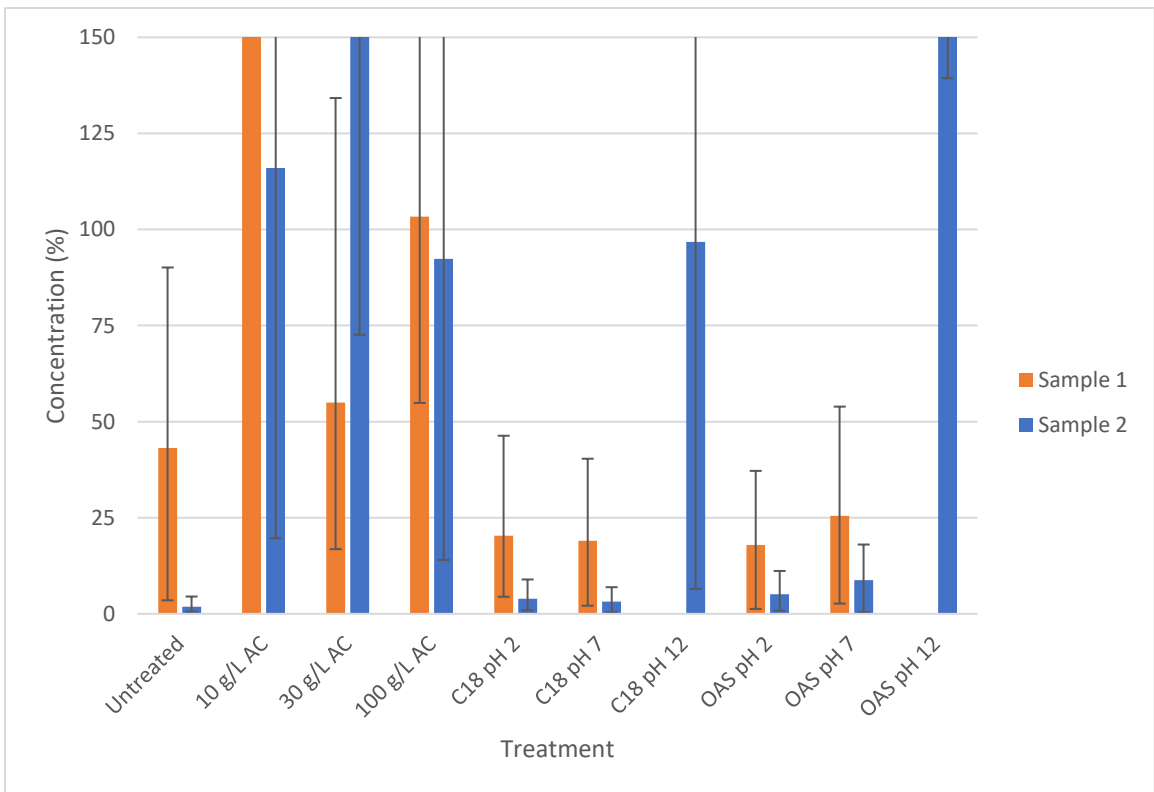


Figure 2: Comparing EC₅₀ values between treatments and samples

Appendix 5: Research Photos



Figure 1: Sample 1, TIE level 1 Aeration setup



Figure 2: 24 hr. End-over-end shaker for TIE treatments

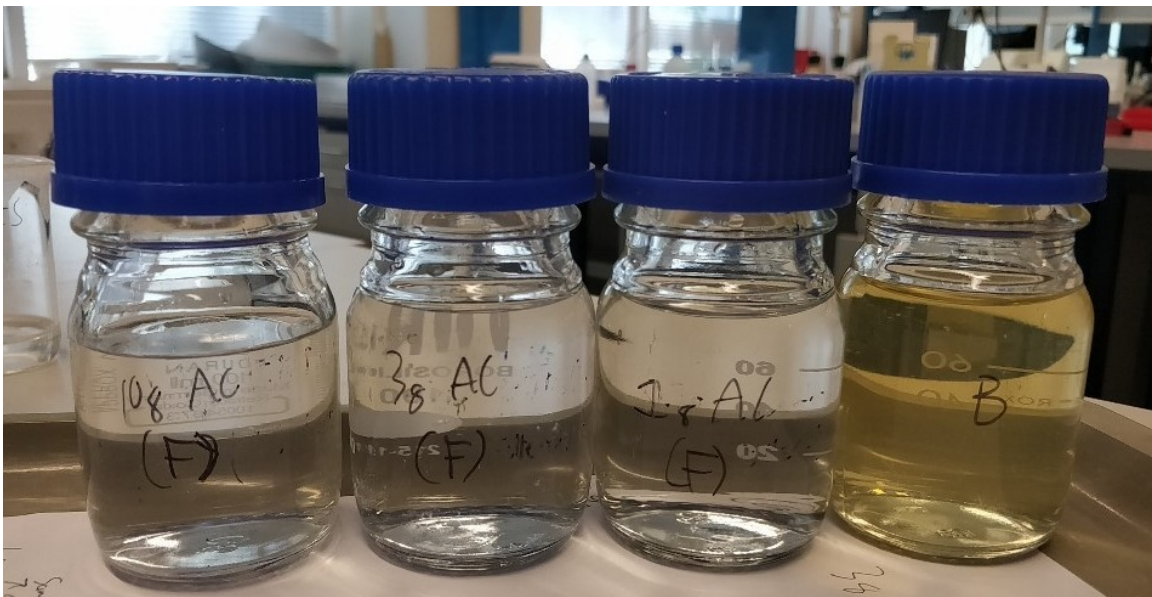


Figure 3: Sample 2 TIE level 2, AC treatments and blank, post-filtration

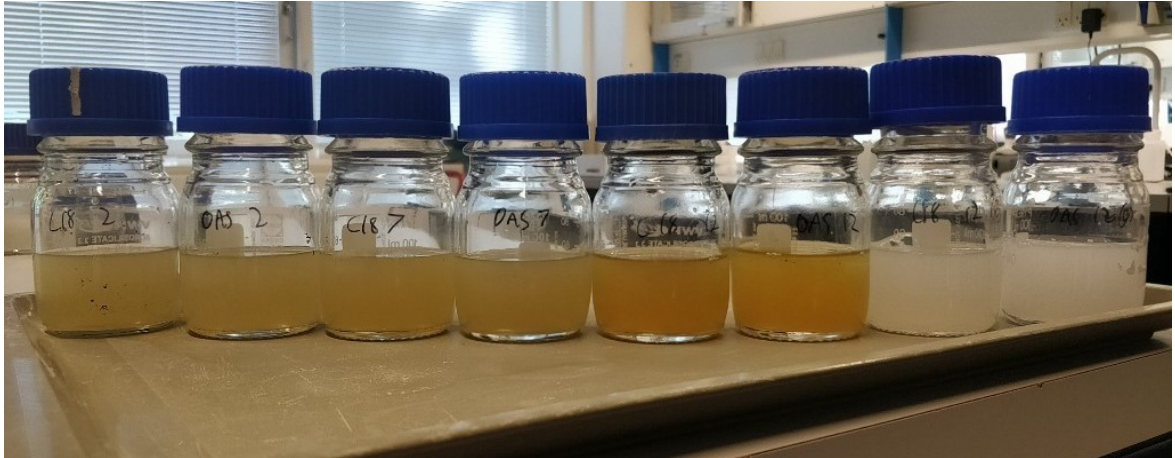


Figure 4: Sample 2 TIE level 3, C18 and OASIS treatments, pre-filtration

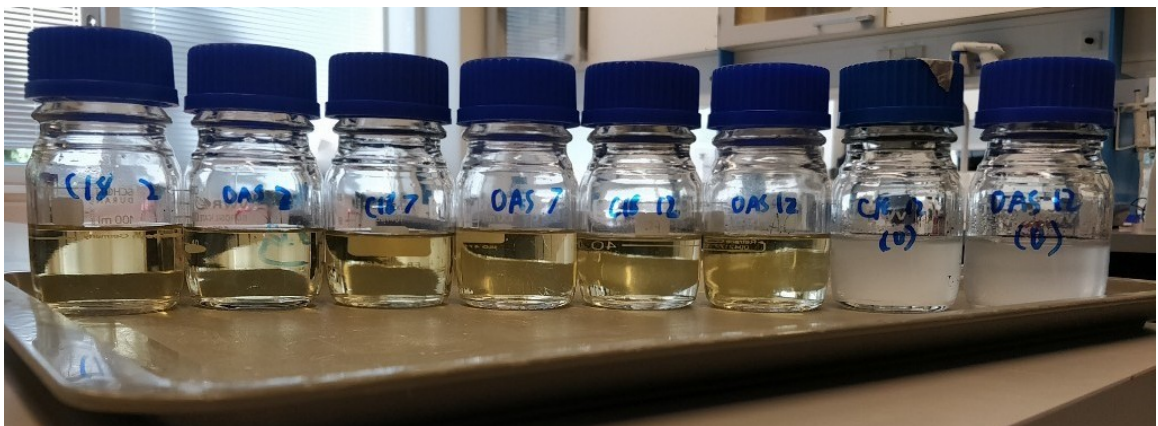


Figure 4: Sample 2 TIE Level 3, C18 and OASIS treatments, post-filtration