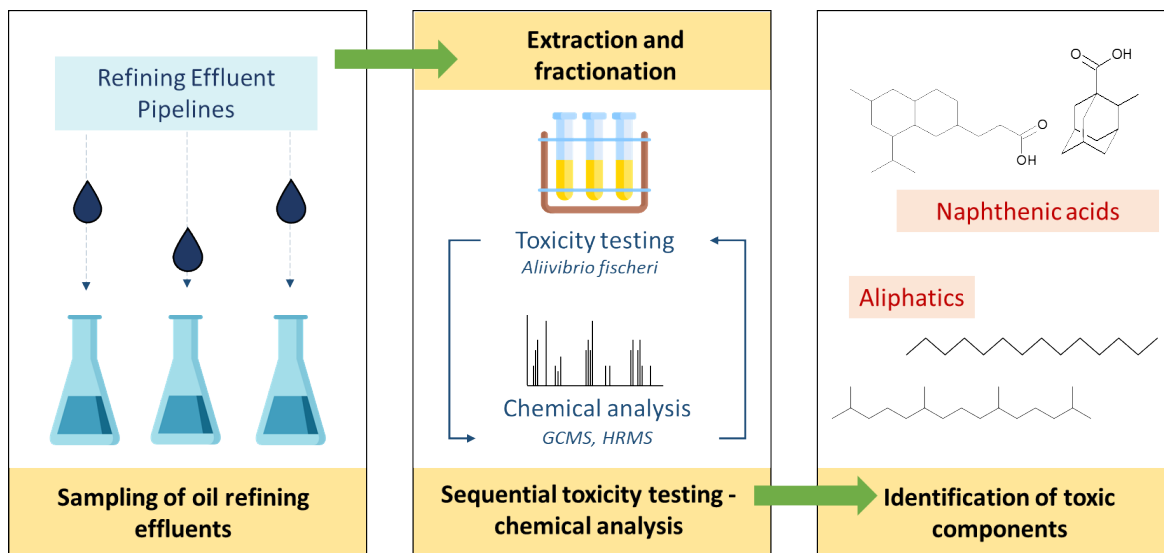# Graphical abstract

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

1 **Naphthenic Acids are Key Contributors to Toxicity of Heavy Oil Refining**

2 **Effluents**

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10

11 **Abstract.** Oil refining produces vast quantities of wastewater with harmful contaminants that

12 can be released back into the environment with a possible risk of toxicity to aquatic wildlife

13 and human populations. Hence the importance of adequate wastewater treatment to achieve

14 safe effluents that protect both ecological and human health. However, some refining

15 effluents are linked to serious pollution problems even after treatment, partly because little

16 progress has been made in determining the causative agents of the observed biological

17 effects, resulting in non-targeted treatment. Here, we followed an effect-directed analysis

18 (EDA) approach using *Aliivibrio fischeri* as biosensor to show that naphthenic acids (NAs)

19 are important components of refining wastewater resulting from the processing of heavy

20 crude oil. Furthermore, we demonstrate that besides mixture effects, NAs have a significant

21 contribution to the toxicity exerted by these effluents. Profiling of the NA mixture was

22 conducted using high resolution liquid chromatography-Orbitrap, which evidenced that O_2

23 NAs corresponded to 90% of the NAs detected. Our findings contrast with previous reports

24 where classic NAs have been found between 15% and 72% and could explain the significant

25 biological effects observed in *A. fischeri*. This study broadens the body of evidence pointing

26 at mixture effects and low-concentration pollutants as the cause of toxicity from RWW, in

27 addition to NAs resulting from the processing of heavy crude oil. Our results can serve as a

28 starting point for setting better effluent discharge standards relevant to oil refining wastewater

29 resulting from heavy crude oil and help improve wastewater treatment plants to reduce

30 effluent toxicity.

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

32 *Aliivibrio fischeri*

33   1. Introduction.

34   The petroleum industry handles more water than oil during their daily operations (IPIECA,

35 2005), especially during oil refining. This makes the refining sector a major water consumer

36 — the refining of 1 m³ of crude oil requires 2 to 2.5 m³ of water (Alva-Argáez et al., 2007;

37 Coelho et al., 2006). As a result, and considering that approximately 13 million m³ of crude

38 oil were refined daily in 2019 (International Energy Agency, 2020), significant volumes of

39 refining wastewater (RWW) are constantly produced worldwide. Hence the importance of

40 RWW quality from the environmental perspective, as refineries are distributed widely around

41 the world and the vast amounts of RWW produced on each site can have hazardous effects on

42 the receiving ecosystems. Wastewater treatment technologies can provide effluents that are

43 environmentally safe, provided continuous monitoring is conducted to follow-up the efficacy

44 of wastewater treatment plants and help identifying effluents of concern. However, there are

45 important discrepancies in relation to quality criteria for industrial effluents around the world,

46 including refining discharges, which mainly derive from different approaches in

47 environmental regulations across countries (Hessel et al., 2007; Jafarinejad and Jiang, 2019;

48 Power and Boumphrey, 2004; Whitehouse, 2001). Some regulations, mainly in low-income

49 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.

74   The tangible outcome of the gap in knowledge in relation to the causal agents of the
75   toxicity exerted by RWW is that treatment processes often fail to fully remove toxicity from
76   effluents. Modern refinery wastewater treatment plants are generally effective in removing
77   suspended oil and suspended solids, but toxic and hydrophilic contaminants are likely to
78   resist treatment and reach waterways (Li et al., 2015). It is, therefore, necessary to redesign
79   treatment plants for these to reduce the concentration of toxic chemicals to non-hazardous
80   levels, but a good understanding of the chemical and toxicological properties of the
81   constituents of wastewater is essential to develop effective treatment systems.
82   Addressing the gap in knowledge of the organic fraction in RWW requires a new approach
83   — the limitations of studying known specific components and TIE methodologies for
84   understanding complex mixtures of organics in environmental samples are well known (Hong
85   et al., 2016; Pessala et al., 2004). An alternative approach to address the problem of
86   environmental diagnostics is conducting an effect-directed analysis (EDA), which includes an
87   extraction step and makes an emphasis on organics as the cause of toxicity, making it an
88   excellent option for the analysis of RWW. This approach, however, has not been successfully
89   applied to RWW before. Therefore, the objective of this study was to identify toxic organics
90   in oil refining effluents following a non-targeted EDA procedure using *Aliivibrio fischeri* as
91   biosensor, thus helping to fill the long-standing gap between chemical composition and
92   toxicity of RWW.

93   2. Materials and Methods

94   2.1. Chemicals and reagents

95   Solvents were obtained from Fisher Scientific. H_2SO_4 and NaOH were purchased from Fluka.
96   Oasis® WAX 6cc (150 mg, 30μm) and HLB 6cc (200 mg) extraction cartridges were
97   obtained from Waters. N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide containing
98   1% t-BDMS-chloride (MTBSTFA) was purchased from Sigma-Aldrich. Stock solutions for

99   spiking (aromatic and aliphatic hydrocarbons; 200 μg/mL in acetone) and the internal

100   standard (IS) solution (1-chlorooctadecane and *o*-terphenyl; 400 μg/mL in acetone) were

101   purchased from Restek UK. Chemicals for the fractionation check solution (naphthalene,

102   bisphenol A, and phenol; 70 mg/L in hexane) were purchased from Sigma-Aldrich. Solutions

103   were stored at 4°C in dark and airtight conditions.

104   For the bioassay, phenol and potassium dichromate were purchased from Sigma-Aldrich.

105   Sodium chloride was purchased from Fischer Scientific and the Microtox® reagent was

106   obtained from Modern Water Inc.

107   2.2. EDA procedure

108   The EDA methodology was conducted in two phases, as shown in Figure 1. Phase I aimed

109   at providing a preliminary characterization, toxicity evaluation, and extraction of effluent

110   samples, all of which are described in the sections 2.5, 2.6, 2.8, and 2.9 (subsection 2.9.1)

111   below. Phase II was carried out subsequently to fractionate the extracts obtained during Phase

112   I (Section 2.7). Fractions were then analyzed to assess toxicity (Section 2.8) and chemical

113   composition (Section 2.9, subsection 2.9.1). Identification and characterization of toxic

114   organics was conducted following the procedures described in Section 2.9, subsections 2.9.2

115   and 2.9.3.

116   2.3. Sampling

117   Effluent samples were collected from 3 pipelines (P1, P2, P3) from an oil refinery located in

118   Barrancabermeja, Colombia, discharging into River Magdalena. Sampling details are

119   provided in Table S1. The temperature and pH of samples were measured *in situ* using an

120   Oakton® portable meter. Samples were acidified to pH 2 and stored at 4°C in airtight

121   conditions until analysis.

122   2.4. Sample Preparation

123   Each sample was divided into two separate sub-batches for chemical analysis and toxicity

124   evaluation, which were processed identically and at the same time. Blanks and analytical

125   quality control (AQC) samples were prepared for quality assurance purposes using Milli-Q®

126   water. Samples for chemical analyses were spiked with aromatic and aliphatic hydrocarbons

127   (final concentration 80 µg/L) and IS (*o*-terphenyl and 1-chlorooctadecane; final

128   concentration100 µg/L).

129   **2.5. Preliminary characterization of effluents**

130   Samples were filtered using 1.2 µm pore size Whatman® filters. Total organic carbon (TOC)

131   of aqueous filtrates was measured by combustion catalytic oxidation/non-dispersive infrared

132   (NDIR) spectrometry using a Shimadzu TOC-VCPN analyzer coupled with a Shimadzu

133   OCT-1 8-port sampler.

134   The total concentration of V, Ni, Zn, As, Se, Hg, Cr, Pb, and Cd was determined using

135   inductively coupled plasma-optical emission spectroscopy (ICP-OES) with a Perkin Elmer

136   Optima 5300 DV spectrophotometer attached to a Perkin Elmer AS 93plus autosampler.

137   Digestion of organic matter was carried out in a CEM MARS 6 microwave digester in

138   accordance with USEPA method 3015A.

139   **2.6. Sample extraction**

140   Sample aliquots (800 mL) were filtered using 1.2 µm pore size Whatman® filters and

141   extracted in duplicate using liquid-liquid extraction (LLE) and solid phase extraction (SPE).

142   Details are provided in the SI.

143   **2.7. Fractionation**

144   LLE and SPE extracts were fractioned using normal phase high-performance liquid

145   chromatography (NP-HPLC) using an Agilent 1260 system on an analytical Waters®

146   SPHERISORB® silica column (4.6 x 100mm, 3-μm particle size). Mobile phases were (A)

147   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

7

173   and Technology (NIST) Mass Spectral Library database 2014 version 2.4.0. For identification

174   of alkanes, positive EI mass spectra and RT were considered, using the aliphatic

175   hydrocarbons present in the spiking solution as reference ($C_9 - C_{40}$).

176   2.9.2. Derivatization with MTBSTFA

177   Derivatization was performed adding 100 μL of the MTBDSTFA reagent to 100 μL of a 5-

178   mg/L solution of the SPE extract (P3) in 1.5 mL capacity glass vials, which were sealed

179   and mixed on a vortex for 1 minute. Vials were transferred to an oven at 60°C for 60

180   minutes to ensure complete ester formation. After this, vials were let to cool to room

181   temperature, and the solvent was evaporated to approximately 10 – 20 μL using a

182   TurboVap® LV concentration evaporator workstation. The volume was then made-up to

183   100 μL with DCM and samples were analyzed using GC-MS under the conditions

184   described in section 2.8.1. 4-Methyl-1-cyclohexanecarboxylic acid (25 mg/mL in DCM)

185   was used as a control to verify the derivatisation process.

186   2.9.3. LC-HRMS

187   High-resolution MS (HRMS) was carried out using a Thermo Accela LC pump and a CTC

188   autosampler interfaced directly to a Thermo Exactive mass spectrometer. Chromatographic

189   separation was conducted on a Varian Pursuit XRs C18 (100 x 3.0 mm, 3 μm, 100 Å)

190   column. The mobile phase consisted of 0.1% NH_4OH in HPLC water (A) and 0.1% NH_4OH

191   in methanol (B), with a flow rate of 600 μL/min. Further details are provided in the SI.

192   Detection was performed in negative ion mode with a scan range of 80 – 500 m/z and the

193   following HESI source conditions: sheath gas flow rate 50 units; spray Voltage 4000 V;

194   capillary temperature 350 °C; capillary voltage 55 V; lens voltage 105 V; skimmer voltage 26

195   V; heater temperature 300 °C.

196   3. Results and Discussion

197   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;

223 data not shown), suggesting that the observed toxicity was caused by organic compounds

224 rather than inorganic chemicals or metals. This indicated that the concentration of metals

225 provided in Table 1 is not sufficient to reduce the light output, or that the metals present may

226 not be bioavailable to compete for a biotic ligand.

227 3.1.3. Chemical analysis

228 The analysis by GC-MS of SPE and LLE blanks revealed that SPE generated more extraction

229 artifacts than LLE (Figure S1 - i and ii), which were predominantly identified as phthalates

230 based on their mass spectra (data not shown) and assumed to originate from the cartridges.

231 Moreover, chromatograms revealed that P3 contained a large unresolved complex mixture

232 (UCM) observed in both LLE/SPE extracts (Figure S1 - iii and iv). The extended retention

233 time (~10 min) suggested that the UCM was composed of multiple co-eluting compounds

234 rather than one compound at a very high concentration. Previous studies have linked 10-

235 minute-long UCMs to naphthenic acids (NAs) (Clemente et al., 2004; John et al., 1998;

236 Merlin et al., 2007), which are of toxicological concern due to their endocrine disruption

237 potential and acute and chronic toxicity to a range of species (Clemente and Fedorak, 2005;

238 Jie et al., 2015; Rogers et al., 2002). Furthermore, the averaged mass spectra for the UCM

239 evidenced the presence of ions 41, 55, 69, 81, 95, 109, 123, 135, 150, 164, 181, and 195 m/z

240 (Figure S2), which have been reported for NAs in EI-MS in almost identical relative

241 abundances (John et al., 1998), hence suggesting that the UCM corresponded to NAs. The

242 relative concentration of the UCM in the aqueous sample was estimated semi-quantitatively

243 following the single point external standard method, using the formula below:

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

245 For calculation purposes, 1-chlorooctadecane was used as IS (spiking concentration 100

246 μg/L; RSD = 14.0% for SPE extracts, RSD = 17.9% for LLE extracts) because it presented

247 lower variability in peak area than *o*-terphenyl (RSD = 110.4% for SPE extracts, RSD =

248   65.7% for LLE extracts). The relative concentration was estimated to be roughly 90 to 135

249   mg/L; the limits of the range correspond to the concentrations calculated with SPE and LLE

250   extracts. This concentration, however, must be interpreted with caution because the detector

251   does not respond identically to 1-chlorooctadecane and NAs, thus an accurate quantitation

252   would require a multiple point standard method using known amounts of the NAs present in

253   the UCM.

254   NAs are naturally present in oil reserves, especially in bitumen (Headley and McMartin,

255   2007), and therefore these have been studied in detail in relation to oil sands process water

256   (OSPW) generated during the extraction of bitumen from the oil sands of northern Alberta,

257   Canada. OSPW are considered an important environmental problem because of the

258   significant health risk they pose to aquatic and mammalian species due to the high content of

259   NAs when compared to background levels in natural waters, which are typically below 1

260   mg/L (CONRAD, 1998). Consequently, Canada has a zero-discharge policy for OSPW and

261   these must be stored in settling ponds (Scott et al., 2005), where NAs are present in

262   concentrations up to 120 mg/L (Holowenko et al., 2001; Kannel and Gan, 2012). However,

263   NAs are not only present in bitumen but also in heavy crude oil (Clemente and Fedorak,

264   2005; Headley and McMartin, 2007). This makes them highly relevant in the context of

265   refining wastewater – especially because NAs are not targeted during treatment of RWW as

266   they are during OSPW treatment. Yet, significantly fewer publications address these

267   pollutants in RWW (Dzidic et al., 1988; Misiti et al., 2013; Wang et al., 2019, 2015; Wong et

268   al., 1996).

269   Colombian heavy crude has been reported to contain significant levels of NAs (Quiroga-

270   Becerra et al., 2012), which would explain their considerable levels in P3. The estimated

271   concentration of NAs in P3 (90 to 135 mg/L) is significantly higher than previous reports of

272   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_{22}, C_{23}, 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

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

327 **3.2.Phase II of EDA**

328 **3.2.1. Toxicity Evaluation**

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

335 **3.2.2. Chemical analysis**

336 Most of the toxic fractions analyzed via GC-MS contained organic acids, methyl/ethyl
337 esters of carboxylic acids, alkanes, and numerous unknowns; no PAHs were detected in full
338 scan mode. From the compounds detected in the toxic fractions, only a handful had reports
339 for single-chemical aquatic toxicity in USEPA ECOTOX Knowledgebase (Figure S3) and
340 their reported $(Log)EC_{50}$ values indicated that, overall, these compounds were less toxic than
341 those detected in extracts. This suggests that mixture effects might be partially responsible for
342 the loss of bioluminescence in *A. fischeri*. The most toxic groups detected were alkanes and
343 NAs, the latter eluting in fractions 2 and 3, both of which showed significant inhibition of
344 luminescence (Figure 3). This coincides with previous studies reporting that NAs are key
345 contributors to biological effects when present in effluents (Clemente et al., 2004; He et al.,
346 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

372   The presence of NAs in the UCM was confirmed via derivatization with MTBSTFA and

373   GC-MS analysis. The ions for t-BDMS derivatives ranged from 213 to 295 m/z, indicating

374   that the NAs in P3 ranged from C_9 to C_{15}, and Z families from 0 to -8, which is in accordance

375   with previous reports of NAs in environmental water samples (Clemente et al., 2003;

376   McKenzie et al., 2014) and commercial mixtures (Clemente et al., 2004; Damasceno et al.,

377   2014). The corresponding NA profile obtained is shown in Figure 4 (A).

378   Subsequent analysis of the NA mixture was conducted using HRMS. After calculating the

379   exact masses of classic NAs fitting the formula $C_nH_{2n+Z}O_2$ for all combinations of $n = 5$ to 35

380   and $Z = 0$ to -12, the predicted ions were searched in the acquired mass spectra, generating

381   the NA profile presented in Figure 4 – B. The resulting profile was similar to that obtained by

382   GC-MS (Figure 4 – A) but differed in the low-intensity ions detected due to the higher

383   sensitivity of HRMS, the latter of which expanded the carbon range to C_{21} and included

384   congeners from $Z = -10$ and -12. Families $Z = 0$ to -6 presented the same proportional

385   contribution ($Z= -2 > -4 > 0 > -6$) in both profiles.

386   Oxidized NAs fitting the formula $C_nH_{2n+Z}O_x$ where $x = 3$ to 5, which result from oxidation

387   of classic NAs via hydroxylated intermediates (Barrow et al., 2009; Grewer et al., 2010; Han

388   et al., 2009), were also detected in the extract, although their intensity was much lower

389   compared to classic NAs (Figure S4). Based on abundance, O_2 NAs corresponded to 89.8%

390   of the NAs detected and oxy-NAs corresponded to 3.5%, 6.5%, and 0.1% for O_3, O_4 and O_5,

391   respectively. These findings corroborate the findings of previous works reporting a

392   predominance of O_2 and O_4 in NA mixtures (Barrow et al., 2009; Grewer et al., 2010) but

393   show a much higher relative abundance of O_2 NAs in relation to other studies, where classic

394   NAs have been found to range between 15% and 72% in groundwater and OSPW (Frank et

395   al., 2014; Grewer et al., 2010; Han et al., 2009; Meshref et al., 2017). Evidence suggests that

396   classic NAs are the most toxic NAs (Morandi et al., 2015) and this could explain the

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

402 **4. Conclusions**

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

418   Finally, our results suggest that (v) the high content of classic NAs (90%) may be linked to
419 the significant inhibition of luminescence observed in *A. fischeri*, as these have been reported
420 to be the most toxic NAs. This has important implications for the further treatment of NA-
421 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.

6. Funding Sources

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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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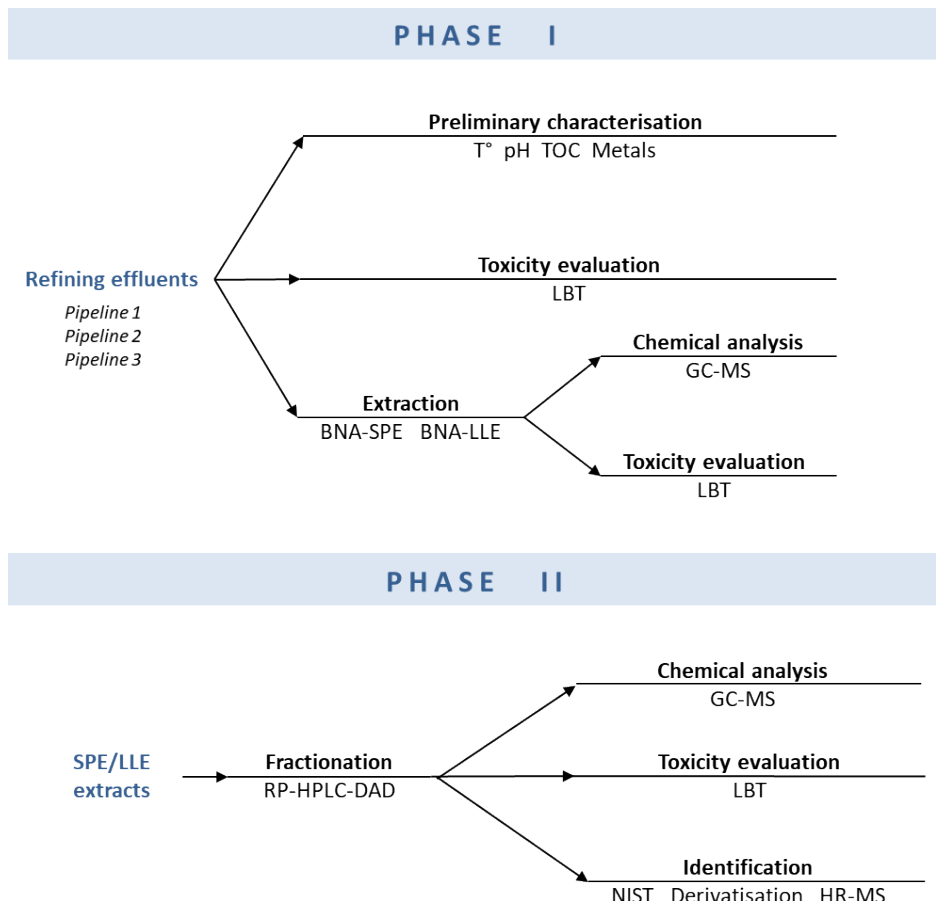
*Corresponding author: acpinzone@gmail.com

Figure 1. Schematic representation of the EDA performed to identify toxic organics in refining effluents
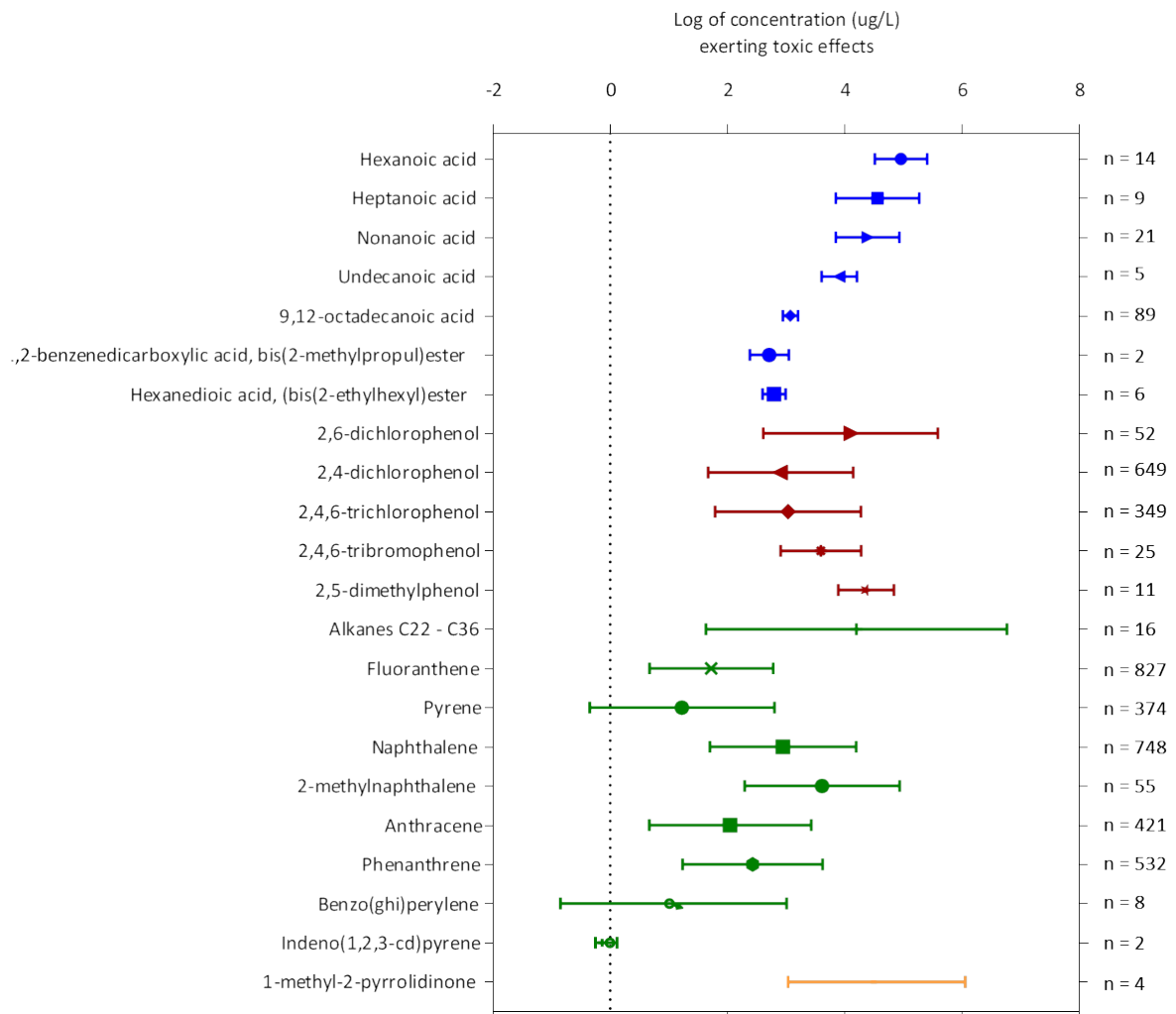
Figure 1. Aquatic toxicity (Log of EC$_{50}$) of compounds detected in RWW extracts, as reported in ECOTOX Knowledgebase, where n = number of reports. Acids and esters are shown in blue, phenols in red, hydrocarbons in green, and ketones in yellow
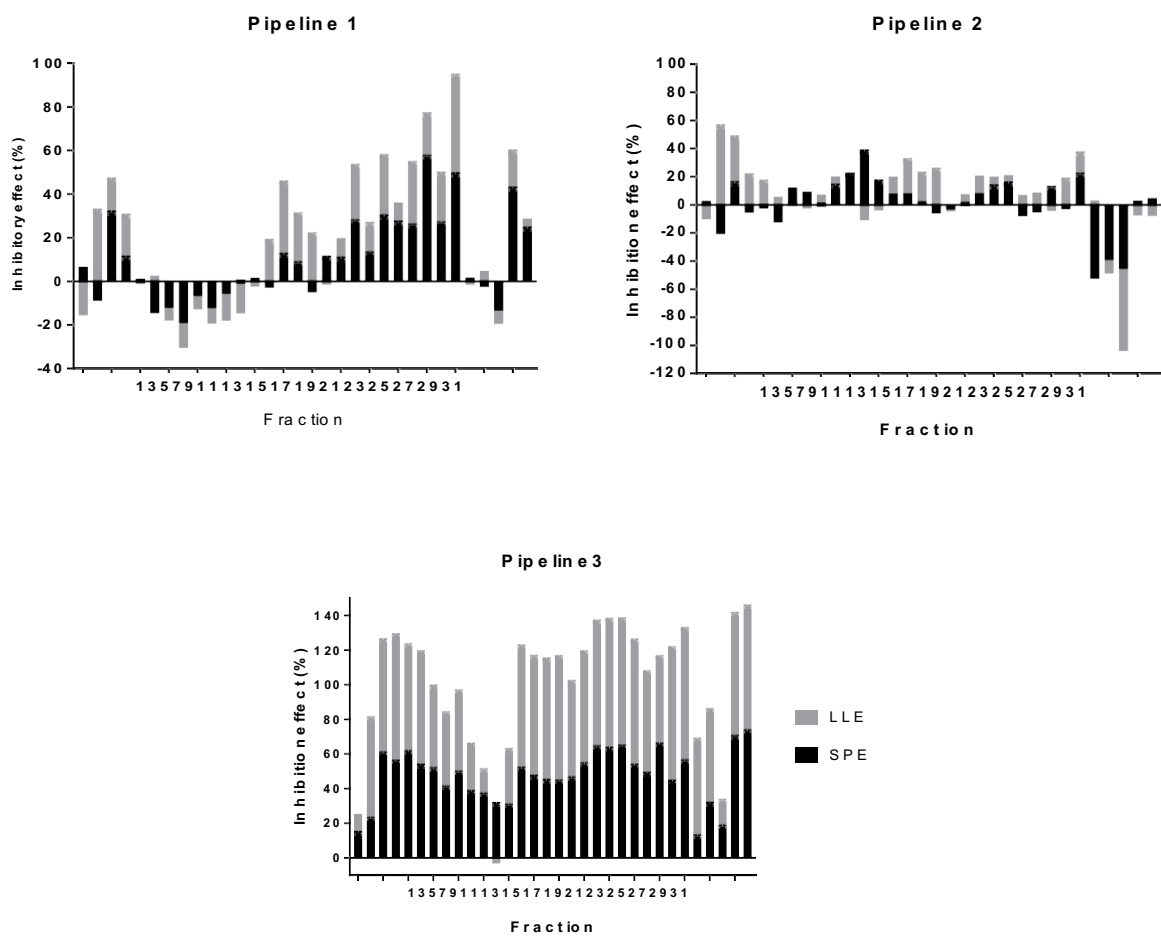
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Figure 2. Toxicity of LLE and SPE fractions from P1, P2, and P3 after the subtraction of toxicity from fractionation blanks
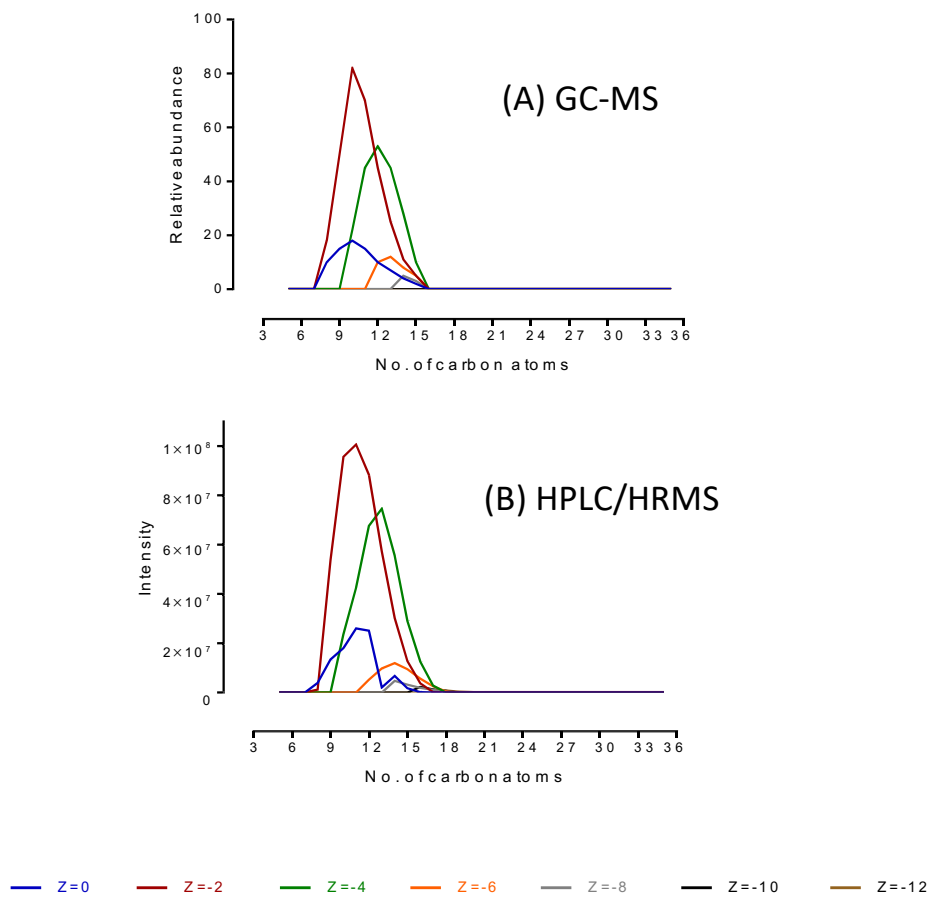
Figure 3. NA profiles for the extract from pipeline 3 analyzed by (A) GC-MS after derivatization with MTBDSTFA, (B) HPLC/HRMS

[Color to be used in print]

Supplementary material for on-line publication only

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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Authors: Angela Pinzon-Espinosa, Rakesh Kanda

CRediT author statement

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