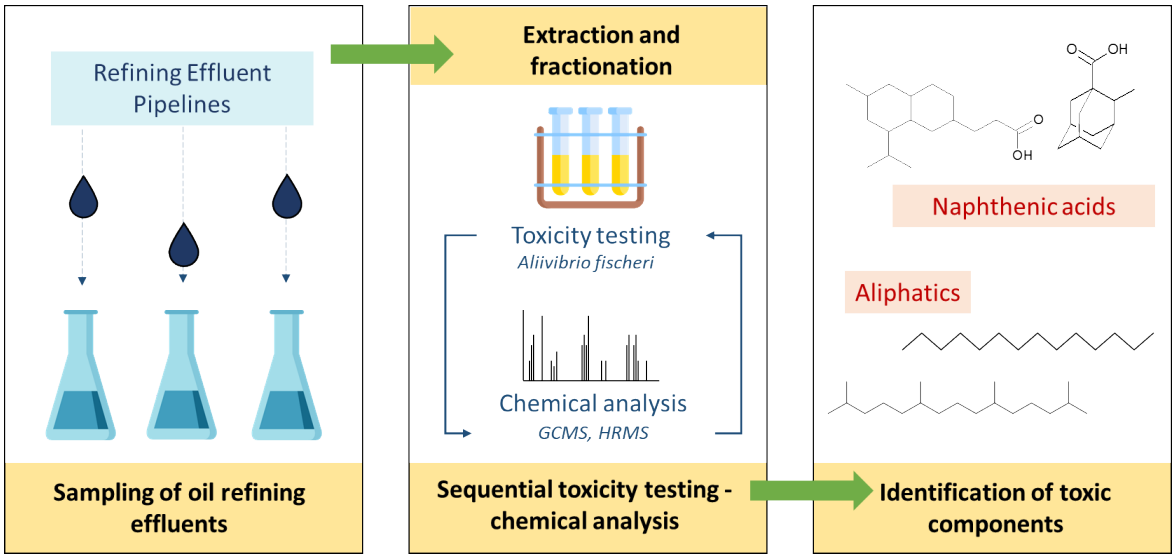
Graphical abstract

Naphthenic Acids are Key Contributors to Toxicity of Heavy Oil Refining Effluents



Highlights

- High complexity of refining effluents has made difficult to link their toxicity and chemistry
- Knowledge gap has led to non-targeted, unsuccessful treatment and unsafe effluents
- An Effect Directed Analysis was applied to refining effluents to detect toxic organics
- Naphthenic acids found to be linked to biological effects on *Aliivibrio fischeri*
- Mixture effects to be key in the toxicity exerted by refining effluents

Naphthenic Acids are Key Contributors to Toxicity of Heavy Oil Refining Effluents

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Abstract. Oil refining produces vast quantities of wastewater with harmful contaminants that can be released back into the environment with a possible risk of toxicity to aquatic wildlife and human populations. Hence the importance of adequate wastewater treatment to achieve safe effluents that protect both ecological and human health. However, some refining effluents are linked to serious pollution problems even after treatment, partly because little progress has been made in determining the causative agents of the observed biological effects, resulting in non-targeted treatment. Here, we followed an effect-directed analysis (EDA) approach using *Aliivibrio fischeri* as biosensor to show that naphthenic acids (NAs) are important components of refining wastewater resulting from the processing of heavy crude oil. Furthermore, we demonstrate that besides mixture effects, NAs have a significant contribution to the toxicity exerted by these effluents. Profiling of the NA mixture was conducted using high resolution liquid chromatography-Orbitrap, which evidenced that O_2 NAs corresponded to 90% of the NAs detected. Our findings contrast with previous reports where classic NAs have been found between 15% and 72% and could explain the significant

biological effects observed in *A. fischeri*. This study broadens the body of evidence pointing at mixture effects and low-concentration pollutants as the cause of toxicity from RWW, in addition to NAs resulting from the processing of heavy crude oil. Our results can serve as a starting point for setting better effluent discharge standards relevant to oil refining wastewater resulting from heavy crude oil and help improve wastewater treatment plants to reduce effluent toxicity.

Keywords. Oil refining, toxicity, effluent, effect-directed analysis, naphthenic acids, *Aliivibrio fischeri*

1. Introduction.

The petroleum industry handles more water than oil during their daily operations (IPIECA, 2005), especially during oil refining. This makes the refining sector a major water consumer — the refining of 1 m^3 of crude oil requires 2 to 2.5 m^3 of water (Alva-Argáez et al., 2007; Coelho et al., 2006). As a result, and considering that approximately 13 million m^3 of crude oil were refined daily in 2019 (International Energy Agency, 2020), significant volumes of refining wastewater (RWW) are constantly produced worldwide. Hence the importance of RWW quality from the environmental perspective, as refineries are distributed widely around the world and the vast amounts of RWW produced on each site can have hazardous effects on the receiving ecosystems. Wastewater treatment technologies can provide effluents that are environmentally safe, provided continuous monitoring is conducted to follow-up the efficacy of wastewater treatment plants and help identifying effluents of concern. However, there are important discrepancies in relation to quality criteria for industrial effluents around the world, including refining discharges, which mainly derive from different approaches in environmental regulations across countries (Hessel et al., 2007; Jafarinejad and Jiang, 2019; Power and Boumphrey, 2004; Whitehouse, 2001). Some regulations, mainly in low-income countries, consider only bulk parameters and metals to establish discharge limits and monitor

50 treatment efficacy, whereas others have a more holistic approach that combines toxicity tests,
51 analytical tools, and biological monitoring (Norberg-King et al., 2018). The latter approach
52 has proven effective to identify effluents of concern and should, in theory, lead to an
53 investigation to determine the causes of biological effects so these can be targeted during
54 treatment (Comber et al., 2015; Sponza, 2002; Vaz Hara and Marin-Morales, 2017).
55 In practice, however, establishing causative agents for biological effects is not
56 straightforward because such effects often result from the interaction of different chemicals
57 or stem from chemicals at concentrations hard to detect. This has been especially true for the
58 refining industry, particularly due to their highly complex nature – harmful effects have been
59 reported for refining effluents in different geographical areas (Atuanyan and Tudararo-
60 Aherobo, 2015; Avci et al., 2005; Bleckmann et al., 1995; Çavas and Ergene-Gözükara,
61 2005; Gupta, A.K.; Ahmad, 2012; Tatem et al., 1978; Wake, 2005) but it is still unclear what
62 exactly is causing these biological effects on receiving environments. Evidence so far
63 indicates that mixture effects and organic chemicals are key, but the existing literature has
64 consistently addressed organic compounds as a whole, without a more in-depth analysis of
65 the organic fraction in RWW. Previous studies aiming at linking toxicity and chemical
66 composition in RWW have evaluated toxicity of known specific components like phenol and
67 chromium (Buikema Jr et al., 1981; Hall Jr et al., 1978), or followed the toxicity
68 identification evaluation (TIE) approach (Ankley et al., 2011; Burks, 1982; Daflon et al.,
69 2017; Dorris et al., 1974). In general, polycyclic aromatic hydrocarbons (PAHs) and metals
70 appear to be behind the observed biological effects in numerous case studies, but the
71 extensive report of "organics" (Ankley et al., 2011; Burks, 1982; Daflon et al., 2017; Dorris
72 et al., 1974) as essential contributors to overall toxicity without shedding much light on their
73 identity demonstrates the need for further research.

The tangible outcome of the gap in knowledge in relation to the causal agents of the toxicity exerted by RWW is that treatment processes often fail to fully remove toxicity from effluents. Modern refinery wastewater treatment plants are generally effective in removing suspended oil and suspended solids, but toxic and hydrophilic contaminants are likely to resist treatment and reach waterways (Li et al., 2015). It is, therefore, necessary to redesign treatment plants for these to reduce the concentration of toxic chemicals to non-hazardous levels, but a good understanding of the chemical and toxicological properties of the constituents of wastewater is essential to develop effective treatment systems.

Addressing the gap in knowledge of the organic fraction in RWW requires a new approach — the limitations of studying known specific components and TIE methodologies for understanding complex mixtures of organics in environmental samples are well known (Hong et al., 2016; Pessala et al., 2004). An alternative approach to address the problem of environmental diagnostics is conducting an effect-directed analysis (EDA), which includes an extraction step and makes an emphasis on organics as the cause of toxicity, making it an excellent option for the analysis of RWW. This approach, however, has not been successfully applied to RWW before. Therefore, the objective of this study was to identify toxic organics in oil refining effluents following a non-targeted EDA procedure using *Aliivibrio fischeri* as biosensor, thus helping to fill the long-standing gap between chemical composition and toxicity of RWW.

2. Materials and Methods

2.1. Chemicals and reagents

Solvents were obtained from Fisher Scientific. H_2SO_4 and NaOH were purchased from Fluka. Oasis® WAX 6cc (150 mg, 30μm) and HLB 6cc (200 mg) extraction cartridges were obtained from Waters. N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide containing 1% t-BDMS-chloride (MTBSTFA) was purchased from Sigma-Aldrich. Stock solutions for

spiking (aromatic and aliphatic hydrocarbons; 200 μg/mL in acetone) and the internal standard (IS) solution (1-chlorooctadecane and *o*-terphenyl; 400 μg/mL in acetone) were purchased from Restek UK. Chemicals for the fractionation check solution (naphthalene, bisphenol A, and phenol; 70 mg/L in hexane) were purchased from Sigma-Aldrich. Solutions were stored at 4°C in dark and airtight conditions.

For the bioassay, phenol and potassium dichromate were purchased from Sigma-Aldrich. Sodium chloride was purchased from Fischer Scientific and the Microtox® reagent was obtained from Modern Water Inc.

2.2. EDA procedure

The EDA methodology was conducted in two phases, as shown in Figure 1. Phase I aimed at providing a preliminary characterization, toxicity evaluation, and extraction of effluent samples, all of which are described in the sections 2.5, 2.6, 2.8, and 2.9 (subsection 2.9.1) below. Phase II was carried out subsequently to fractionate the extracts obtained during Phase I (Section 2.7). Fractions were then analyzed to assess toxicity (Section 2.8) and chemical composition (Section 2.9, subsection 2.9.1). Identification and characterization of toxic organics was conducted following the procedures described in Section 2.9, subsections 2.9.2 and 2.9.3.

2.3. Sampling

Effluent samples were collected from 3 pipelines (P1, P2, P3) from an oil refinery located in Barrancabermeja, Colombia, discharging into River Magdalena. Sampling details are provided in Table S1. The temperature and pH of samples were measured *in situ* using an Oakton® portable meter. Samples were acidified to pH 2 and stored at 4°C in airtight conditions until analysis.

2.4. Sample Preparation

Each sample was divided into two separate sub-batches for chemical analysis and toxicity evaluation, which were processed identically and at the same time. Blanks and analytical quality control (AQC) samples were prepared for quality assurance purposes using Milli-Q® water. Samples for chemical analyses were spiked with aromatic and aliphatic hydrocarbons (final concentration 80 µg/L) and IS (*o*-terphenyl and 1-chlorooctadecane; final concentration100 µg/L).

2.5. Preliminary characterization of effluents

Samples were filtered using 1.2 µm pore size Whatman® filters. Total organic carbon (TOC) of aqueous filtrates was measured by combustion catalytic oxidation/non-dispersive infrared (NDIR) spectrometry using a Shimadzu TOC-VCPN analyzer coupled with a Shimadzu OCT-1 8-port sampler.

The total concentration of V, Ni, Zn, As, Se, Hg, Cr, Pb, and Cd was determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) with a Perkin Elmer Optima 5300 DV spectrophotometer attached to a Perkin Elmer AS 93plus autosampler. Digestion of organic matter was carried out in a CEM MARS 6 microwave digester in accordance with USEPA method 3015A.

2.6. Sample extraction

Sample aliquots (800 mL) were filtered using 1.2 µm pore size Whatman® filters and extracted in duplicate using liquid-liquid extraction (LLE) and solid phase extraction (SPE). Details are provided in the SI.

2.7. Fractionation

LLE and SPE extracts were fractioned using normal phase high-performance liquid chromatography (NP-HPLC) using an Agilent 1260 system on an analytical Waters® SPHERISORB® silica column (4.6 x 100mm, 3-μm particle size). Mobile phases were (A) hexane 100% and (B) hexane:methanol:IPA 10:25:65 (v/v/v) flowing at 1 mL/min. Further

148 details are provided in the SI.

149 2.8. Toxicity evaluation

150 Testing was performed using a modified version of the LBT methodology described in BS
151 EN ISO 11348-3:2008, adapting the procedure to 96-well plates (Blaise et al., 1994) to
152 reduce sample requirements. Contact time was 15 minutes, 1% methanol was used as carrier
153 solvent, Cr(VI) was used as positive control, and phenol (expected EC_{50}: 13 – 26 mg/L) was
154 used as reference substance. Light output was measured in a Promega GloMax™
155 luminometer and incubation was performed in an Aqualytic thermostatic cabinet at 15°C
156 ±0.3. The full modified procedure is described in the SI.

157 The standard assay was applied to aqueous samples and extracts, which were tested in
158 duplicate. The increased sensitivity assay was used for fractions but due to limitations in the
159 amount of sample, only one replicate was performed; EC_{50} values for phenol, f_{kt} values, and
160 RSD for the positive control (≤ 3%) within each batch were used as criteria of validity.
161 Toxicity was expressed as toxicity units (TU), where $TU = 100/EC_{50}$.

162 2.9. Chemical analysis

163 **2.9.1. GC-MS**

164 GC-MS analysis was performed using a Perkin Elmer Clarus® 500 instrument equipped with
165 a DB-5 capillary column (30 m x 0.25 mm I.D) coated with 0.25 μm 5% phenyl
166 polysilphenylene siloxane film. High purity helium was used as carrier gas flowing at 1
167 ml/min. The inlet was held at 250°C, and the injection volume was 1 μL. The column was
168 held at 35°C for 4 minutes, ramped at 8 °C/min to 310°C, and held for 10 minutes, for a total
169 run time of 48 minutes. The mass spectrometer was operated in electron ionization (EI) mode
170 with ionization energy of 70 eV. The scan range was 50 to 600 amu.

171 Identification of individual compounds was conducted by probability-based matching
172 (match and reversed match ≥ 800) with mass spectra in the National Institute of Standards

and Technology (NIST) Mass Spectral Library database 2014 version 2.4.0. For identification of alkanes, positive EI mass spectra and RT were considered, using the aliphatic hydrocarbons present in the spiking solution as reference (C_9 – C_{40}).

2.9.2. Derivatization with MTBSTFA

Derivatization was performed adding 100 μL of the MTBDSTFA reagent to 100 μL of a 5-mg/L solution of the SPE extract (P3) in 1.5 mL capacity glass vials, which were sealed and mixed on a vortex for 1 minute. Vials were transferred to an oven at 60°C for 60 minutes to ensure complete ester formation. After this, vials were let to cool to room temperature, and the solvent was evaporated to approximately 10 – 20 μL using a TurboVap® LV concentration evaporator workstation. The volume was then made-up to 100 μL with DCM and samples were analyzed using GC-MS under the conditions described in section 2.8.1. 4-Methyl-1-cyclohexanecarboxylic acid (25 mg/mL in DCM) was used as a control to verify the derivatisation process.

2.9.3. LC-HRMS

High-resolution MS (HRMS) was carried out using a Thermo Accela LC pump and a CTC autosampler interfaced directly to a Thermo Exactive mass spectrometer. Chromatographic separation was conducted on a Varian Pursuit XRs C18 (100 x 3.0 mm, 3 μm, 100 Å) column. The mobile phase consisted of 0.1% NH_4OH in HPLC water (A) and 0.1% NH_4OH in methanol (B), with a flow rate of 600 μL/min. Further details are provided in the SI. Detection was performed in negative ion mode with a scan range of 80 – 500 m/z and the following HESI source conditions: sheath gas flow rate 50 units; spray Voltage 4000 V; capillary temperature 350 °C; capillary voltage 55 V; lens voltage 105 V; skimmer voltage 26 V; heater temperature 300 °C.

3. Results and Discussion

3.1. Phase I of EDA

3.1.1. Preliminary characterization

Table 1 presents the results for the preliminary characterization of aqueous samples and compares these with the maximum national discharge limits as stated in the relevant national legislation (Resolution 0631/2015; Ministry of Environment and Sustainable Development of Colombia). Data show that P3 was discharged at a temperature that exceeds the maximum national discharge limit (40°C), and had by far the greatest TOC content, being considerably higher than levels previously reported for treated RWW ranging from 6 to 70 mg/L (Daflon et al., 2017; Gillenwater et al., 2012; Nogueira et al., 2016; Pessala et al., 2004; Thakur et al., 2014). Moreover, P3 contained detectable yet legally compliant levels of Ni, Zn, and As.

3.1.2. Toxicity Evaluation

The EC_{50} values obtained for phenol using the adapted test were all within the reported range of 13 – 26 mg/L, which confirmed the suitability of the modified LBT procedure. Toxicity data of aqueous samples and extracts are presented in Table 2, showing that results correlated with TOC values provided in Table 1. P3 (aqueous sample) was the most toxic sample (5.0 TU; EC_{50} = 20%); the EC_{50} value was comparable to previous reports of RWW, although the high chemical variability in RWW has resulted in a wide range of EC_{50} values reported in the scientific literature (Aruldoss and Viraraghavan, 1998; Chang et al., 1981). Extracts obtained from LLE and SPE from P1 showed similar toxicity (4.4 vs 4.1 TU), whereas for P2 the SPE extract showed higher toxicity than the LLE extract (8.4 vs 3.0 TU). However, the TUs corresponding to P3 were remarkably higher than those of P1 and P2 — the LLE extract from P3 was almost 350 times more toxic than that of P2 and nearly 250 times more toxic than that of P1. As for SPE extracts, the P3 extract was nearly 80 times higher than P2 and 150 times higher than P1. These results suggested a different composition of P3 when compared to P1 and P2, with chemicals impacting significantly the light output in *A. fischeri*.

For all three samples, light output stabilised after 15 minutes (monitored up to 90 minutes;

data not shown), suggesting that the observed toxicity was caused by organic compounds rather than inorganic chemicals or metals. This indicated that the concentration of metals provided in Table 1 is not sufficient to reduce the light output, or that the metals present may not be bioavailable to compete for a biotic ligand.

3.1.3. Chemical analysis

The analysis by GC-MS of SPE and LLE blanks revealed that SPE generated more extraction artifacts than LLE (Figure S1 - i and ii), which were predominantly identified as phthalates based on their mass spectra (data not shown) and assumed to originate from the cartridges. Moreover, chromatograms revealed that P3 contained a large unresolved complex mixture (UCM) observed in both LLE/SPE extracts (Figure S1 - iii and iv). The extended retention time (~10 min) suggested that the UCM was composed of multiple co-eluting compounds rather than one compound at a very high concentration. Previous studies have linked 10-minute-long UCMs to naphthenic acids (NAs) (Clemente et al., 2004; John et al., 1998; Merlin et al., 2007), which are of toxicological concern due to their endocrine disruption potential and acute and chronic toxicity to a range of species (Clemente and Fedorak, 2005; Jie et al., 2015; Rogers et al., 2002). Furthermore, the averaged mass spectra for the UCM evidenced the presence of ions 41, 55, 69, 81, 95, 109, 123, 135, 150, 164, 181, and 195 m/z (Figure S2), which have been reported for NAs in EI-MS in almost identical relative abundances (John et al., 1998), hence suggesting that the UCM corresponded to NAs. The relative concentration of the UCM in the aqueous sample was estimated semi-quantitatively following the single point external standard method, using the formula below:

Relative concentration of UCM = [(Area of UCM) / (Area of IS)] x Concentration of IS

For calculation purposes, 1-chlorooctadecane was used as IS (spiking concentration 100 μg/L; RSD = 14.0% for SPE extracts, RSD = 17.9% for LLE extracts) because it presented lower variability in peak area than *o*-terphenyl (RSD = 110.4% for SPE extracts, RSD =

65.7% for LLE extracts). The relative concentration was estimated to be roughly 90 to 135 mg/L; the limits of the range correspond to the concentrations calculated with SPE and LLE extracts. This concentration, however, must be interpreted with caution because the detector does not respond identically to 1-chlorooctadecane and NAs, thus an accurate quantitation would require a multiple point standard method using known amounts of the NAs present in the UCM.

NAs are naturally present in oil reserves, especially in bitumen (Headley and McMartin, 2007), and therefore these have been studied in detail in relation to oil sands process water (OSPW) generated during the extraction of bitumen from the oil sands of northern Alberta, Canada. OSPW are considered an important environmental problem because of the significant health risk they pose to aquatic and mammalian species due to the high content of NAs when compared to background levels in natural waters, which are typically below 1 mg/L (CONRAD, 1998). Consequently, Canada has a zero-discharge policy for OSPW and these must be stored in settling ponds (Scott et al., 2005), where NAs are present in concentrations up to 120 mg/L (Holowenko et al., 2001; Kannel and Gan, 2012). However, NAs are not only present in bitumen but also in heavy crude oil (Clemente and Fedorak, 2005; Headley and McMartin, 2007). This makes them highly relevant in the context of refining wastewater – especially because NAs are not targeted during treatment of RWW as they are during OSPW treatment. Yet, significantly fewer publications address these pollutants in RWW (Dzidic et al., 1988; Misiti et al., 2013; Wang et al., 2019, 2015; Wong et al., 1996).

Colombian heavy crude has been reported to contain significant levels of NAs (Quiroga-Becerra et al., 2012), which would explain their considerable levels in P3. The estimated concentration of NAs in P3 (90 to 135 mg/L) is significantly higher than previous reports of NAs in treated RWW (2.8 to 11.6 mg/L) (Misiti et al., 2013) and more in the range of levels

273 reported in OSPW. Despite the similarities of the hazardous potential of effluents in both
274 scenarios, wastewater management practices are entirely different. Refining effluents are
275 treated and discharged under effluent guidelines that do not require reporting of NAs, so
276 these are masked under the bulk parameters of BOD, COD, or TOC, which means that only
277 toxicity tests can suggest their presence. Such toxicity tests are not mandatory in many
278 regulatory systems, including the Colombian legislation.
279 Table 3 shows the broad range of chemicals detected in extracts and the number of reports
280 for single-chemical aquatic toxicity (algae, bacteria, crustaceans, fish, amphibians, and
281 invertebrates) as reported by the US EPA ECOTOX Knowledgebase. Not only the structural
282 diversity in RWW samples is evident from the table, but also the fact that only a third of the
283 compounds identified have been toxicity-tested as pure substances, with evident differences
284 between types of compounds. Phenols and PAHs have numerous reports of aquatic toxicity,
285 in contrast to alkanes and carboxylic acids/esters. Within alkanes, only C_{12}, C_{13}, and C_{28} had
286 reports of aquatic toxicity, for a total of 18 reports. Ketones, on the other hand, have been
287 barely reported regarding their single-chemical aquatic toxicity; these are expected to exert
288 baseline toxicity because of the electron-withdrawing carbonyl moiety (Cronin and Schultz,
289 1998). Within the miscellaneous organics, which included amides and ethers, no reports were
290 found in the database. This might be the result of methodological challenges to toxicity-test
291 certain compounds, different risks of exposure among chemicals, or simply trends in
292 research. Regardless of the cause, the lack of ecotoxicological data complicates the linking of
293 chemical composition and observed toxicity, and the selection of target chemicals with
294 environmental relevance for treatment and monitoring.
295 The EC_{50} (Log of μg/L) values reported in ECOTOX Knowledgebase for compounds
296 identified in the extracts are provided in Figure 2. The figure shows that toxicity of acids
297 increases with chain length as a result of an increase in hydrophobicity as it drives their

298 partitioning into lipid membranes (Mayer and Reichenberg, 2009). In the case of phenols,
299 substituted phenols were detected in P1 and P3; these have been previously reported in RWW
300 and are likely to originate from the added chemicals during exploration and pre-treatment of
301 crude, both of which occur before the refining stage (Hashemi et al., 2015). Figure 2 shows
302 that not only the type and degree of substitution are key factors for the toxic action of
303 phenols, but also the pattern of substitution. This is observed with 2,6-dichlorophenol and
304 2,4-dichlorophenol, the latter being more toxic. As the hydroxyl group of phenols interact
305 with the π-electrons of the aromatic ring, phenols can generate stable phenoxy radicals that
306 are involved in the formation of intermediate metabolites that interact with biomolecules.
307 However, chlorines in *ortho* position form hydrogen bonds and shield the ─OH group (Boyd
308 et al., 2001), impacting the formation of such radicals. Moreover, the distribution of toxicity
309 data shows that PAHs are the most toxic group, whose toxicity also depends on their
310 hydrophobicity (Barata et al., 2005).
311 Interestingly, petroleum refining effluent guidelines tend not to regulate specific organic
312 toxicants – regardless of their single-chemical environmental toxicity – but rather include all
313 organic contaminants within 5-day BOD, COD, oil and grease, and phenolic compounds. In
314 particular, the Colombian guidelines for refining effluents require the analysis and report of
315 PAHs, BTEX, and adsorbable organic halogens (AOX), but there are no maximum discharge
316 limits established. Within the European context, the Integrated Pollution Prevention and
317 Control (IPCC) directive (2010/75/EU) does not set discharge limits either, but rather focuses
318 on the application of best available technologies. The situation is the same in the US, where
319 the concentration of PAHs, methylphenols, and other toxic organics in RWW has been found
320 to be consistently below treatable levels (US EPA, 2004), thus these are not considered
321 pollutants of concern and no maximum discharge limits have been set. The question is
322 whether current regulations are protecting humans and wildlife from RWW, considering that

the behavior of chemicals in a mixture may not be as predictable as that of pure compounds. Consequently, assessing compounds separately may underestimate the biological effects of RWW because chemicals can interact and generate mixture effects, even when each chemical is present at concentrations considered safe (Kortenkamp et al., 2009).

3.2.Phase II of EDA

3.2.1. Toxicity Evaluation

Numerous fractions from extraction blanks induced a significant reduction of luminescence, which suggested the presence of phthalates and was later confirmed by MS (data not shown). Consequently, toxicity of fractions was estimated after subtracting that of blanks (Figure 3), revealing that fractions from P3 showed markedly higher toxicity. Fractions with the highest toxicity values for each sample and extraction method (using the 75th percentile as the cut-off value) were further analyzed using GC-MS.

3.2.2. Chemical analysis

Most of the toxic fractions analyzed via GC-MS contained organic acids, methyl/ethyl esters of carboxylic acids, alkanes, and numerous unknowns; no PAHs were detected in full scan mode. From the compounds detected in the toxic fractions, only a handful had reports for single-chemical aquatic toxicity in USEPA ECOTOX Knowledgebase (Figure S3) and their reported (Log)EC_{50} values indicated that, overall, these compounds were less toxic than those detected in extracts. This suggests that mixture effects might be partially responsible for the loss of bioluminescence in *A. fischeri*. The most toxic groups detected were alkanes and NAs, the latter eluting in fractions 2 and 3, both of which showed significant inhibition of luminescence (Figure 3). This coincides with previous studies reporting that NAs are key contributors to biological effects when present in effluents (Clemente et al., 2004; He et al., 2012; Kannel and Gan, 2012; Quinlan and Tam, 2015),.

In a few toxic fractions, no peaks were observed other than compounds known to correspond to column bleed and the IS used for AQC, suggesting that some of the compounds impacting bioluminescence could be thermally labile or have low volatility and therefore were not amenable to GC-MS analysis.

Overall, our results are comparable to other studies in the sense that the chemical analysis of toxic fractions does not point at single toxicants but rather to groups of chemicals. This confirms the importance of mixture effects in RWW, but also identifies key groups when it comes to biological effects – this is the case of NAs. It is noteworthy that there is a low number of publications involving TIE/EDA of refining effluents, which might be related to publication bias stemming from the "disappointing" outcome of not finding an evident chemical, or few chemicals, causing all the observed effects. Dorris et al., 1974 reported that none of the compounds identified in toxic fractions, which included aliphatic hydrocarbons, *m*-cresol, and dioctyl phthalate, could fully account for the acutely lethal effects observed on *Daphnia magna*. Similarly, Ankley et al., 2011 indicated that attempts to assign toxicity in RWW to single chemicals were unsuccessful and usually faced a broad distribution of toxicity among multiple fractions, complicating the establishment of a causation relationship. Leonards et al., 2011 found that narcotic effects play an essential role in the toxicity of RWW, but these could not explain the observed toxicity for several samples, suggesting an analysis of individual organic contaminants to help establishing causative factors. These outcomes suggest that (i) toxicity might be linked to compounds that are not amenable for GC-MS detection or present in concentrations below the LoD, (ii) the observed toxicity is the result of the aggregate effect of various compounds, also known is mixture effect, or (iii) stems from the numerous unknowns that could not be identified using the NIST library, the latter of which suggests that the range of identification could be increased using LC-MS.

3.2.2.1.Characterization of NAs

The presence of NAs in the UCM was confirmed via derivatization with MTBSTFA and GC-MS analysis. The ions for *t*-BDMS derivatives ranged from 213 to 295 *m/z*, indicating that the NAs in P3 ranged from C_9 to C_{15}, and *Z* families from 0 to -8, which is in accordance with previous reports of NAs in environmental water samples (Clemente et al., 2003; McKenzie et al., 2014) and commercial mixtures (Clemente et al., 2004; Damasceno et al., 2014). The corresponding NA profile obtained is shown in Figure 4 (A).

Subsequent analysis of the NA mixture was conducted using HRMS. After calculating the exact masses of classic NAs fitting the formula $C_nH_{2n+Z}O_2$ for all combinations of n = 5 to 35 and Z = 0 to -12, the predicted ions were searched in the acquired mass spectra, generating the NA profile presented in Figure 4 – B. The resulting profile was similar to that obtained by GC-MS (Figure 4 – A) but differed in the low-intensity ions detected due to the higher sensitivity of HRMS, the latter of which expanded the carbon range to C_{21} and included congeners from Z = -10 and -12. Families Z = 0 to -6 presented the same proportional contribution (Z= -2 > -4 > 0 > -6) in both profiles.

Oxidized NAs fitting the formula $C_nH_{2n+Z}O_x$ where x = 3 to 5, which result from oxidation of classic NAs via hydroxylated intermediates (Barrow et al., 2009; Grewer et al., 2010; Han et al., 2009), were also detected in the extract, although their intensity was much lower compared to classic NAs (Figure S4). Based on abundance, O_2 NAs corresponded to 89.8% of the NAs detected and oxy-NAs corresponded to 3.5%, 6.5%, and 0.1% for O_3, O_4 and O_5, respectively. These findings corroborate the findings of previous works reporting a predominance of O_2 and O_4 in NA mixtures (Barrow et al., 2009; Grewer et al., 2010) but show a much higher relative abundance of O_2 NAs in relation to other studies, where classic NAs have been found to range between 15% and 72% in groundwater and OSPW (Frank et al., 2014; Grewer et al., 2010; Han et al., 2009; Meshref et al., 2017). Evidence suggests that classic NAs are the most toxic NAs (Morandi et al., 2015) and this could explain the

significant inhibition of luminescence observed with P3. This has important implications for the treatment of RWW, particularly at refineries processing heavy oils, where it is unlikely that residence times at treatment plants would be sufficient to biodegrade the toxic fraction. These findings will therefore aid future work to refine, optimize or redesign wastewater treatment processes to ensure effluent discharges are not toxic to the receiving environments.

4. Conclusions

The findings of this study confirm that organics are key players in the toxicity exerted by RWW, highlighting the relevance of an EDA approach for understanding the complexity of these effluents. Our results suggest that (i) mixture effects are important for the biological effects observed in *A. fischeri*, but that (ii) some of the organics involved in such biological effects might be present at concentrations below the detection limits of analytical instruments. Additionally, (iii) two key groups were identified to have a significant contribution to the biological effects observed – aliphatic hydrocarbons and NAs. The latter group is a high priority finding because NAs are not usually included in effluent guidelines for the refining sector, and therefore represent a hazard for human populations and wildlife due to their reported toxicity and their resistance to treatment. The fact that heavy crude oil has high contents of NAs makes heavy oil refining effluents a significant source for NAs into aquatic ecosystems, if not tackled appropriately from the regulatory and technological perspectives. Our results also indicate that (iv) the concentration of NAs in heavy RWW can be as high as that reported in OSPW, highlighting the need for further treatment of NA-containing RWW.

Finally, our results suggest that (v) the high content of classic NAs (90%) may be linked to the significant inhibition of luminescence observed in *A. fischeri*, as these have been reported to be the most toxic NAs. This has important implications for the further treatment of NA-containing RWW, as oxidation of classic NAs could lead to a decreased toxicity.

5. Supplementary information

This section contains details on sampling, extraction, fractionation, toxicity assessment. Also, results from chemical analysis (TICs of LLE/SPE extracts; NA profiles) and toxicity assessment.

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8. References

Alva-Argáez, A., Kokossis, A., Smith, R., 2007. The design of water-using systems in petroleum refining using a water-pinch decomposition. Chem. Eng. J. 128, 33–46.

Ankley, G.T., Hockett, J.R., Mount, D.I., Mount, D.R., 2011. Early evolution of the toxicity identification evaluation process: Contributions from the United States environmental protection agency effluent testing program, in: Brack, W. (Ed.), Effect-Directed Analysis of Complex Environmental Contamination. Springer, pp. 1–18.

Aruldoss, J.A., Viraraghavan, T., 1998. Toxicity testing of refinery wastewater using microtox. Bull. Environ. Contam. Toxicol. 60, 456–463.

444 Atuanyan, E., Tudararo-Aherobo, L., 2015. Ecotoxicological effects of discharge of Nigerian
445 petroleum refinery oily sludge on biological sentinels. African J. Environ. Sci. Technol.
446 9, 95–103.

447 Avci, A., Kacmaz, M., Durak, I., 2005. Peroxidation in muscle and liver tissues from fish in a
448 contaminated river due to a petroleum refinery industry. Ecotoxicol. Environ. Saf. 60,
449 101–105.

450 Barata, C., Calbet, A., Saiz, E., Ortiz, L., Bayona, J.M., 2005. Predicting single and mixture
451 toxicity of petrogenic polycyclic aromatic hydrocarbons to the copepod Oithona davisae.
452 Environ. Toxicol. Chem. 24, 2992–2999.

453 Barrow, M.P., Headley, J. V., Peru, K.M., Derrick, P.J., 2009. Data Visualization for the
454 Characterization of Naphthenic Acids within Petroleum Samples. Energy & Fuels 23,
455 2592–2599.

456 Blaise, C., Forghani, R., Legault, R., Guzzo, J., Dubow, M.S., 1994. A bacterial toxicity
457 assay performed with microplates, microluminometry and Microtox reagent.
458 Biotechniques 16, 932–937.

459 Bleckmann, C.A., Rabe, B., Edgmon, S.J., Fillingame, D., 1995. Aquatic toxicity variability
460 for fresh- and saltwater species in refinery wastewater effluent. Environ. Toxicol. Chem.
461 14, 1219–1223. https://doi.org/10.1002/etc.5620140714

462 Boyd, E., Killham, K., Meharg, A., 2001. Toxicity of mono-, di- and tri-chlorophenols to lux
463 marked terrestrial bacteria, Burkholderia species Rasc c2 and Pseudomonas fluorescens.
464 Chemosphere 43, 157–166.

465 Buikema Jr, A.L., Niederlehner, B.R., Cairns Jr, J., B.R., N., J., C.J., 1981. The effects of a

466 simulated refinery effluent and its components on the estuarine crustacean, Mysidopsis
467 bahia. Arch. Environ. Contam. Toxicol. 10, 231–239.

468 Burks, S.L., 1982. Review of pollutants in petroleum refinery wastewaters and effect upon
469 aquatic organisms. Environ. Impact Pet. Refin. 7, 271–283.
470 https://doi.org/http://dx.doi.org/10.1016/0160-4120(82)90117-9

471 Çavas, T., Ergene-Gözükara, S., 2005. Induction of micronuclei and nuclear abnormalities in
472 Oreochromis niloticus following exposure to petroleum refinery and chromium
473 processing plant effluents. Aquat. Toxicol. 74, 264–271. https://doi.org/S0166-
474 445X(05)00177-3 [pii]

475 Chang, J.C., Taylor, P.B., Leach, F.R., 1981. Use of the Microtox assay system for
476 environmental samples. Bull. Environ. Contam. Toxicol. 26, 150–156.

477 Clemente, J.S., Fedorak, P.M., 2005. A review of the occurrence, analyses, toxicity, and
478 biodegradation of naphthenic acids. Chemosphere 60, 585–600.

479 Clemente, J.S., Mackinnon, M., Fedorak, P.M., 2004. Aerobic biodegradation of two
480 commercial naphthenic acids preparations. Environ. Sci. Technol. 38, 1009–1016.

481 Clemente, J.S., Prasad, N.G.N., Mackinnon, M., Fedorak, P.M., 2003. A statistical
482 comparison of naphthenic acids characterized by gas chromatography–mass
483 spectrometry. Chemosphere 50, 1265–1274.

484 Coelho, A., Castro, A. V, Dezotti, M., Sant'Anna Jr, G.L., 2006. Treatment of petroleum
485 refinery sourwater by advanced oxidation processes. J. Hazard. Mater. 137, 178–184.
486 https://doi.org/S0304-3894(06)00078-1 [pii]

487 Comber, M., Girling, A., den Haan, K., Whale, G., 2015. Oil refinery experience with the

488 assessment of refinery effluents and receiving waters using biologically based methods.
489 Integr. Environ. Assess. Manag. 11, 653–665.

490 CONRAD, 1998. Naphthenic acids background information: discussion report. Edmonton,
491 AB.

492 Cronin, M., Schultz, W., 1998. Structure–Toxicity Relationships for Three Mechanisms of
493 Action of Toxicity to Vibrio fischeri. Ecotoxicol. Environ. Saf. 39, 65–69.

494 Daflon, S.D.A., Guerra, I.L., Reynier, M. V, Cerqueira, A.C., Botta, C.R., Campos, J.C.,
495 2017. Toxicity identification and evaluation of a petroleum refinery wastewater. J.
496 Environ. Sci. Heal. - Part A 52, 842–848.

497 Damasceno, F.C., Gruber, L.D.A., Geller, A.M., Vaz de Campos, M.C., Gomes, A.O.,
498 Guimaraes, R.C.L., Péres, V.F., Jacques, R.A., Caramao, E.B., 2014. Characterization of
499 naphthenic acids using mass spectroscopy and chromatographic techniques: study of
500 technical mixtures. Anal. Methods 6, 807–816.

501 Dorris, T.C., Burks, S.L., Waller, G.R., 1974. Effects of residual toxins in oil refinery
502 effluents on aquatic organisms. Washington D.C.

503 Dzidic, I., Somerville, A.C., Raia, J.C., Hart, H. V, 1988. Determination of Naphthenic acids
504 in California crudes and refinery wastewaters by fluoride ion chemical ionization mass
505 spectrometry. Anal. Chem. 60, 1318–1323.

506 E.G., L.P., F., P.J., Mike, C., Graham, W., George, S., 2011. Impact of biodegradation on the
507 potential bioaccumulation and toxicity of refinery effluents. Environ. Toxicol. Chem.
508 30, 2175–2183. https://doi.org/10.1002/etc.628

509 Frank, R.A., Roy, J.W., Bickerton, G., Rowland, S.J., Headley, J. V, Scarlett, A.G., West,

510 C.E., Peru, K.M., Parrott, J.L., Conly, F.M., Hewitt, L.M., 2014. Profiling Oil Sands
511 Mixtures from Industrial Developments and Natural Groundwaters for Source
512 Identification. Environ. Sci. Technol. 48, 2660–2670. https://doi.org/10.1021/es500131k

513 Gillenwater, P.S., Ungun-Demirtas, M., Negri, M.C., Snyder, W., 2012. Comparative
514 evaluation of As, Se and V removal technologies for the treatment of oil refinery
515 wastewater. Water Sci. Technol. 65, 112–118.

516 Grewer, D.M., Young, R.F., Whittal, R.M., Fedorak, P.M., 2010. Naphthenic acids and other
517 acid-extractables in water samples from Alberta: What is being measured? Sci. Total
518 Environ. 408, 5997–6010.

519 Gupta, A.K.; Ahmad, M.., 2012. Assessment of cytotoxic and genotoxic potential of refinery
520 waste effluent using plant, animal and bacterial systems. J. Hazard. Mater. 201–202, 92–
521 99.

522 Hall Jr, L.W., Buikema Jr, A.L., Cairns Jr, J., 1978. The effects of a simulated refinery
523 effluent on the grass shrimp, Palaemonetes pugio. Arch. Environ. Contam. Toxicol. 7,
524 23–35.

525 Han, X., MacKinnon, M.D., Martin, J.W., 2009. Estimating the in situ biodegradation of
526 naphthenic acids in oil sands process waters by HPLC/HRMS. Chemosphere 76, 63–70.
527 https://doi.org/10.1016/J.CHEMOSPHERE.2009.02.026

528 Hashemi, W. Al, Maraqa, M.A., Rao, M. V, Hossain, M.M., 2015. Characterization and
529 removal of phenolic compounds from condensate-oil refinery wastewater. Desalin.
530 Water Treat. 54, 660–671.

531 He, Y., Patterson, S., Wang, N., Hecker, M., Martin, J.W., El-Din, M.G., Giesy, J.P.,

532 Wiseman, S.B., 2012. Toxicity of untreated and ozone-treated oil sands process-affected
533 water (OSPW) to early life stages of the fathead minnow (Pimephales promelas). Water
534 Res. 46, 6359–6368. https://doi.org/https://doi.org/10.1016/j.watres.2012.09.004

535 Headley, J., McMartin, D., 2007. A review of the ocurrence and fate of naphthenic acids in
536 aquatic environments. J. Environ. Sci. Heal. - Part A 39, 1989–2010.

537 Hessel, C., Allegre, C., Maisseu, M., Charbit, F., Moulin, P., 2007. Guidelines and legislation
538 for dye house effluents. J. Environ. Manage. 83, 171–180.
539 https://doi.org/https://doi.org/10.1016/j.jenvman.2006.02.012

540 Holowenko, F., Mackinnon, M., Fedorak, P., 2001. Naphthenic acids and surrogate
541 naphthenic acids in methanogenic microcosms. Water Res. 35, 2595–2606.

542 Hong, S., Giesy, J.P., Lee, J.-S., Lee, J.-H., Khim, J.S., 2016. Effect-directed analysis:
543 Current status and future challenges. Ocean Sci. J. 51, 413–433.
544 https://doi.org/10.1007/s12601-016-0038-4

545 International Energy Agency, 2020. Oil market report.

546 IPIECA, 2005. Water resource management in the petroleum industry.

547 Jafarinejad, S., Jiang, S.C., 2019. Current technologies and future directions for treating
548 petroleum refineries and petrochemical plants (PRPP) wastewaters. J. Environ. Chem.
549 Eng. 7, 103326. https://doi.org/https://doi.org/10.1016/j.jece.2019.103326

550 Jie, W., Xiaofeng, C., Yi, H., Xiaoyan, T., 2015. Developmental toxicity and endocrine
551 disruption of naphthenic acids on the early life stage of zebrafish (Danio rerio). J. Appl.
552 Toxicol. 35, 1493–1501. https://doi.org/10.1002/jat.3166

553 John, W., Rughani, J., Green, S., McGinnis, G., 1998. Analysis and characterization of

554 naphthenic acids by gas chromatography–electron impact mass spectrometry of tert.-
555 butyldimethylsilyl derivatives. J. Chromatogr. A 807, 241–251.
556 https://doi.org/10.1016/S0021-9673(98)00085-5

557 Kannel, P.R., Gan, T.Y., 2012. Naphthenic acids degradation and toxicity mitigation in
558 tailings wastewater systems and aquatic environments: A review. J. Environ. Sci. Heal. -
559 Part A 47, 1–21.

560 Kortenkamp, A., Backhaus, T., Faust, M., 2009. State of the art: Report on mixture toxicity.

561 Li, Y., Fang, Z., He, C., Zhang, Y., Xu, C., Chung, K., Shi, Q., 2015. Molecular
562 characterization and transformation of dissolved organic matter in refinery wastewater
563 from water treatment processes: characterization by Fourier transform ion cyclotron
564 resonance mass spectrometry. Energy & Fuels 29, 6556–6963.

565 Mayer, P., Reichenberg, F., 2009. Can highly hydrophobic organic substances cause aquatic
566 baseline toxicity and can they contribute to mixture toxicity? Environ. Toxicol. Chem.
567 25, 2639–2644.

568 McKenzie, N., Yue, S., Liu, X., Ramsay, B., Ramsay, J., 2014. Biodegradation of naphthenic
569 acids in oils sands process waters in an immobilized soil/sediment bioreactor.
570 Chemosphere 109, 164–172. https://doi.org/10.1016/J.CHEMOSPHERE.2014.02.001

571 Merlin, M., Guigard, S., Fedirakm Phillip, 2007. Detecting naphthenic acids in waters by gas
572 chromatography–mass spectrometry. J. Chromatogr. A 1140, 225–229.
573 https://doi.org/10.1016/J.CHROMA.2006.11.089

574 Meshref, M.N.A., Chelme-Ayala, P., Gamal El-Din, M., 2017. Fate and abundance of
575 classical and heteroatomic naphthenic acid species after advanced oxidation processes:

576 Insights and indicators of transformation and degradation. Water Res. 125, 62–71.
577 https://doi.org/10.1016/J.WATRES.2017.08.007

578 Misiti, T.M., Tezel, U., Pavlostathis, S.G., 2013. Fate and effect of naphthenic acids on oil
579 refinery activated sludge wastewater treatment systems. Water Res. 47, 460.

580 Morandi, G.D., Wiseman, S.B., Pereira, A., Mankidy, R., Gault, I.G.M., Martin, J.W., Giesy,
581 J.P., 2015. Effects-Directed Analysis of Dissolved Organic Compounds in Oil Sands
582 Process-Affected Water. Environ. Sci. Technol. 49, 12395–12404.
583 https://doi.org/10.1021/acs.est.5b02586

584 Nogueira, A.A., Bassin, J.P., Cerqueira, A.C., Dezotti, M., 2016. Integration of biofiltration
585 and advanced oxidation processes for tertiary treatment of an oil refinery wastewater
586 aiming at water reuse. Environ. Sci. Pollut. Res. 23, 9730–9741.
587 https://doi.org/10.1007/s11356-015-6034-x

588 Norberg-King, T.J., Embry, M.R., Belanger, S.E., Braunbeck, T., Butler, J.D., Dorn, P.B.,
589 Farr, B., Guiney, P.D., Hughes, S.A., Jeffries, M., Journel, R., Lèonard, M., McMaster,
590 M., Oris, J.T., Ryder, K., Segner, H., Senac, T., Van Der Kraak, G., Whale, G., Wilson,
591 P., 2018. An International Perspective on the Tools and Concepts for Effluent Toxicity
592 Assessments in the Context of Animal Alternatives: Reduction in Vertebrate Use.
593 Environ. Toxicol. Chem. 37, 2745–2757. https://doi.org/10.1002/etc.4259

594 Pessala, P., Schultz, E., Nakari, T., Joutti, A., Herve, S., 2004. Evaluation of wastewater
595 effluents by small-scale biotests and a fractionation procedure. Ecotoxicol. Environ. Saf.
596 59, 263–272. https://doi.org/http://dx.doi.org/10.1016/j.ecoenv.2003.10.002

597 Power, E.A., Boumphrey, R.S., 2004. International trends in bioassay use for effluent
598 management. Ecotoxicology 13, 377–398.

599 Quinlan, P.J., Tam, K.C., 2015. Water treatment technologies for the remediation of
600 naphthenic acids in oil sands process-affected water. Chem. Eng. J. 279, 696–714.
601 https://doi.org/10.1016/J.CEJ.2015.05.062

602 Quiroga-Becerra, H., Mejía-Miranda, C., Laverde-Catano, D., Hernández-López, M., Gómez-
603 Sánchez, M., 2012. A kinetic study of esterification of naphthenic acids from a
604 Colombian heavy crude oil. Ciencia, Tecnol. y Futur. 4, 21–31.

605 Rogers, V. V, Wickstrom, M., Liber, K., Mackinnon, M., 2002. Acute and subhronic
606 mammalian toxicity of naphthenic acids from oil sands tailings. Toxicol. Sci. 66, 347–
607 355.

608 Scott, A.C., Mackinnon, M., Fedorak, P.M., 2005. Naphthenic acids in Athabasca oil sands
609 tailings waters are less biodegradable than commercial naphthenic acids. Environ. Sci.
610 Technol. 39, 8388–8394.

611 Sponza, D.T., 2002. Necessity of Toxicity Assessment in Turkish Industrial Discharges
612 (Examples from Metal and Textile Industry Effluents). Environ. Monit. Assess. 73, 41–
613 66. https://doi.org/10.1023/A:1012663213153

614 Tatem, H.E., Cox, B.A., Anderson, J.W., H.E., T., B.A., C., J.W., A., 1978. The toxicity of
615 oils and petroleum hydrocarbons to estuarine crustaceans. Estuar. Coast. Mar. Sci. 6,
616 365–373. https://doi.org/http://dx.doi.org/10.1016/0302-3524(78)90128-7

617 Thakur, C., Srivastava, V.C., Mall, I.D., 2014. Aerobic degradation of petroleum refinery
618 wastewater in sequential batch reactor. J. Environ. Sci. Heal. - Part A 49, 1436–1444.

619 US EPA, 2004. Technical support document for the 2004 effluent guidelines program plan.
620 Section 7: Petroleum Refining. US EPA, Washington DC, United States of America.

621 Vaz Hara, R., Marin-Morales, M.A., 2017. In vitro and in vivo investigation of the genotoxic
622 potential of waters from rivers under the influence of a petroleum refinery (Sao Paulo
623 State - Brazil). Chemosphere 174, 321–330.

624 Wake, H., 2005. Oil refineries: A review of their ecological impacts on the aquatic
625 environment. Estuar. Coast. Shelf Sci. 62, 131–140.

626 Wang, B., Cui, H., Liu, H., Wan, Y., 2019. Derivatization for Nontargeted Screening of
627 Acids in Oilfield Refinery Wastewater: Identification and Behaviors of Recalcitrant
628 Chlorinated Naphthenic Acids. Environ. Sci. Technol. 53, 1022–1030.
629 https://doi.org/10.1021/acs.est.8b05310

630 Wang, B., Wan, Y., Gao, Y., Zheng, G., Yang, M., Wu, S., Hu, J., 2015. Occurrences and
631 Behaviors of Naphthenic Acids in a Petroleum Refinery Wastewater Treatment Plant.
632 Environ. Sci. Technol. 49, 5796–5804. https://doi.org/10.1021/es505809g

633 Whitehouse, P., 2001. Measures for protecting water quality: Current approaches and future
634 developments. Ecotoxicol. Environ. Saf. 50, 115–126.

635 Wong, D., van Compernolle, R., Nowlin, J., O'Neal, D., Johnson, G., 1996. Use of
636 supercritical fluid extraction and fast ion bombardment mass spectrometry to identify
637 toxic chemicals from a refinery effluent adsorbed onto granular activated carbon.
638 Chemosphere 32, 1669–1679. https://doi.org/10.1016/0045-6535(96)00065-3

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Tables

Naphthenic Acids are Key Contributors to Toxicity of Heavy Oil Refining Effluents

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Table 1. Results for the preliminary characterization of effluent samples from Barrancabermeja, Colombia, showing compliance with discharge limits as stated in Resolution 0631/2015

Parameter		**Sample**			**Maximum discharge limits***
		P1	**P2**	**P3**	
pH		7.36	7.30	6.47	6.0 – 9.0
Temperature (°C)		32.30	30.40	60.40	< 40
TOC (mg/L)		39.59	22.65	127.50	Not specified
Total content (mg/L) (*n*=3)	V	< 0.02	< 0.02	< 0.02	1.00
	Ni	<0.01	<0.01	0.045 ± 0.001	0.50
	Zn	0.027 ± 0.001	0.078 ± 0.002	0.027 ± 0.004	3.00
	As	< 0.02	< 0.02	0.086 ± 0.000	0.10
	Se	< 0.01	< 0.01	< 0.01	0.20
	Hg	< 0.01	< 0.01	< 0.01	0.01
	Cr	<0.04	<0.04	<0.04	0.50
	Pb	<0.04	<0.04	<0.04	0.10
	Cd	<0.04	<0.04	<0.04	0.10

* Resolution 0631/2015 from the Ministry of Environment and Sustainable Development of Colombia

Table 2. Toxicity results (EC_{50} values and TU) for aqueous samples and extracts

Sample	Type of sample	EC_{50}	TU
P1	Aqueous	No inhibition observed	
	SPE	22.6 REF	4.4
	LLE	24.1 REF	4.1
P2	Aqueous	No inhibition observed	
	SPE	11.9 REF	8.4
	LLE	33.4 REF	3.0
P3	Aqueous	20.0%	5.0
	SPE	0.2 REF	666.6
	LLE	0.1 REF	1000.0

REF: Relative enrichment factor = Enrichment factor (Volume of sample / Volume of extract) x Dilution factor (Volume of extract added to assay / total volume of assay)

Table 3. Organic compounds identified in SPE and LLE extracts from RWW samples

Type of compound	Compound	Sample	Extract	No. of reports in ECOTOX Knowledgebase
Organic acids and esters	Hexanoic acid	P1	LLE	15
	Heptanoic acid	P1	LLE	10
	Nonanoic acid	P1	LLE	23
	4-acetylbutiric acid	P1	SPE	No reports
	Undecanoic acid	P2	LLE	5
	9,12-octadecadienoic acid	P3	LLE	90
	4-methyl-3-pentenoic acid	P3	LLE	No reports
	2,2,4-trimethyl-3-carboxy isopropyl pentanoic acid, isobutyl ester	P1	LLE	No reports
	2,2,4-trimethyl-1,3-pentanoic acid, diisobutyl ester	P1	LLE	No reports
	Hexanedioic acid, bis(2-ethylhexyl)ester	P1	LLE	No reports
	Tetradecanoic acid, methyl ester	P1, P2	SPE	No reports
	Pentadecanoic acid, methyl ester	P1	SPE	No reports
	Hexadecanoic acid, methyl ester	P1	SPE	No reports
	Octadecanoic acid, methyl ester	P1	SPE	No reports
	Octadecanoic acid, ethyl ester	P1	SPE	No reports

Type of compound	Compound	Sample	Extract	No. of reports in ECOTOX Knowledgebase
Organic acids and esters	cis-butenedioic acid, bis(2-ethylhexyl) ester	P1	LLE	No reports
	1,2-benzenedicarboxylic acid, bis(2-methylpropyl)ester	P3	LLE	2
	Hexanedioic acid, bis(2-ethylhexyl)ester	P3	LLE	9
	2-isopropylphenyl oxalic acid, pentyl ester	P3	SPE	No reports
Phenols	2,6-dichlorophenol	P1	LLE	60
	2,4-dichlorophenol	P1	LLE	756
	2,6-dichloro-4-(1,1-dimethylethyl)phenol	P1	LLE	No reports
	2,4,6-trichlorophenol	P1	LLE	433
	2,4,6-tribromophenol	P1	SPE	25
	2,5-dimethylphenol	P3	LLE, SPE	21
Hydrocarbons	Alkanes $C_{22} - C_{36}$	P1, P2, P3	LLE, SPE	18
	1,2-epoxyhexadecane	P1	LLE	No reports
	1,2-epoxynonadecane	P1	LLE	No reports
	1,2-dichlorooctane	P2	LLE	No reports
	1,5,5-trimethyl-6-acetylmethylcyclohexene	P2	SPE	No reports
	Nonadecene	P1	LLE	No reports

Type of compound	Compound	Sample	Extract	No. of reports in ECOTOX Knowledgebase
Hydrocarbons	Docosene	P1	LLE	No reports
	Fluoranthene	P1	LLE	1067
	Pyrene	P1	LLE	502
	Naphthalene	P3	LLE	1179
	2-methylnaphthalene	P3	LLE	62
	Anthracene/phenanthrene	P3	LLE, SPE	511/611
	Benzo(*ghi*)perylene	P3	LLE	10
	Indeno(1,2,3-*cd*)pyrene	P3	LLE	3
Ketones	1-methyl-2-pyrrolidinone	P1	LLE	4
	2,6-di-tert-butylbenzoquinone	P1	LLE	No reports
	4,6-dimethyl-2H-pyran-2-one	P1	SPE	No reports
	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	P1	LLE	No reports
	Benzophenone	P1, P2	LLE	No reports
	3,5-dimethyl-2-furyl methyl ketone	P2	SPE	No reports
	5-hydroxy-4,5-dimethyl-2,5-dihydrofuran-2-one	P2	SPE	No reports
	4,6-dimethyl-2H-pyran-2-one	P2	SPE	No reports

Type of compound	Compound	Sample	Extract	No. of reports in ECOTOX Knowledgebase
Ketones	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	P2	SPE	No reports
	3',8,8'-trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone	P2	SPE	No reports
	1-nitro-2-octanone	P3	SPE	No reports
Miscellaneous	Tetrahydro-1,1-dioxide thiophene	P1, P3	LLE	No reports
	Vinyl lauryl ether	P1	LLE	No reports
	2-Ethoxyethyl ether	P1	LLE	No reports
	Tetradecanamide	P1	LLE	No reports
	Diethyltoluamide	P2	LLE	No reports
	N-butyl-benzenesulfonamide	P2	LLE	No reports
	N-propylbenzamide	P3	LLE	No reports
	Isocyanatobenzene	P3	LLE	No reports
	Benzenethiol	P3	LLE	No reports
	3-mercaptopropionitrile	P3	LLE	No reports
	Triacetin (1,2,3-triacetoxypropane)	P2	LLE	No reports

Figures

Naphthenic Acids are Key Contributors to Toxicity of Heavy Oil Refining Effluents

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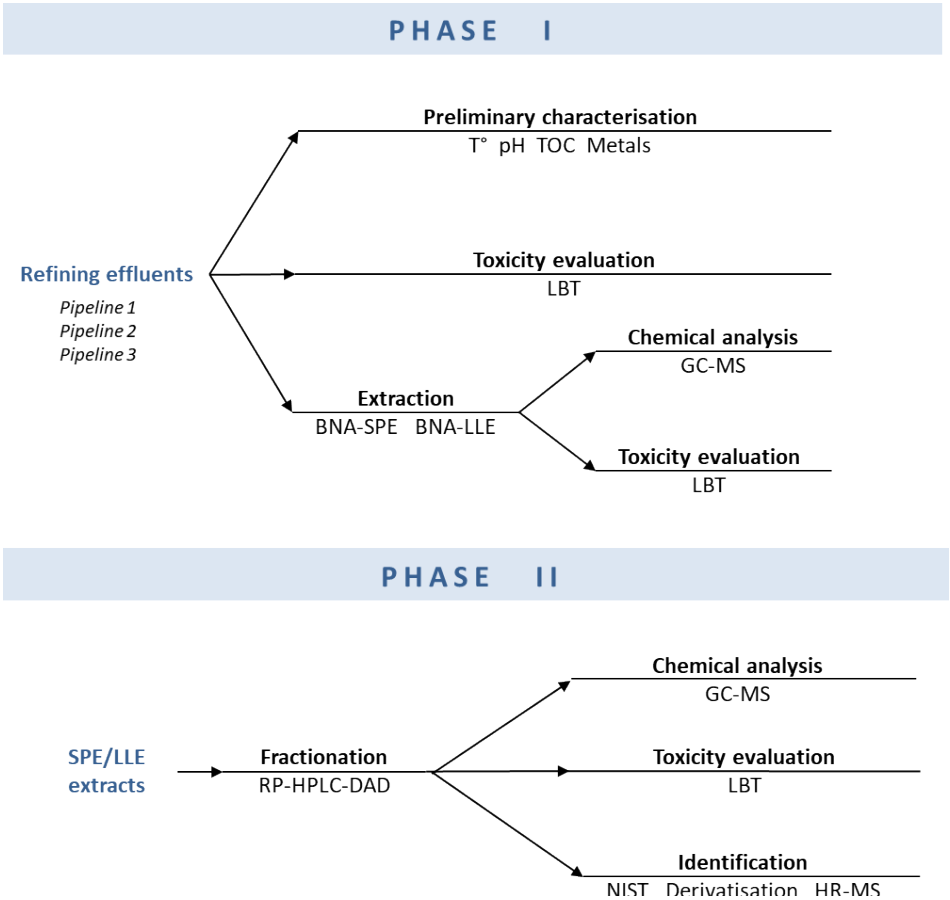

Figure 1. Schematic representation of the EDA performed to identify toxic organics in refining effluents

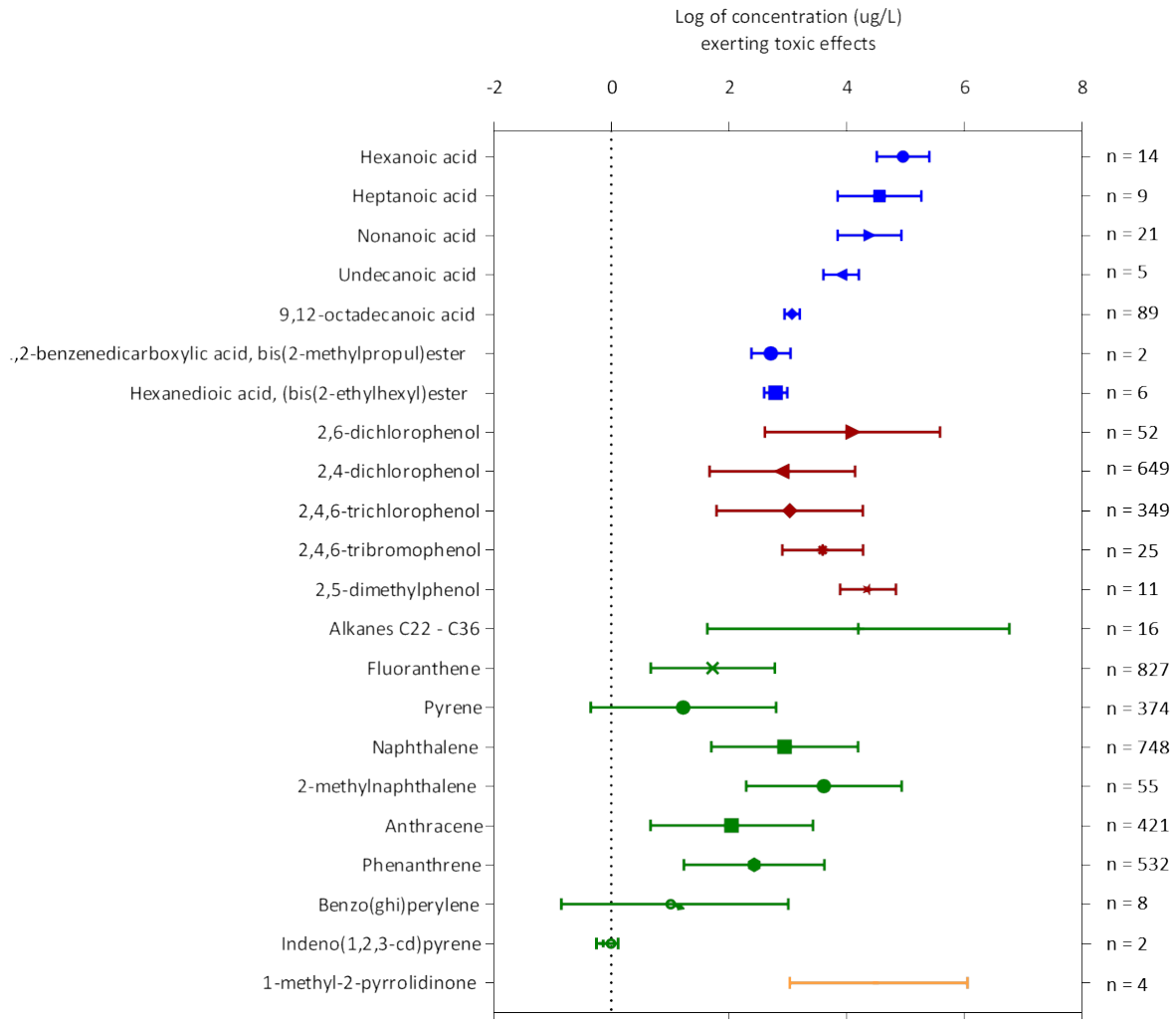

Figure 1. Aquatic toxicity (Log of EC_{50}) of compounds detected in RWW extracts, as reported in ECOTOX Knowledgebase, where n = number of reports. Acids and esters are shown in blue, phenols in red, hydrocarbons in green, and ketones in yellow

[Color to be used in print]

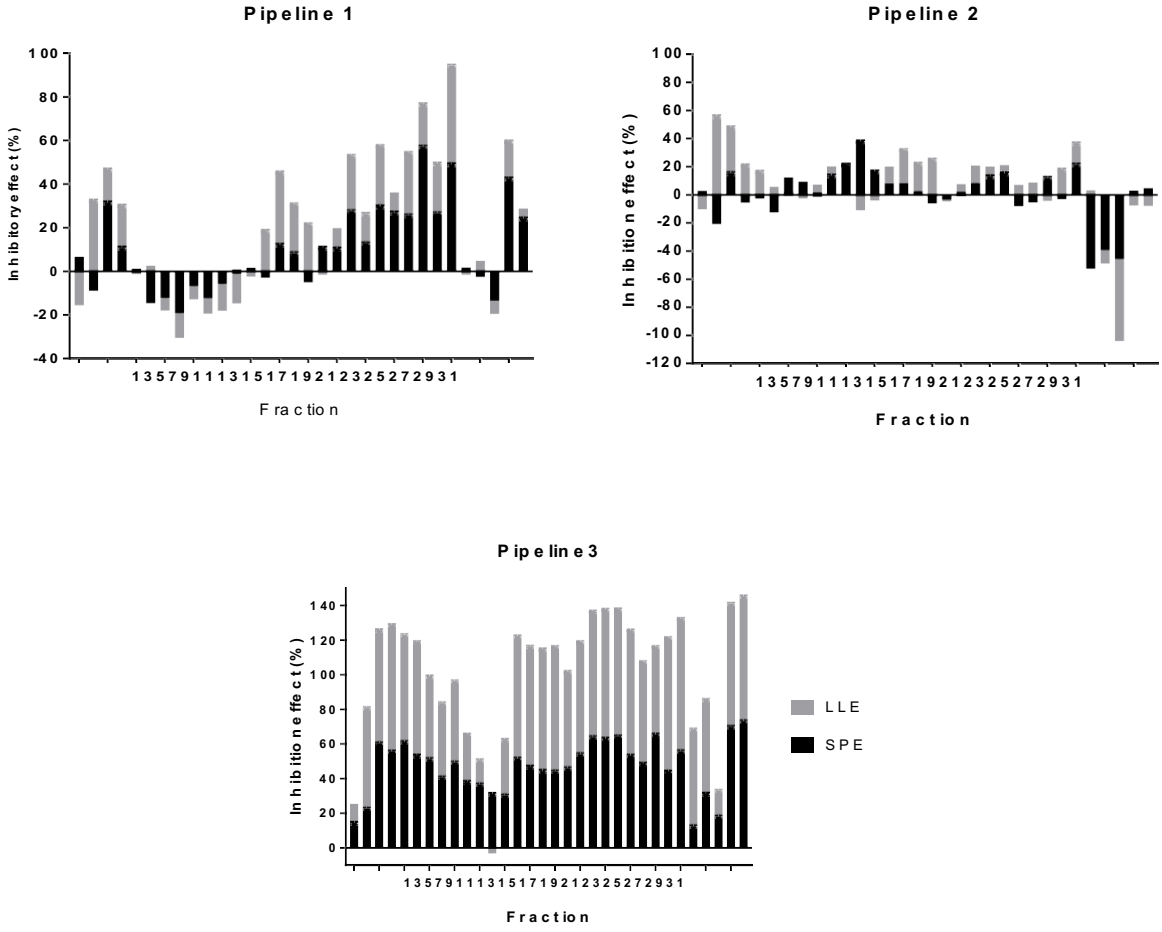

Figure 2. Toxicity of LLE and SPE fractions from P1, P2, and P3 after the subtraction of toxicity from fractionation blanks

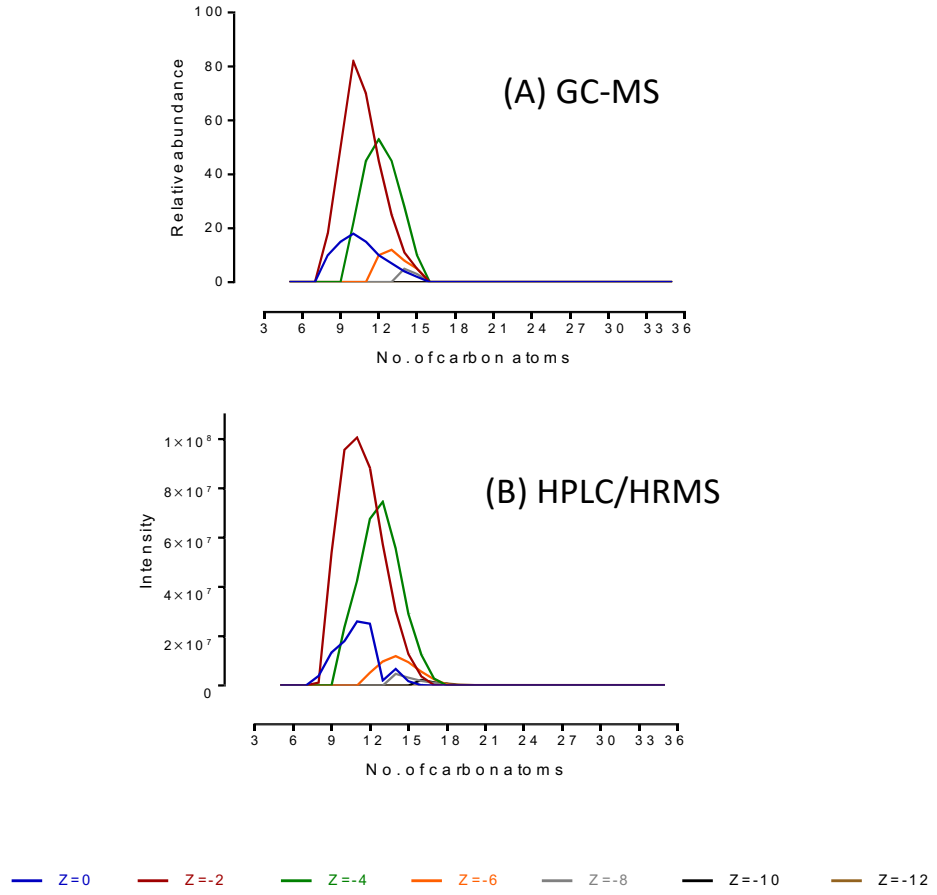

Figure 3. NA profiles for the extract from pipeline 3 analyzed by (A) GC-MS after derivatization with MTBDSTFA, (B) HPLC/HRMS

[Color to be used in print]

Supplementary material for on-line publication only

Click here to download Supplementary material for on-line publication only: Supplementary data_Pinzon & Kanda_revised.doc

Manuscript title: Naphthenic Acids are Key Contributors to Toxicity of Heavy Oil Refining Effluents

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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CRediT author statement

Author	**Contribution**
Angela Pinzón-Espinosa	Conceptualization Investigation Writing - Original Draft Funding acquisition
Rakesh Kanda	Conceptualization Writing - Review & Editing Supervision